UNIVERSITY OF SOUTHAMPTON

DIETARY INFLUENCE ON MATERNAL VASCULAR FUNCTION IN PREGNANCY IN THE RAT

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ABSTRACT

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Pregnancy is a state characterized by significant cardiovascular adaptive responses to meet the metabolic needs of the mother and fetus. Maternal blood volume and cardiac output are increased, while total vascular resistance and arterial pressure tend to decrease. Maternal cardiac output and plasma volume are substantially reduced in pregnant rats fed a low protein diet or fed 50% of their normal daily food intake throughout gestation, but it is not known whether vascular function is also compromised. This thesis investigates the effect of a low protein diet (9% casein) on systemic nitric oxide production and vascular function in virgin and pregnant rats.

Systemic nitric oxide (NO) production was assessed through urinary nitrate excretion. Urinary nitrate excretion was reduced on day 18 of gestation on the low protein diet ($P = 0.04$), although overall the data were inconclusive. To investigate the influence of a 9% casein diet on vascular reactivity, contractile responses to phenylephrine (PE), or acetylcholine (ACh)-mediated relaxation and the contribution of NO to these mechanisms were studied in virgin and pregnant rats. Virgin and pregnant Wistar rats were fed either the 18% casein or 9% casein diets for 18-19 days; in the pregnant rats, the diets were given from day 1 of pregnancy. 3 mm segments of a third-order mesenteric artery (~ 300 μm in diameter) were excised and mounted on fine tungsten wires on a small vessel myograph. There were no differences in contractile sensitivity to PE in these vessels from virgin ($P = 0.6$) and pregnant rats ($P = 0.3$) fed the 18% casein and the 9% casein diet. Significant reductions in the sensitivity to ACh were found in vessels from virgin ($P = 0.03$) and pregnant ($P = 0.02$) rats that had consumed the 9% casein diet. Inhibition of cyclooxygenase and NO synthesis with a combination of indomethacin (INDO) and N^{ω}-nitro-L-arginine methyl ester (L-NAME) significantly decreased ACh-induced dilations in all groups, whereas indomethacin alone did not affect the ACh response. In arteries from the virgin rats on the low protein diet there was also a significant reduction in the sensitivity ($P = 0.0003$) and maximum relaxation ($P = 0.009$) to the NO donor spermine NONOate (SPN) whereas the vasodilator potency of SPN did not differ between pregnant 18% and 9% casein-fed rats. Mean placental and fetal weights were significantly lower in the rats fed on 9% casein ($P < 0.0001$ and $P = 0.005$ respectively).

The results show that low protein diets impair vasodilator responses in female rats. These effects may contribute to the poor cardiovascular adaptation to pregnancy and lower fetal weights associated with restricted protein intake.

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ABBREVIATIONS

AA	arachidonic acid
ACh	acetylcholine
ADMA	asymmetrical dimethylarginine
Ang II	angiotensin II
Arg	L-arginine
BK	bradykinin
8-Br-cGMP	8-bromoguanosine-3':5'-cyclic monophosphate
bp	base pairs
^{0}**C**	temperature in degrees centigrade
Ca^{2+}	calcium
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
cNOS	constitutive nitric oxide synthase
CO_2	carbon dioxide
COX	cyclooxygenase
CR	creatinine
Da	Dalton
DAG	diacylglycerol
EC	endothelial cells
EC_{50}	the agonist dose yielding 50% of the maximum response, expressed as -log mol/L = pEC_{50}
$_{ec}$NOS	endothelial constitutive nitric oxide synthase
EDCF	endothelium-derived contracting factor
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
ERPF	effective renal plasma flow
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFR	glomerular filtration rate
GTP	guanosine triphosphate
h	hour
^{125}I	radioactive isotope of iodine

INDO	indomethacin
iNOS	inducible nitric oxide synthase
K^+	potassium
KCl	potassium chloride
kDa	kilodaltons
kPa	kilopascals
KPSS	physiological saline solution with substitution of KCl for NaCl
l	litre
L-NAME	N^{ω}-nitro-L-arginine methyl ester
L-NMMA	N^{G}-monomethyl-L-arginine
LPS	lipopolysaccharide
M	molar concentration
MAP	mean arterial pressure
mg	milligram
µg	microgram
µl	microlitre
min	minutes
µM	micromolar
mM	millimolar
ml	millilitre
ml/min	millilitre per minute
mmol/L	millimol per litre
mN/mm	millinewton per millimetre
mRNA	messenger ribonucleic acid
NA	noradrenaline
NAK	noradrenaline in potassium
nmol/L	nanomol per litre
NO	nitric oxide
NOx	nitrite and nitrate
nNOS	neuronal nitric oxide synthase
NP	nonpregnant
O_2^-	superoxide anion
P	pregnant
PE	phenylephrine

pEC_{50}	the negative logarithm to base 10 of the EC_{50}
PI 3-K/Akt	phosphoinositide 3-kinase/Akt signalling pathway
PIP_2	PI (4,5) bis-phosphate
PGI_2	prostacyclin
PLC	phospholipase C
pmol/L	picomol per litre
PREG pregnant	
PSS	physiological saline solution
RIA	radioimmunoassay
RT-PCR	reverse transcription-polymerase chain reaction
SDMA	symmetric dimethylarginine
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sGC	soluble guanylate cyclase
SNGFR	single nephron glomerular filtration rate
SPN	spermine NONOate
TGs	triacylglycerols
TXs	thromboxanes
$U_{cGMP}V$	urinary excretion of cGMP
U_{CR}	urinary creatinine
$U_{CR}V$	urinary excretion of creatinine
U_{NOx}	urinary nitrite and nitrate
$U_{NOx}V$	urinary excretion rate of nitrite and nitrate
VEGF vascular endothelial growth factor	
VSMCs	vascular smooth muscle cells
w/v	weight/volume

CHAPTER 1
INTRODUCTION

1.1 Cardiovascular and renal changes during pregnancy in humans

Pregnancy is accompanied by haemodynamic and body fluid alterations that are believed to be initiated through peripheral arterial vasodilatation. Arterial vasodilatation leads to decreased blood pressure and enhanced cardiac output in association with decreased systemic and pulmonary vascular resistance during the first trimester (Duvekot and Peeters, 1994; Clapp and Capeless, 1997), activation of the renin-angiotensin-aldosterone system, and renal sodium and water retention (Schrier and Briner, 1991). The haemodynamic changes found in human pregnancy have been proposed to be primarily due to systemic vasodilatation (Schrier and Briner, 1991) similar to other sodium and water retaining states (Abraham and Schrier, 1997). The peripheral vasodilatation found early in human gestation is also seen in the baboon species prior to volume expansion (Phippard *et al*, 1986).

The most obvious adaptive change to the state of pregnancy is the rise in blood volume, which increases approximately 10% by the twelfth week of gestation; at term, the intravascular volume exceeds the nonpregnant value by 45-55%, having reached a plateau at approximately 34 weeks (Rovinsky and Jaffin, 1965). This is attributable to both an increase in plasma volume of the order of 1250 ml above nonpregnant levels (Hytten and Paintin, 1963) and in erythrocyte mass (Pritchard and Adams, 1960), but the increase in plasma volume is proportionately greater and, therefore, leads to a fall in haematocrit. That this particular haemodynamic adaptation is important for pregnancy is emphasized by the high incidence of defective plasma volume expansion in pregnancies complicated by preeclampsia or poor fetal growth (Pirani *et al*, 1973).

The rise in blood volume is associated with an increase in cardiac output of

about 40% during the first trimester, largely due to increases in both stroke volume (35%) and heart rate (15%) (Robson *et al*, 1989). By the second trimester, increments in cardiac output of approximately 30% to 60% can be demonstrated, whereas during the final weeks of pregnancy cardiac output may decrease (Robson *et al*, 1989).

Maternal blood pressure decreases in normal pregnancy. There is a small fall in systolic arterial blood pressure and a more marked decrease in diastolic blood pressure, reaching a nadir at around 20 weeks of gestation, with a progressive increase thereafter (Robson *et al*, 1989).

The lowering of the blood pressure is achieved by a substantial reduction in the peripheral vascular resistance. The fall in peripheral vascular resistance reaches a nadir at 20 weeks when values are 34% below those calculated prior to conception (Robson *et al*, 1989). As a result of maternal vasodilatation, peripheral blood flow increases significantly. The uterus can be regarded as the central target of the increased circulation of pregnancy. The blood supply to the uterus increases remarkably from 50 ml/min in early pregnancy to approximately 500 ml/min at term (Assali, 1989). This dramatic rise in flow maintains normal oxygen and nutrient delivery to the fetoplacental unit.

The changes in systemic haemodynamics are detectable by 6-week of gestation in pregnant women (Chapman *et al*, 1998). The adaptive changes in maternal haemodynamics have been proposed to precede changes in renal function and the related changes in volume homeostasis in early human pregnancy (Duvekot *et al*, 1993). Duvekot and coworkers (1993) reported that by the fifth week of human pregnancy, an overall fall in systemic vascular tone is the very first adaptive change of the cardiovascular system, giving rise to both an increased vascular capacity and a decreased filling state. The decrease in systemic vascular tone results in a rapid fall in preload and afterload, leading to a compensatory increase in the heart rate. Subsequently, cardiac output increases because of a rise in stroke volume, which develops because the vascular filling state normalizes. The increased vascular capacity of the uterus, placenta, and other enlarged organs of the woman's body increases the blood volume (see below) to fill the extra vascular capacity. Duvekot *et al*, (1993) found a

biphasic rise in heart rate in the first trimester. They found the increase in the heart rate to be evident by the fifth week of gestation, when values are approximately 15% above those found prior to conception (from 66 to 76 beats/min), changing a little between five and eight weeks, and resuming its increase after the eighth week to a maximum of about 85 beats/min in the thirty-fifth week, a total rise that is comparable with data reported by others (Clapp *et al*, 1988, Robson *et al*, 1989). The rise in the heart rate by the fifth week of gestation, and the increase in stroke volume between the fifth and eighth weeks of gestation, together with a rise in the fractional contribution of the stroke volume to the concomitant increase in cardiac output between week five and eight of pregnancy, is indicative of a rise in the vascular filling state in this period (Duvekot *et al*, 1993).

The renal vascular bed participates in the vasodilator response to pregnancy. A significant increase in both effective renal plasma flow (ERPF) and glomerular filtration rate (GFR) is evident as early as 6-week gestation in pregnant women, with maximal increases of 40-80% above preconception values during the late first or early second trimester (Conrad and Lindheimer, 1999). As mentioned above, the cardiac output increases progressively from the first trimester and starts to decrease in the third trimester of a normal pregnancy. Recent evidence suggests that the maximal increase of GFR precedes the maximal increase of cardiac output because the GFR increase is caused by maximal renal vasodilatation (Varga *et al*, 2000). The GFR increases in parallel with changes in renal plasma flow and to a lesser extent, thus resulting in a decrease in filtration fraction early in pregnancy. The increase in renal plasma flow during pregnancy occurs prior to maximal plasma volume expansion. In pregnant women, glomerular hyperfiltration persists throughout pregnancy.

1.2 Cardiovascular and renal adaptations in rats

Cardiovascular and renal changes during rat pregnancy, such as increased uterine blood flow, decreased mean arterial pressure and increased GFR are similar to those observed in pregnant women. In the rat, blood volume increases approximately 60% above that of nonpregnant animals by term (Barron et *al*, 1984). Cardiac output increases about 40% above the nonpregnant levels in late-pregnant animals, a significant portion of which is received by the uterine circulation at term (0.5% of cardiac output in nonpregnant *versus* 25% of cardiac output in late-pregnant animals (Bruce, 1976). Thus, uterine blood flow is enhanced substantially, in some cases as much as 100-fold during gestation. This dramatic alteration in the local blood flow can only be achieved by a profound decrease in vascular resistance, because blood pressure does not increase and is, in fact, lower at term (Bruce, 1976). The peripheral vasodilatation found early in human gestation is also seen in pseudopregnant rats where vaginal stimulation results in alterations in maternal hormonal responses identical to normal pregnancy in the absence of a fetal-placental unit (Baylis, 1982).

In chronically instrumented conscious rats, a significant increase in both ERPF and GFR is evident by gestational day six with a peak of 20-40% above preconception values during gestational days 12-16 (Conrad, 1984). (Rat gestation lasts 22 days). Micropuncture studies of changes in glomerular filtration and renal plasma flow in the rat revealed that glomerular filtration rate and renal plasma flow were elevated by approximately 26% and 30%, respectively, above nonpregnant controls on the twelfth day of gestation (Baylis, 1980). Conrad (1984) demonstrated a significant rise in the GFR and ERPF as early as day six of gestation achieving peak values at 11 to 15 days of gestation. These data agree in part with data from Baylis in which whole kidney GFR and single nephron glomerular filtration rate (SNGFR) were found to be elevated in anaesthetised pregnant and pseudopregnant Munich-Wistar rats on gestational day nine (Baylis, 1982) and day 12 (Baylis, 1980). Total renal plasma flow rate and single glomerular plasma flow rate were also elevated in 9-day pregnant and

pseudopregnant rats compared to virgins (Baylis, 1982). Conrad's data (1984) confirms the studies of Atherton and Pirie, performed in anaesthetised, acutely prepared rats infused with isotonic saline. These authors reported that GFR was significantly elevated above control levels during early (gestational days five to 7) and mid, but not late gestation (Atherton and Pirie, 1981). Thus, while many workers have reported increased GFR relatively early in gestation, at days five to seven (Atherton *et al*, 1982; Conrad, 1984), days nine to 16 (Baylis, 1980; Atherton *et al*, 1982), others have reported a significant rise in GFR only during late gestation, days 17-22 (Garland and Green, 1982; Davison and Lindheimer, 1980). The inconsistencies in the literature may have arisen from the variable effects of anaesthesia and acute surgery upon renal haemodynamics. The interventions of surgery and anaesthesia have been shown to reduce plasma volume in rats. Although the maximal rise of renal haemodynamics achieved during midgestation in rats (25-30%) is not as great as that seen in humans (40-60%) (Davison and Dunlop, 1980), the peak occurs at a similar stage of pregnancy in both species.

1.3 The effects of dietary changes on maternal cardiovascular adaptations in pregnant rats

The maternal cardiovascular adaptations to pregnancy in the rat include significant increases in plasma volume and cardiac output (Rosso and Streeter, 1979). In the rat, increases in plasma volume are observed between days 12 and 21 (Knopp *et al*, 1975), or even as early as day five (Atherton *et al*, 1982). The increase in maternal plasma volume, the increases in cardiac output and the fractional distribution of cardiac output to the uterus (Ahokas *et al*, 1983a) serve to establish and maintain a high uteroplacental blood flow that permits adequate fetal growth.

Previous studies have demonstrated that pregnant rats fed 50% of their normal daily food intake from day five of gestation had a marked reduction (50%) in cardiac output compared with rats fed *ad libitum* a standard diet (Rosso and Kava, 1980). In the same study, Rosso and Kava reported no change in the fraction of the cardiac output

distributed to the uterus, whilst Ahokas and coworkers (1983a) observed that both the total cardiac output and the fraction of the cardiac output delivered to the uterus were decreased by 30% and 40% respectively in the 50% diet-restricted pregnant rats.

Dietary restriction, whether is imposed during the last week of gestation only or throughout gestation, has been reported to cause an inadequate expansion of the maternal cardiac output. Ahokas *et al* (1983b) measured cardiac output in pregnant rats fed ad libitum, fed a 50% restricted diet from day 14 of gestation on, and fed a 50% restricted diet from day five of gestation on. Cardiac output in both groups of diet-restricted dams was 30% lower than that of the *ad libitum*-fed pregnant dams, and not significantly increased above that of the nonpregnant rats. In another study, Ahokas *et al*, (1984) measured the distribution of cardiac output to other maternal organs in *ad libitum*-fed or 50% diet-restricted, term pregnant rats, and in *ad libitum*-fed nonpregnant rats. They found that the diet-restricted dams had a 33% lower total cardiac output than the *ad libitum*-fed dams, but it was not significantly different from that of the nonpregnant rats (Ahokas *et al*, 1984). In the above studies the technique of radioactively labelled microspheres was used to measure cardiac output and the rats were anaesthetized.

It has also been shown that pregnant rats fed 50% of their normal daily food intake or fed 6% casein *ad libitum* (protein restricted) throughout gestation had significantly lower plasma volume at term compared with rats fed *ad libitum* a standard diet or fed 25% casein *ad libitum*, respectively (Rosso and Streeter, 1979). A similar trend is apparent in the work of Lederman and Rosso (1989) where 55% food restricted or 6% casein-fed pregnant rats had significantly lower plasma volume on day 19 compared with well-nourished pregnant rats (25% casein). The decrease in plasma volume demonstrated at term in undernourished rats (Rosso and Streeter, 1979), has been reported to be manifested earlier in pregnancy as well (Lederman and Rosso, 1989). Low protein-fed pregnant rats (6% casein) had lower plasma volume than food-restricted rats on days 12, 19 and 21 of gestation (Lederman and Rosso, 1989). The pattern of plasma volume change, with maximum mean values on day 19, (compared to

days 5, 12, 19 and 21 of gestation) was not altered by undernutrition (Lederman and Rosso, 1989).

Fasting between days 17 and 19 of pregnancy in the rat has been shown to result in a reduction in the total body water in both *ad libitum*-fed and 50% food-restricted pregnant rats (Lederman and Rosso, 1981) but in the fasted restricted pregnant rats, total body water fell below that of the fasted *ad libitum*-fed nonpregnant rats and was not significantly different from fasted restricted nonpregnant rats (Lederman and Rosso, 1981). Decreased body water may reflect a decrease in blood volume. As indicated before, food or protein restrictions reduce the blood volume increase that normally occurs in pregnancy (Rosso and Streeter, 1979) and placental blood flow is also reduced (Rosso and Kava, 1980).

In normal pregnancy the maternal uteroplacental vasculature undergoes considerable adaptive remodelling to accommodate the increase in blood flow required to sustain fetal growth (Osol and Cipolla, 1993). Vascular remodelling that results in a larger lumen diameter will directly decrease vascular resistance and will increase uterine blood flow. The enhanced production of endogenous vasodilators from the vessel wall may also decrease vascular resistance and thereby increase uterine blood flow (Magness, 1991).

Dietary restriction to 50% of *ad libitum* intake throughout gestation caused a 40-50% reduction in uteroplacental blood flow, the supply line of nutrients available for placental transfer to the fetus (Rosso and Kava, 1980; Ahokas *et al*, 1983a; Ahokas *et al*, 1986). Uterine and preplacental vascular resistance was 63% and 55% higher in the 50% diet-restricted dams than in *ad libitum*-fed (Ahokas *et al*, 1986). Myometrial blood flow in the 50% diet-restricted rats was lower than that in *ad libitum*-fed (Ahokas *et al*, 1986). The lower uteroplacental blood flow rate is the result of a decreased cardiac output, and a decreased fraction of the cardiac output delivered to the uterus (Ahokas *et al*, 1983a). The percentage of cardiac output distributed to the maternal hepatic-portal circulation was increased, while that to other organs was maintained (Ahokas *et al*, 1984). The increase in the percentage of cardiac output to the

hepatic-portal circulation is probably a protective mechanism to ensure a continued supply of glucose when exogenous dietary supplies are lacking. Increased hepatic gluconeogenesis has been observed in fasting pregnant rats (Metzger *et al*, 1971) and sheep (Morriss *et al*, 1980). Ahokas *et al* (1986) provided evidence that the reduced uteroplacental blood flow associated with diet restriction was the result of increased uteroplacental α-adrenergic vasomotor tone. Fasting during the last four days of pregnancy in the rat has been shown to cause a fourfold increase in the urinary epinephrine and norepinephrine excretion rates (Young and Landsberg, 1979). When dietary nutrients are in short supply, the maternal metabolism, rather than the developing fetus is protected. During periods of malnutrition the cardiac output in the mother was redistributed to maximise the mother's own use of available nutrients and to protect her against severe depletion of nutrient stores by the developing fetus.

1.4 The mesenteric circulation in rats

The studies described in Chapters 4 and 5 of this thesis were performed using resistance-sized mesenteric arteries from virgin and late pregnant (18-19 days) Wistar rats. These vessels were chosen for study because the splanchnic circulation receives a significant portion of the cardiac output and is a major determinant of total peripheral vascular resistance (Christensen and Mulvany, 1993). The systemic resistance vasculature of the rat is remodelled during pregnancy as evidenced by significant alterations in the passive mechanical properties and extracellular matrix proteins in mesenteric arteries. Mackey *et al*, (1992) reported that compared with mesenteric arteries of nonpregnant rats, arteries of 20-day pregnant rats exhibited decreased distensibility and decreased hydroxyproline/leucine (index of collagen) and desmosine/leucine (elastin concentration). Arteries of pregnant rats were less stiff by late gestation. The significant changes in passive mechanics and in extracellular protein content support the notion that arterial wall remodelling in the peripheral vasculature may be one component of the cardiovascular adaptations during pregnancy.

1.4.1. Small artery physiology

The rapid flow of blood throughout the body is produced by pressures created by the pumping action of the heart. Once the heart has ejected an amount of blood, that blood has to be channelled into the immediate area of cells in the body for effective exchange of O_2, CO_2, nutrients, waste products and hormones. The circulatory system is a closed system of different types of blood vessels which serve as the transport conduit for the blood (Fig. 1.1).

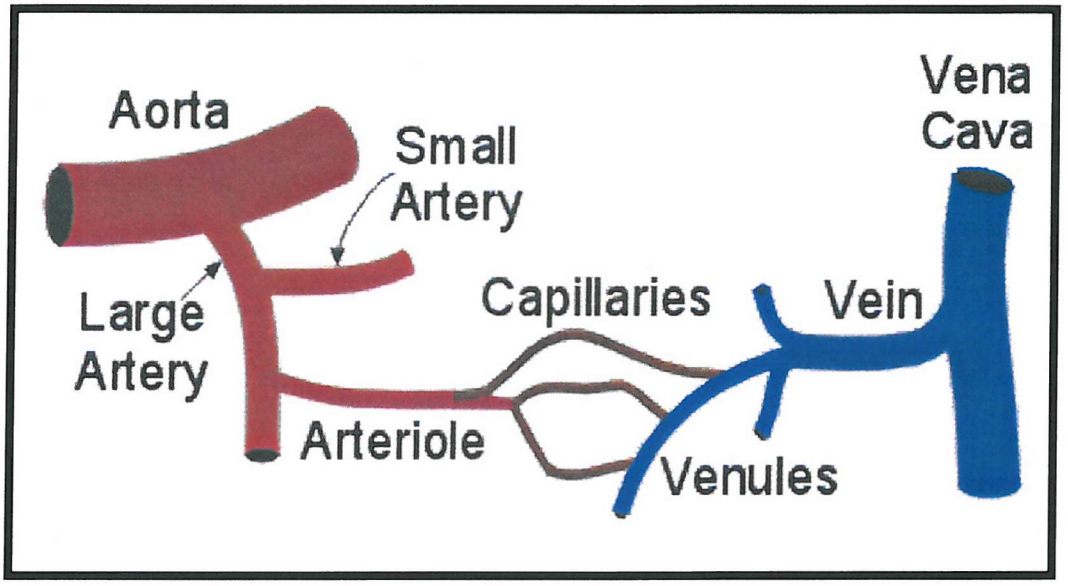


Fig. 1.1.: The vascular network.

The arteries of the systemic circulation branch off the aorta, dividing into progressively smaller vessels. The smallest arteries branch into arterioles, which are not elastic but are the major sites of resistance to flow and are responsible for the pattern of blood flow distribution to the various organs. Together, the small arteries and the arterioles represent the primary vessels that are involved in the regulation of arterial blood pressure. The arterioles branch into a huge number (estimated at 10 billion) of very small vessels, the capillaries, which unite to form larger diameter vessels, the venules. The arterioles, capillaries and venules are termed the microcirculation. The

venules in the systemic circulation then unite to form larger vessels, the veins, which serve as low-resistance conduits for transport of blood from the tissues back to the heart (Fig. 1.1).

The main role of the precapillary vessels within the cardiovascular system is to distribute the blood in such a way that each capillary is provided with blood in the correct amount, and at the correct pressure which allows appropriate diffusion across the capillaries. This role is attained through the ability of the cardiovascular system to change the resistance of each vessel by controlling its lumen diameter. The lumen diameter is, in turn, a function of the amount and arrangement of the vascular wall materials, and, in particular, of the level of tone of the vascular smooth muscle (Mulvany and Aalkjær, 1990). The vessels that contribute substantially to the precapillary resistance are the small resistance arteries which are prearteriolar vessels with diameters in the region of 50-500 μm (Mulvany and Aalkjær, 1990).

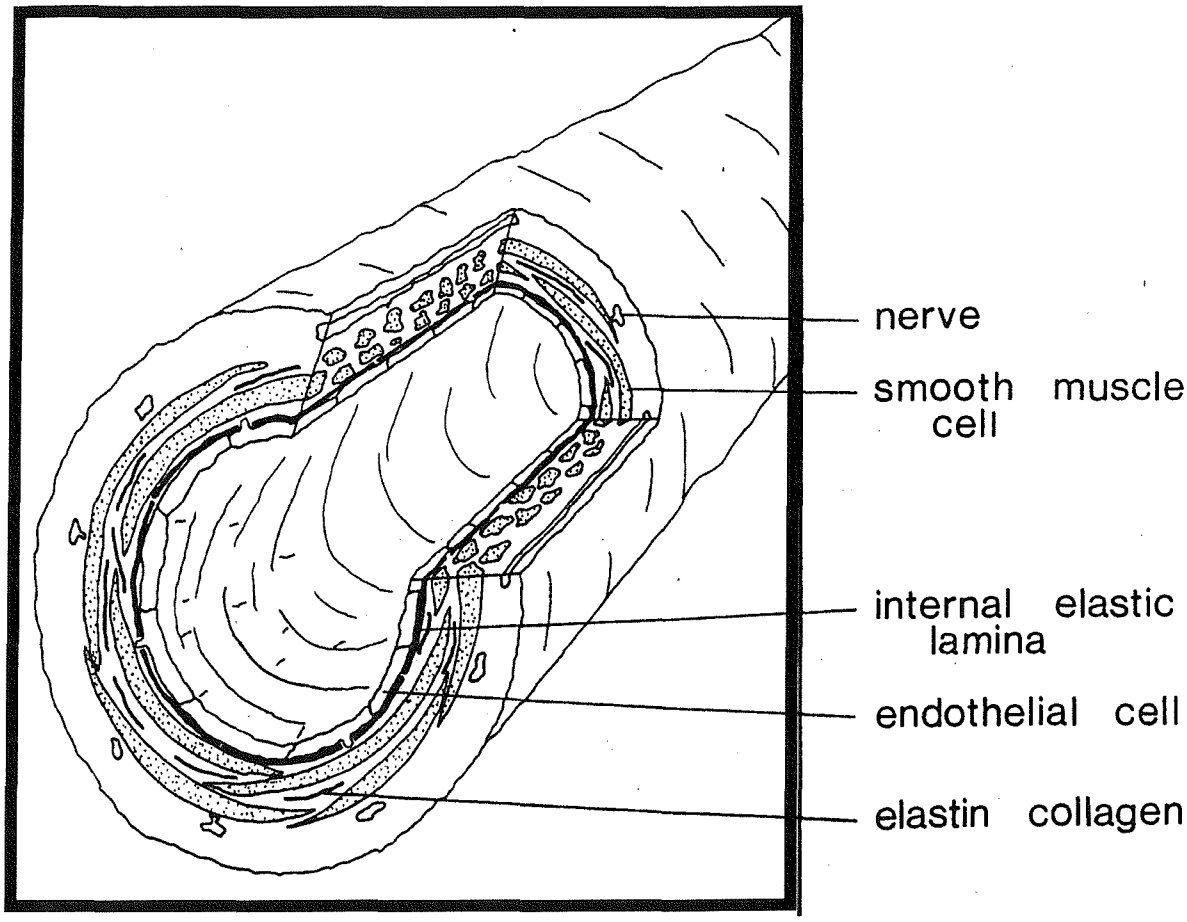


Figure 1.2.: General architecture of a small artery. (Mulvany and Aalkjaer, Physiol. Rev. 70: 921-961; 1990).

1.4.2 Small artery structure

The vascular wall of small arteries, similar to other blood vessels, consists of three major layers, an inner tunica intima, a middle tunica media and an outer tunica adventitia (Fig. 1.2). The tunica intima is composed of two primary layers. The inner layer consists of endothelium which serves as a physical lining of the vessel to which blood cells do not normally adhere. The outer layer of the tunica intima is composed of connective tissue containing elastic and collagen fibres. The tunica media of small arteries, as in larger arteries, is bounded on the luminal side by a well-defined internal elastic lamina, which separates the media from the intima, but the external elastic lamina

is fragmented or absent, and there are no elastic laminae within the media (Lee *et al*, 1983; Mulvany and Aalkjær, 1990). Typically, a small resistance artery will have 2-3 layers of smooth muscle cells arranged circumferentially around the lumen in the tunica media. The smooth muscle cells are electrically coupled by gap junctions in a syncytium (Hirst *et al*, 1997) and are confined in an extracellular matrix of connective tissue proteins such as collagen, elastin and a number of other glycoproteins. The adventitia of the small arteries, as in larger arteries, contains connective tissue (elastin and collagen), fibroblasts, mast cells, macrophages, and occasional Schwann cells with associated nerve axons (Lee *et al*, 1983).

1.4.3 Vascular resistance

Resistance (R) is the measurement of hindrance to blood flow through a vessel due to friction between the blood and the vessel walls. Resistance to blood flow within a vascular network is determined by the size of individual vessels (length and diameter), the organization of the vascular network (series and parallel arrangements), physical characteristics of the blood (viscosity) and extravascular mechanical forces acting upon the vasculature.

Of the above factors, changes in the vessel diameter are most important quantitatively for regulating blood flow within an organ, as well as for regulating arterial pressure. Changes in vessel diameter, particularly in small arteries and arterioles, enable organs to adjust their own blood flow to meet the metabolic requirements of the tissue. Therefore, if an organ needs to adjust its blood flow, cells surrounding these blood vessels release vasoactive substances that can either constrict or dilate the resistance vessels.

Poiseuille established that the resistance (R) to a steady laminar flow (Q) of blood within a blood vessel is directly proportional to the viscosity (η) of the blood and to the length (L) of the vessel. It is inversely proportional to the fourth power of the

vessel's radius (r^4), which is the major variable controlling changes in resistance. The following equation defines the contributions of these three determinants:

$$R = (\eta L/r^4)\ (8/\pi)$$

where $8/\pi$ is a constant.

By combining this definition of resistance with Ohm's law of flow ($Q = \Delta P/R$) in which Q is the blood flow, ΔP is the pressure difference (P_1-P_2) between the two ends of the vessel, and R is the resistance, one can derive the following formula, known as Poiseuille's law:

$$Q = \frac{\Delta P \pi r^4}{8\eta L}$$

This equation describes the crucial interrelationships between blood flow (Q), blood pressure gradient (ΔP) and vascular resistance (R) and features the important point that small changes in the vessel radius or small changes in the vascular smooth muscle tone will produce large changes in intraluminal blood flow.

1.4.4 Control of vascular tone (Figure 1.3).

The active tension exerted by vascular smooth muscle in a segment of wall is called vessel 'tone'. All resistance and capacitance vessels under basal conditions retain some degree of contraction that determines the diameter, and hence tone, of the vessel. Basal vascular tone is different among organs. Those organs having the capacity to produce large increases in blood flow such as skeletal muscle and the splanchnic circulation, have a high basal vascular tone whereas organs having low vasodilatory capacity have a low vascular tone. Vascular tone is determined by many different competing vasoconstrictor influences that tend to depolarize the cells and open their calcium channels, and vasodilator influences such as tonic secretion of nitric oxide by endothelial cells. These influences can be separated into intrinsic factors that originate

from the vessel itself or the surrounding tissue and extrinsic factors that originate from outside of the organ or tissue where the blood vessel is located. Intrinsic factors include physical factors (temperature, pressure), the myogenic response, locally secreted hormones and chemical substances such as arachidonic acid metabolites, histamine, and bradykinin which can either increase or decrease tone, and endothelial secretions such as nitric oxide and endothelin, which can either increase or decrease tone. The extrinsic factors are the autonomic nerves and circulating endocrine secretions which serve the function of regulating arterial blood pressure by altering systemic vascular resistance. They increase the vascular tone and cause vasoconstriction (Fig. 1.3).

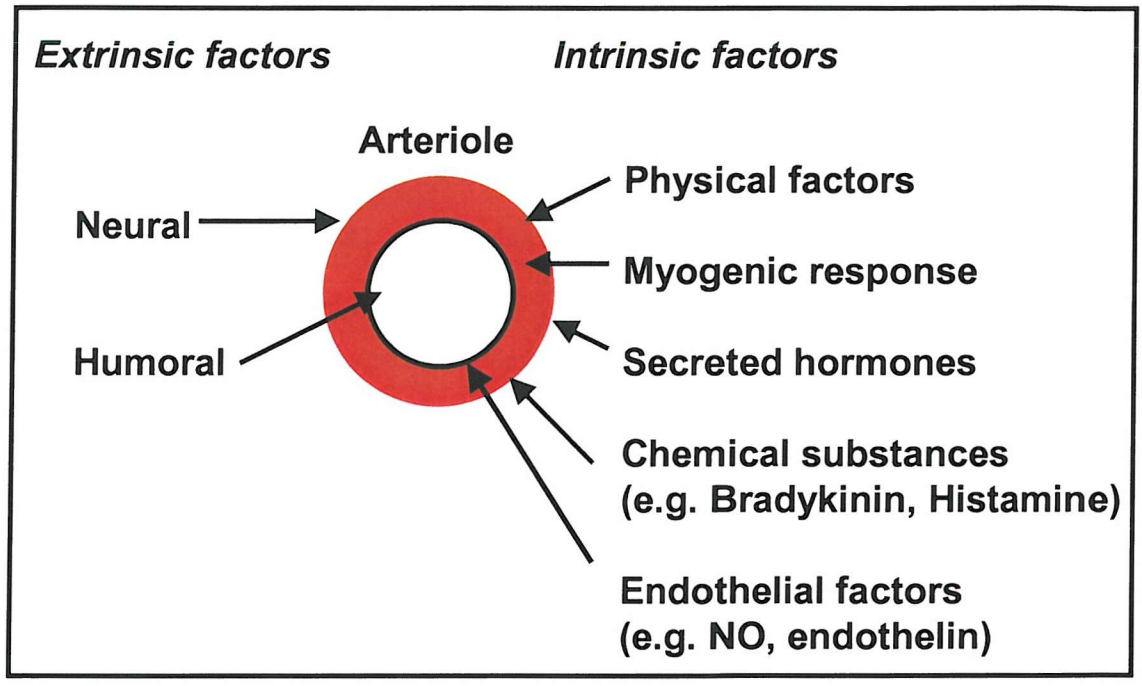


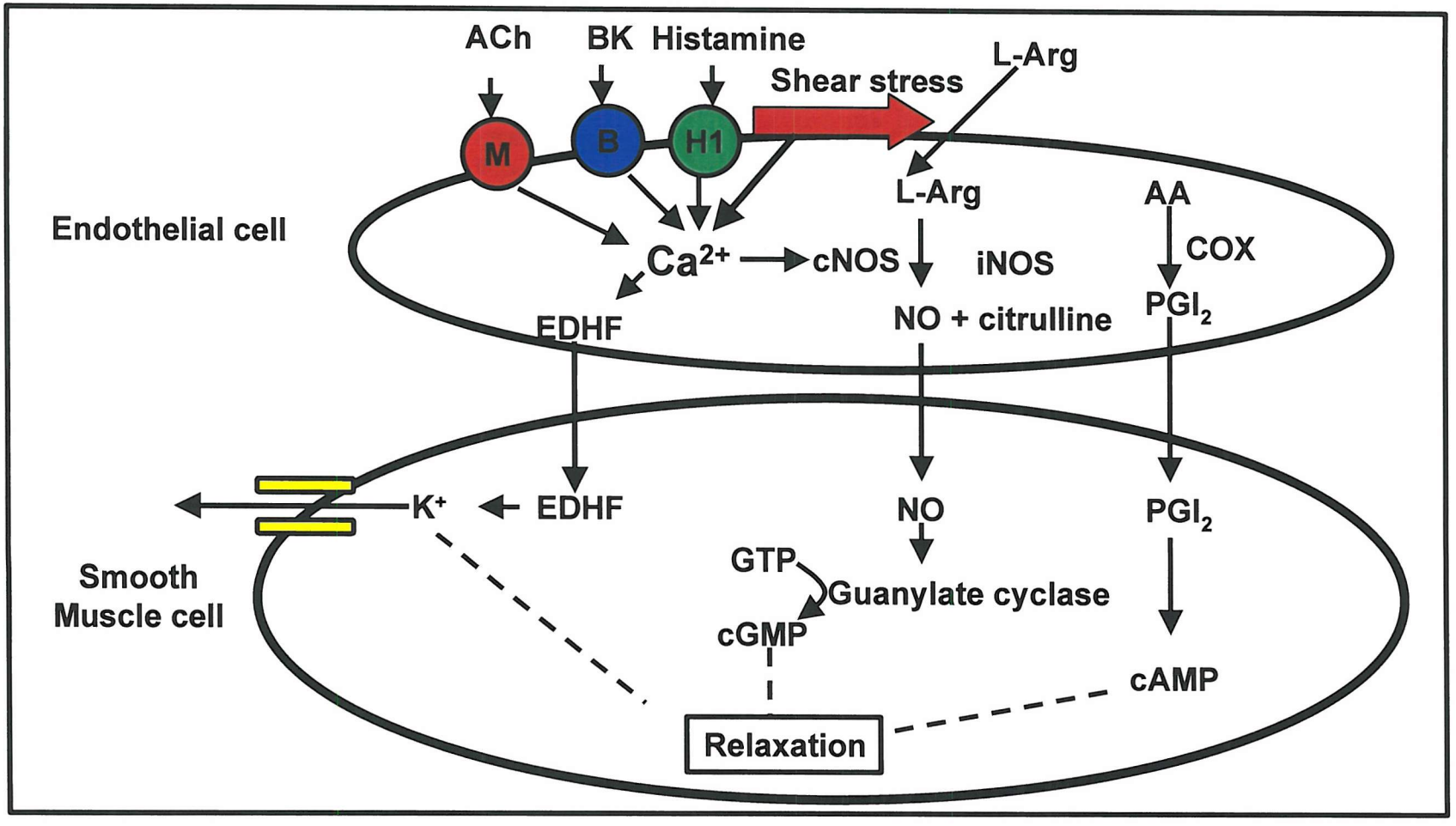
Figure 1.3.: Control of vascular tone.

1.5 Endothelial control of vascular tone in pregnancy

The endothelium is a common structural component of the entire cardiovascular system and consists of a smooth, single-celled layer of cells which line the inner surface of the vessels. Capillaries consist only of endothelium. The endothelium was first described several hundred years ago, and until relatively recently was thought to be a passive barrier between circulating blood and the underlying vascular smooth muscle, but it is now known to have a large number of active functions. Endothelial cells regulate the transport of macromolecules (e.g. proteins, nucleic acids and polysaccharides) and other substances between plasma and interstitial fluid, mediate angiogenesis, play a central role in vascular remodeling, synthesize active hormones from inactive precursors, regulate leukocyte and platelet adhesion and platelet activation, secretion and aggregation (Vander *et al*, 1998).

The vascular endothelium plays a prominent role in the homeostatic regulation of vascular tone by secreting various biologically active substances that act on adjacent vascular smooth muscle cells; these include vasodilators prostacyclin (PGI_2; see section 1.5.1: *Vasodilatory prostaglandins*), NO (see section 1.5.2: *Nitric oxide*), the endothelium-derived hyperpolarizing factor (EDHF) (Figure 1.4.) [see section 1.5.3: *Endothelium-derived hyperpolarizing factor (EDHF)*] and vasoconstrictors endothelin-1, platelet-activating factor (PAF) and angiotensin. The experiments performed in this thesis to investigate vascular function examined agonist-mediated events *in vitro* but there are also methods that utilize flow to induce shear stress-mediated release of endothelium-dependent vasodilators (see *Flow-mediated responses*).

Figure 1.4.: Endothelium-derived mediators of vasorelaxation. Schematic diagram of the principal pathways leading to formation and release of vasoactive mediators in endothelial cells such as nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF), in response to agonist-receptor binding or shear stress: ACh acetylcholine, BK bradykinin. M, B, H1, refers to receptors for acetylcholine, bradykinin, histamine respectively. The vasoactive substances released from the endothelium act on the adjacent vascular smooth muscle cells to evoke relaxation.


ACh
BK
Histamine
Shear stress
L-Arg
M
B
H1
L-Arg
AA
Endothelial cell
Ca2+
cNOS
iNOS
COX
EDHF
NO + citrulline
PGI2
K+
EDHF
NO
PGI2
Smooth
Muscle cell
GTP
Guanylate cyclase
cGMP
cAMP
Relaxation

1.5.1 Vasodilatory prostaglandins

Endothelium, smooth muscle, leukocytes, platelets are capable of producing various vasoactive substances that are products of arachidonic acid (AA) metabolism. Among these are prostaglandins (PGE_2, $PGF_{2\alpha}$), PGI_2, thromboxanes (TXs) and leukotrienes. Membrane phospholipids, acted upon by phospholipase A_2 or C, form AA which serves as a precursor for prostaglandins, PGI_2, and thromboxanes, which are derived from the cyclooxygenase (COX) pathway. COX is an enzyme that exists in two forms: COX-1 and COX-2. COX-1 is found in most cells as a constitutive enzyme (i.e. it is always present) and it is thought that the prostanoids it produces are involved in normal homeostasis (e.g. regulating vascular responses and coordinating the actions of circulating hormones). COX-2 is induced in inflammatory cells by inflammatory stimuli. COX acts on AA to produce cyclic endoperoxides (PGG_2, PGH_2). These can then be acted upon by prostacyclin synthase in the endothelium to form PGI_2. ACh-induced relaxation releases both constrictor and dilator prostanoids to different extents in different tissues, synthesis of which can be blocked by a non-specific COX inhibitor e.g. indomethacin. AA can also be metabolised by 5-lipoxygenase, primarily in leukocytes, to give rise to various leukotrienes.

PGI_2 and prostaglandin E_2 (PGE_2) are powerful vasodilators. Their synthesis is induced by humoral and mechanical stimuli via discrete regulated pathways (Meade *et al*, 1996). Once formed, PGI_2 has relatively short half life, one of the features that limits the magnitude of its signals and exerts control over its biologic activity (Campbell and Halushka, 1996). PGI_2 acts on IP-receptors present on vascular smooth muscle to cause vasodilatation. PGE_2 acts on EP_2-receptors to cause relaxation of vascular smooth muscle. PGI_2 and PGE_2 cause vasodilatation by the stimulation of adenylate cyclase which increases cAMP and decreases smooth muscle Ca^{2+}. Although IP-receptors are present in the arterial vascular wall, PGI_2 is not constitutively produced and does not appear to regulate basal systemic vascular tone. Rather, PGI_2 synthesis is induced at sites of vascular perturbation, where it may regulate vasoconstriction (Topper *et al*, 1996).

Vasodilator prostaglandins are unlikely to mediate the haemodynamic changes of rat pregnancy except during certain unphysiological circumstances (Conrad and Colpoys, 1986; Danielson and Conrad, 1995), since administration of the COX inhibitor indomethacin, did not increase blood pressure. In an early study in humans, measurement of circulating concentrations of 6-oxo-$PGF_{1\alpha}$ in the maternal plasma revealed that its levels are too low for PGI_2 to function as a circulating hormone in this situation, although there was a significant upward trend during pregnancy (Barrow *et al*, 1983). PGI_2 biosynthesis may be increased locally in the uterine and renal circulations during pregnancy, or in the placenta, perhaps in response to increased circulating levels of angiotensin II (Magness *et al*, 1992) as intravenous angiotensin II caused a concentration-dependent increase in the uterine arteriovenous difference of 6-oxo-$PGF_{1\alpha}$ in pregnant ewes.

1.5.2 Nitric oxide

Furchgott and Zawadzki (1980) demonstrated that acetylcholine could cause relaxation of strips of rabbit aorta precontracted with noradrenaline only in the presence but not in the absence of endothelial cells, thus providing evidence of the existence of an Endothelium-Derived Relaxing Factor (EDRF). Palmer *et al*, (1987) showed that precontracted bioassay strips, denuded of endothelium, relaxed when exposed to isolated cultured endothelial cells. It was also shown that circulating and local hormones such as bradykinin, histamine and serotonin as well as substances such as ADP and ATP, growth factors such as platelet derived growth factor, could stimulate the release of this relaxing factor. The above observations and further work led to conclusion that the endothelial cell layer is the source of the endothelium derived relaxing factor. The identification of EDRF as nitric oxide by using a sensitive chemiluminescent method (Palmer *et al*, 1987), has led to a better understanding of the role of the vascular endothelium in regulating vascular tone.

NO is constantly formed by the intact endothelium through the conversion of L-Arginine to L-Citrulline in a five-electron oxidation reaction catalyzed by a group of enzymes, nitric oxide synthases (NOS). There are two general forms of NOS: constitutive and inducible (see section 1.5.2.1). NO is continuously produced by constitutive NOS (cNOS). The activity of cNOS is modulated by calcium that is released from subsarcolemmal storage sites in response to the binding of certain ligands to their receptors. Substances such as ACh, BK, histamine, insulin, and substance P regulate NO production by this mechanism. The EDRF/NO system is tonically active in resistance vessels under basal conditions, providing a physiological vasodilator mechanism that influences peripheral vascular resistance and hence systemic blood pressure (Rang *et al*, 1999). NO diffuses out of the endothelial cell into the adjacent smooth muscle cells where it binds to the haeme moiety on guanylyl cyclase and activates this enzyme to produce cGMP from the triphosphate GTP. cGMP activates cGMP-dependent protein kinase that subsequently leads to the inhibition of calcium influx into the smooth muscle cell, and decreased calcium-calmodulin stimulation of myosin light chain kinase. This in turn causes dephosphorylation of myosin light chains, resulting in relaxation of smooth muscle and consequent vasodilatation.

1.5.2.1 Nitric Oxide Synthase (NOS) isoforms

Endothelium-derived nitrogen monoxide (nitric oxide, NO) is an important component of vascular homeostasis through regulation of vascular tone (Lefroy *et al*, 1993) arterial pressure (Rees *et al*, 1989), platelet and leukocyte adhesion to the endothelial cell surface and vascular smooth muscle cell proliferation (Garg and Hassid, 1989). NO is cleaved from the terminal guanidino nitrogen of L-arginine (Schmidt *et al*, 1988) by NO synthases (NOS) in a complex reaction requiring molecular oxygen, reducing equivalents from NADPH as cosubstrates, tetrahydrobiopterin (BH_4), a cytochrome P-450-type heme moiety, calmodulin, flavin adenine dinucleotide (FAD)

and flavin mononucleotide (FMN) (Bredt and Snyder, 1994). To date, all sequenced NOS complementary DNAs fall into the cytochrome P-450 reductase-like family. NO synthases in the cytochrome P-450 reductase-like family are of three known types. Two constitutive NO synthases were termed cNOSs to reflect their strict dependency on intracellular calmodulin and Ca^{2+} levels, produce 'puffs' of NO for signalling purposes and are expressed in a limited tissue distribution. One cNOS, endothelial cNOS ($_{ec}$NOS), is expressed in endothelium, neurons, cardiac myocytes and certain subsets of respiratory epithelial cells. The other is found in central and peripheral neurons, neuroblastomas, platelets, skeletal muscle, epithelial cells of bronchioli, alveoli, uterus and stomach (Nathan and Xie, 1994). It is called neuronal cNOS ($_{nc}$NOS). The isoforms differ in subunit molecular mass (133,000 for purified $_{ec}$NOS, 162,000 for $_{nc}$NOS). In contrast to these isozymes, the third isoform is inducible (iNOS) and is expressed in virtually all nucleated cells subjected to immunologic or certain nonimmunologic stimuli. Once expressed, iNOS remains tonically activated producing large amounts of NO for cytoactive purposes. Although its activity was originally believed to be Ca^{2+}/calmodulin independent, owing to its tight binding to calmodulin, studies with purified, recombinant iNOS protein indicate that iNOS is twice as active in the presence of Ca^{2+} as in its absence (Venema *et al*, 1996).

Highly homologous complementary DNAs encoding $_{ec}$NOS were first cloned from bovine and human endothelial cells and a partial $_{ec}$NOS cDNA cloned from rat kidney has been characterised (Mohaupt *et al*, 1994). The $_{ec}$NOS NH_2 terminus lacks a 220 amino acid domain present in $_{nc}$NOS. The NH_2 terminus of $_{ec}$NOS contains a motif of myristoylation and reversible palmitoylation, modifications unique to $_{ec}$NOS in this gene family that contribute to its membrane association (Robinson *et al*, 1995). These modifications may also represent an additional mechanism of regulation of enzyme activity. Mutagenesis of the sequence gives rise to a mutant cDNA, which, when transfected, yields a product that no longer undergoes myristoylation and is cytosolic (Sessa, 1994). The human $_{ec}$NOS gene on chromosome 7 is organized into 26 exons and spans ~ 21 kb (Marsden *et al*, 1993). $_{ec}$NOS deficient mutant mice are

hypertensive and more susceptible to global cerebral ischemia than wild-type littermates (Huang *et al*, 1995).

All reported $_{nc}$NOS and $_{ec}$NOS isozymes are regulated by Ca^{2+} via calmodulin. Thus Ca^{2+} influx into neural or endothelial cells prompted by acetylcholine, bradykinin or shear stress for example, will stimulate NO biosynthesis. In addition, a host of physiological and pathophysiological stimuli have been shown to regulate the expression, intracellular distribution, or activity of the various NOS isozymes through transcriptional, posttranscriptional, translational, and/or posttranslational mechanisms. In vivo data indicate that $_{ec}$NOS gene expression can be modulated by chronic hypoxia (Shaul *et al*, 1995). The latter study showed upregulation of $_{ec}$NOS expression in the pulmonary vasculature of the chronically hypoxic rat. Endothelial cell culture studies have demonstrated that transforming growth factor-β1 (Inoue *et al*, 1995), protein kinase C (Ohara *et al*, 1995) tumour necrosis factor-α, lysophosphatidylcholine and the degree of cellular proliferation (Arnal *et al*, 1994) all influence the level of $_{ec}$NOS gene expression via changes in the rates of gene transcription and/or mRNA stability. Recent evidence indicates that $_{ec}$NOS is regulated, in part, through the coordinated phosphorylation and dephosphorylation of the amino acid residues Ser-1177 and Thr-495 (human sequence) (Harris *et al*, 2001, Michell *et al*, 2001). For example, growth factors such as vascular endothelial growth factor (VEGF) (Fulton *et al*, 1999), oestrogen (Haynes *et al*, 2000), and the viscous drag generated by the streaming blood on the endothelial layer (shear stress) (Dimmeler *et al*, 1999) activate the phosphoinositide 3-kinase/Akt signalling pathway (PI 3-K/Akt), resulting in promotion of $_{ec}$NOS activity through increased Ser-1177 phosphorylation. Inhibition of the phosphatidylinositol-3-OH kinase/Akt pathway or mutation of the Akt site on $_{ec}$NOS protein (at serine 1177) attenuates the serine phosphorylation and prevents the activation of $_{ec}$NOS. Mimicking the phosphorylation of Ser 1177 directly enhances enzyme activity and alters the sensitivity of the enzyme to Ca^{2+}, rendering its activity maximal at sub-physiological concentrations of Ca^{2+}. Thus, phosphorylation of $_{ec}$NOS by Akt represents a Ca^{2+}-independent regulatory mechanism for activation of $_{ec}$NOS (Dimmeler *et al*,

1999). Studies in porcine aortic endothelial cells have shown that hydrogen peroxide activates $_{ec}$NOS through coordinated phosphorylation and dephosphorylation mediated by PI 3-K activation (Thomas *et al*, 2002).

Most of the cellular $_{ec}$NOS is contained within the Golgi apparatus in cultured endothelial cells as well as in intact blood vessels. At present, no clear suggestion has been presented on the function of the Golgi localisation of $_{ec}$NOS. $_{ec}$NOS is transported together with caveolin-1 to the caveolae at the plasma membrane via vesicles. Caveolae are specialized invaginations of the plasma membrane and are present in most cell types, with the highest numbers being present in endothelial cells, adipocytes, fibroblasts, and smooth muscle cells. This membrane domain harbors many signal transduction pathways, and evidence is accumulating that signal transduction pathways ascending from various stimuli from outside of the cell converge at this specific spot (Liu *et al*, 1997). Within the caveolae, $_{ec}$NOS is bound to caveolin-1, which inhibits $_{ec}$NOS activity. Caveolae-localized $_{ec}$NOS also interacts with the bradykinin receptor and with the cationic amino acid transporter CAT-1. CAT-1 is involved in the transfer of the $_{ec}$NOS substrate arginine across the membrane. Receptors for estrogen (Kim *et al*, 1999) and VEGF as well as a calcium pump are also present in caveolae. The receptors for bradykinin and acetylcholine are not constitutively present in caveolae but may translocate to caveolar membranes on agonist stimulation (De Weerd and Leeb-Lundberg, 1997, Feron *et al*, 1997). These 7-transmembrane-spanning receptors activate G-proteins which in turn act upon phospholipase C_β (PLC_β) in the plasma membrane. Receptor-mediated activation of PLC_β results in the cleavage of PI (4,5) bis-phosphate (PIP_2), forming diacylglycerol (DAG) and inositol (1,4,5) tris-phosphate (IP_3), both of which function as second messengers. IP_3 acts by releasing calcium from intracellular stores. Intracellular calcium is stored in the endoplasmic reticulum. IP_3 binds to a specific receptor present in the membrane of the endoplasmic reticulum, which is coupled to a calcium-selective ion channel. Opening these channels in the endoplasmic reticulum membrane releases a flood of calcium into the cell and raises the free concentration 10- to 100-fold (Berridge 1997). Furthermore, G-protein-coupled receptors activate calcium channels at the

caveolae. This calcium flux induces binding of calmodulin to $_{ec}$NOS, whereas the $_{ec}$NOS-caveolin-1 interaction is disrupted. At the same time, $_{ec}$NOS is depalmitoylated (only the myristoyl moiety remains) and therefore is released from the plasma membrane into the cytosol. On binding of Ca^{2+} to calmodulin, $_{ec}$NOS is stimulated. Binding of a 90-KD heat shock protein (Hsp90) to $_{ec}$NOS in response to histamine, VEGF, or shear stress increases $_{ec}$NOS activity by facilitating the calmodulin-induced displacement of caveolin from $_{ec}$NOS (Gratton *et al*, 2000).

1.5.2.2 Evidence that NO-NOS are increased in rat pregnancy

1.5.2.2.1 Changes in cGMP and in nitrite-nitrate excretion

Results available in the literature strongly suggest that endogenous NO production is increased in pregnant rats. Plasma levels and the urinary excretion rates of guanosine 3', 5'-cyclic monophosphate (cGMP), a cellular mediator of vascular smooth muscle relaxation are increased during rat pregnancy. These findings most probably indicate increased tissue production of cGMP. A metabolic study showed increased entry of cGMP into the plasma compartment, rather than decreased clearance (Conrad and Vernier, 1989). Pseudopregnant rats also exhibit augmented urinary cGMP excretion, suggesting that the proliferative activity that accompanies fetoplacental maturation, as well as placental hormones, are not necessary for the rise in urinary excretion of cGMP. Although these findings are regarded as indirect evidence of increased NO-NOS activity during rat pregnancy and pseudopregnancy, it is worth noting that other mediators, such as atrial natriuretic peptide, may also produce increase of cGMP and vasodilatation during pregnancy.

Conrad *et al* (1993), investigated the plasma concentration and the urinary excretion of the stable nitric oxide metabolites nitrite and nitrate (NOx). The urinary excretion rate and plasma levels of nitrite and nitrate, were elevated in pregnant and pseudopregnant rats, paralleling the rise in urinary cGMP excretion. The increased levels of cGMP and urinary NOx excretion may reflect local increases in NO production in specific vascular beds such as the uterine or renal. This was further substantiated by the demonstration that chronic treatment with the NO synthase inhibitor L-N^{ω}- arginine methyl ester (L-NAME) during pregnancy reduced the gestational increase in urinary NOx excretion (Conrad *et al*, 1993). Nitric oxide haemoglobin was detected by electron paramagnetic resonance spectroscopy in the red blood cells of pregnant rats but was not detected in the red blood cells of nonpregnant rats.

These results demonstrated that endogenous NO production is increased in pregnant rats and may contribute to the systemic arterial vasodilatation in normal pregnancy.

1.5.2.2.2 Nitric oxide synthases during pregnancy

Increases in basal NO production have been reported systemically in pregnant rats (Conrad *et al*, 1993; Conrad and Vernier, 1989; Danielson and Conrad, 1995) and locally in uterine arteries from women (Nelson, *et al*, 1998), guinea pigs (Weiner *et al*, 1994), and sheep (Magness and Rosenfeld, 1993). Several studies provide convincing evidence demonstrating that NOS expression and activity increase during pregnancy. Goetz *et al*, (1994) reported a twofold increase in mRNA transcripts for $_{ec}$NOS in the aorta of pregnant rats. Uterine artery, but not systemic artery endothelial, $_{ec}$NOS protein (Magness *et al*, 1997; Magness *et al*, 2001; Nelson *et al*, 2000;) and mRNA (Bird *et al*, 2000; Magness *et al*, 2001) are elevated by pregnancy. Joyce *et al* (2002) reported increased $_{ec}$NOS protein expression in the smaller uterine artery from midpregnant ewes which was followed by a decreasing expression gradient in late gestation.

Increased basal NOS specific activity has been shown in endothelium intact uterine artery preparations from pregnant women (Nelson *et al*, 2000), guinea pigs (Weiner *et al*, 1994) and sheep (Magness *et al*, 1996). Magness *et al* (1996) reported that the NOS specific activity was nearly 1.8-fold greater in uterine arteries from pregnant compared with nonpregnant sheep. This increase was specific to the uterine artery because no difference was found in NOS activity among omental (systemic) arteries due to pregnancy status. The NOS activity was inhibited 70-80% by L-NAME. This study also demonstrated that the uterine artery endothelium was the primary source of the NOS specific activity, because denuding the uterine arteries decreased NOS specific activity by as much as 77%, a decrease consistent with the fall in cGMP production observed in arteries after endothelium removal. The remaining 'putative NOS

specific activity' in denuded vessels was near the detection limit of this assay and could not be inhibited further by L-NAME. Thus the endothelium contains virtually all of the NOS activity in the uterine vascular wall.

The increase in uterine artery endothelium-derived NOS activity during pregnancy may be a function of increased total $_{ec}$NOS, nNOS (Ca^{2+} sensitive, constitutive), and/or iNOS enzyme content. Magness *et al* (1997) addressed this issue by testing the Ca^{2+} sensitivity of uterine and omental artery NOS activity and cGMP production in uterine arteries from pregnant sheep. They found that both uterine and omental artery NOS activity and cGMP production in the Ca^{2+}-containing media were inhibited (80%) by removal of Ca^{2+} plus the presence of the Ca^{2+} chelator EGTA, suggesting the presence of $_{ec}$NOS and/or nNOS rather than iNOS. The same study reported the presence of $_{ec}$NOS, but not nNOS, in the uterine and systemic artery endothelium by Western blotting. Additionally, the expression of $_{ec}$NOS protein by the uterine artery endothelium, but not the vascular smooth muscle, was increased in pregnancy. This pregnancy-related response appeared to be unique to the uterine vasculature, since the endothelium of the systemic arteries showed little or no changes in $_{ec}$NOS with pregnancy (Magness *et al*, 1997).

The data obtained from the latter study were in contrast with those of Weiner *et al*, (1994) who observed that mRNAs encoding both $_{ec}$NOS and nNOS were increased in skeletal muscle during guinea pig pregnancy. Magness *et al*, (1997) were in agreement with Weiner *et al*, (1994) that Ca^{2+}-dependent NOS specific activity was elevated in pregnancy.

Oestrogen treatment of ovariectomized ewes has been reported to result in an increase in $_{ec}$NOS protein expression in uterine, but not mammary, or systemic (omental and renal) artery endothelium (Vagnoni *et al*, 1998). In guinea pigs, five days of oestrogen treatment also increased calcium-sensitive NOS activity and $_{ec}$NOS mRNA expression in skeletal muscle (Weiner *et al,* 1994), thus supporting the idea that the increase in NOS activity is under oestrogen influence.

Nerve fibres containing NOS have been localized in mid-term pregnant rat uterus; Buhimschi *et al*, (1996) demonstrated that the rat cervix expresses all three NOS isoforms: iNOS, nNOS and $_{ec}$NOS. It has been shown that NOS activity increases in rat uterus during pregnancy and falls at term (Natuzzi *et al*, 1993). The presence of iNOS staining has been shown in cells at the fetal-maternal interface of the rat placenta (Purcell *et al*, 1997). Using immunohistochemical techniques, Riemer and coworkers (1997) showed the expression of two NOS isoforms in the pregnant rat uterus: $_{ec}$NOS, localized in the vascular endothelium, and iNOS expressed in the myometrial and vascular smooth muscle, as well as in the decidual epithelium; the expression of both isoforms fell at term. Similarly, NOS activity, estimated by measuring the difference in radiolabelled arginine to citrulline conversion, decreased between days 15 and 21 of gestation (Sladek and Roberts, 1996).

The changes in placental and uterine NOS expression and activity suggest a paracrine role for NO in regulating uterine smooth muscle cell contractility, blood flow and immunosuppression required for the maintenance of pregnancy. NO withdrawal at term may also be involved in the initiation of labour. A brief summary of the evidence indicating increased NO biosynthesis during pregnancy in women, rats and sheep is provided in Table 1.1.

Table 1.1.

Evidence of increased nitric oxide biosynthesis during pregnancy.

Finding	Species	Reference
Increased plasma cGMP levels	Rat	Conrad and Vernier, 1989
Increased urinary excretion of cGMP	Rat	Conrad and Vernier, 1989
	Rat	Conrad *et al*, 1993
Increased urinary excretion and plasma levels of nitrite and nitrate	Rat	Conrad *et al*, 1993
Increased $_{ec}$NOS mRNA expression in aorta	Rat	Goetz *et al*, 1994
Increased NOS activity in different tissues	Guinea pig	Weiner *et al*, 1994
NOS expression in uterus and placenta changes towards parturition	Rat	Natuzzi *et al*, 1993
	Rat	Purcell *et al*, 1997
	Rat	Riemer *et al*, 1997
Increased endothelial, $_{ec}$NOS protein expression in uterine artery	Sheep	Magness *et al*, 1997
	Sheep	Magness *et al*, 2001
	Sheep	Joyce *et al*, 2002
	Human	Nelson *et al*, 2000
Increased $_{ec}$NOS mRNA expression in uterine artery	Human	Bird *et al*, 2000
	Sheep	Magness *et al*, 2001

1.5.2.3 Nitric oxide in human pregnancy

The reduction in total peripheral vascular resistance in early pregnancy plays an important part in determining a woman's vascular adaptation to pregnancy (McCarthy *et al*, 1994). Considerable evidence from studies performed in experimental animals have suggested that increased biosynthesis of NO has an important role in creating the vasodilatation of early pregnancy (see section 1.5.2.2: *Evidence that NO-NOS are increased in rat pregnancy*). In guinea pigs, endothelial NOS is upregulated in a variety of tissues during pregnancy (Weiner *et al*, 1991). This upregulation may occur after oestradiol-mediated induction of $_{ec}$NOS (Weiner *et al*, 1994). Indirect observations in humans suggest that NO activity may be increased during normal pregnancy; serum levels of nitrite and cGMP are increased (Boccardo *et al*, 1996). Further evidence that NO may mediate pregnancy-induced vasodilatation is provided by the findings of increased NO activity in the dorsal hand vein of puerperal women (Ford *et al*, 1996) and in the hand circulation during pregnancy (Williams *et al*, 1997).

Nonetheless, care should be taken when extrapolating the results of animal studies to the human state, as it is recognised that there is intra- and inter- species variation in the contribution of NO to vasorelaxation (Gillham *et al*, 2003). Studies looking into NO production in human pregnancy rely on the measurement of its two relatively stable metabolites, nitrite and nitrate (NOx) using the Griess reaction. Shaamash *et al* (2000), reported a significant increase in serum NOx concentrations in normal pregnant women compared with age-matched normal nonpregnant women, thus suggesting that serum NO production is increased in normal pregnancy compared with the nonpregnant state. In the study by Shaamash *et al* (2000), total NOx levels were found to be significantly higher in the maternal sera of the pre-eclamptic and eclamptic women, compared with those of normal pregnant women. Also, fetal blood levels of total NOx were significantly increased in pre-eclampsia and eclampsia compared with those of normal pregnancy. This group concluded that the increased maternal and fetal serum NO levels in pre-eclampsia and eclampsia, possibly represent a compensatory/protective mechanism to maintain blood flow and

limit platelet aggregation in the fetal-maternal circulations (Shaamash *et al* 2000). Most recently, Choi *et al* (2003) demonstrated an increase in serum NOx concentrations during normal pregnancy compared with the nonpregnant state. Elevated NOx concentrations during pregnancy returned to nonpregnant levels within 12 weeks after delivery (Choi *et al* 2003). In contrast, other groups of investigators have reported no change in serum concentrations or urinary excretion of nitrate during normal human pregnancy compared with the nonpregnant state (Brown *et al*, 1995; Smarason *et al*, 1997).

These inconsistent results may be explained by the differing methodology. In the aforesaid studies of human pregnancy, the ingestion of nitrate was not controlled. The dietary intake of nitrate can significantly affect the plasma level and urinary excretion of NOx, and consistent estimates of nitrate, and hence NO production, may be obtained when dietary intake is reduced and controlled. Conrad *et al*, (1999), got round this methodological difficulty by measuring the plasma level and 24-h urinary excretion of NOx during normal pregnancy and pre-eclampsia in women on a diet containing reduced NOx. In this way, the plasma and urinary levels mainly reflected endogenous production rather than dietary intake (Conrad *et al*, 1999). Conrad and co-workers (1999), demonstrated that plasma levels and urinary excretion of NOx does not increase in normal pregnancy. The studies by Conrad *et al*, (1999), have not provided clear evidence for reduced NO production in pre-eclampsia.

Evidence against an increase in NO activity in human pregnancy is provided by the study of McCarthy *et al* (1994), who investigated endothelium-dependent relaxation directly by examining acetylcholine-mediated relaxation in small arteries isolated from the subcutaneous fat layer of women undergoing either gynaecological surgery or Caesarean section. McCarthy *et al* (1994), using small vessel myography, measured tension in resistance arteries of normal pregnant and nonpregnant women and assessed the contributions of vasodilatory prostanoids and endothelium-derived relaxing factor on endothelium-dependent relaxation, as elicited by acetylcholine (1 nmol/L to 10 μmol/L) after precontraction with 3 μmol/L norepinephrine. Endothelium-dependent relaxation was similar in arteries from pregnant and

nonpregnant women. Furthermore, the response to sodium nitroprusside (SNP, a donor of NO)-induced vasodilatation did not differ in vessels from either group of women. Ashworth *et al*, (1996) using wire myography, studied endothelium-dependent relaxation in response to bradykinin in both myometrial and omental resistance arteries in normotensive pregnant and nonpregnant women. Ashworth *et al*, (1996), demonstrated that the endothelium-dependent relaxation to bradykinin was less in myometrial resistance arteries than in omental resistance arteries. There appeared to be no intrinsic difference between vessels from nonpregnant and pregnant women in endothelium-dependent response to bradykinin (Ashworth *et al*, 1996).

Pascoal and Umans (1996), using the same method, assessed mechanisms of acetylcholine- and bradykinin-induced relaxations in human omental resistance vessels dissected from omental biopsies obtained from women at laparotomy (nonpregnant), or at caesarean delivery (pregnant). Maximal ACh- or BK-induced relaxations in human omental microvessels were not decreased by either N^G-nitro-L-arginine, or indomethacin (Pascoal and Umans 1996). By contrast, BK failed to relax vessels that had been preconstricted with potassium gluconate. In the combined presence of N^G-nitro-L-arginine and indomethacin, addition of charybdotoxin, a selective antagonist of some calcium-sensitive K^+ channels, did not inhibit maximal BK-induced relaxation (Pascoal and Umans 1996). By contrast with results in the rat (Conrad *et al*, 1993; Molnar and Hertelendy, 1992), the results by Pascoal and Umans (1996) suggested no important contributions by either endothelium-derived NO or a factor acting at charybdotoxin-sensitive K^+ channels to either maximal ACh- or BK-induced relaxation in these human vessels. Pascoal and Umans (1996) did not comment on the role of NO in the response to intermediate concentrations of either agonist, as these were not evaluated. However, a previous study in human arteries from another vascular bed (see below), in which NO plays an obvious contributory role in the response to endothelium-dependent vasodilators has suggested that reduced responses to maximally effective concentrations of vasodilators accompany any decrements in response to intermediate concentrations of vasodilators after NO synthase inhibition (McCarthy *et al*, 1994).

In another study, Pascoal *et al* (1998) extended the above findings by showing that NOS inhibition failed to shift ACh and BK dose-response curves in normal gravidas. Their protocols were conducted in the absence of flow, a major stimulus for endothelial NO production *in vivo*, suggesting caution in mechanistic comparison of their data with those from whole animal or perfused vessel systems.

Pregnancy increases endothelium-dependent relaxation of some rat vessel preparations, but not others (Pascoal *et al*, 1995), perhaps by increasing sensitivity to flow or by favoring contributions of NO (Cockell and Poston, 1997a). Studies of human resistance vessels *in vitro* are more limited; they suggest increased shear stress and contributions by a novel hyperpolarizing vasodilator, but no augmentation of ACh or BK effect (Pascoal and Umans, 1996; Cockell and Poston, 1997). There are no data concerning mechanisms that might effect these gestational changes or account for species differences.

The unaltered ACh-mediated relaxation in small subcutaneous arteries from normal pregnant women demonstrated by McCarthy and coworkers (1994), contrasts with the increased BK-mediated relaxation shown by Knock and Poston (1996) in the small subcutaneous arteries from normotensive pregnant compared with those from nonpregnant women. In contrast to ACh, widely used in the laboratory as an endothelium-dependent vasodilator but with little proved physiologic role in the peripheral vasculature, BK is a physiologically active agonist synthesized in human vascular smooth muscle and endothelium (Graf *et al*, 1994; Knock and Poston, 1996). Knock and Poston, (1996), using wire myography, showed that in arteries from women with pre-eclampsia, BK responses were blunted compared with those in arteries from normotensive pregnant women. Relaxation in pregnant, nonpregnant and pre-eclamptic groups was attenuated in the presence of the NO synthase inhibitor N^{ω}-nitro-L-arginine so that it became similar in the three groups. Indomethacin had a small but significant inhibitory effect on BK-induced relaxation, but this component of relaxation was no different among groups. Sensitivity of arteries to norepinephrine and sodium nitroprusside showed no significant differences in the three groups of women. This study by Knock and Poston (1996), provided evidence for an increase in BK-mediated NO synthesis from the vascular

endothelium of small arteries from the peripheral circulation of normotensive pregnant women and a relative reduction in women with pre-eclampsia. In turn, these changes may contribute to vasodilatation in normal pregnancy and elevation of the blood pressure in pre-eclampsia (Knock and Poston 1996).

The finding by Knock and Poston (1996), that the sensitivity to norepinephrine was unchanged in subcutaneous arteries from pregnant compared with nonpregnant women, is in agreement with a study by Steele and coworkers (1993), in isolated uterine radial arteries from pregnant women and from an in vivo investigation of pressor responses (Ramsay *et al*, 1993).

In a recent study, Kenny *et al*, (2002), using pressure myography, compared responses to the endothelium-dependent vasodilator, BK, in myometrial arteries from normal pregnant and nonpregnant women and women with pre-eclampsia, in order to assess the relative contributions of NO, endothelium-derived hyperpolarizing factor (EDHF) and prostanoids in mediating endothelium-dependent vasodilatation. BK-induced concentration-dependent relaxation in arteries isolated from the three subject groups did not differ with regard to sensitivity or maximum response. Responses to BK in all three groups were unaffected by cyclo-oxygenase inhibition alone, and were similarly unaffected by partial depolarization. The NO synthase inhibitor, N-nitro-L-arginine methyl ester, significantly attenuated the responses to BK in arteries from nonpregnant women and almost abolished responses in arteries from women with pre-eclampsia, thus suggesting that the responses to BK in arteries isolated from nonpregnant women and women with pre-eclampsia were mediated largely by NO. However, in arteries from normal pregnant women, BK-induced responses were maintained in the presence of NOS inhibition. Inhibition of NOS combined with partial depolarization, abolished responses to BK in these vessels. These results suggest that, "in the absence of NO, an EDHF can mediate vasodilatation to BK and vice versa, so offering redundancy in endothelium-dependent mechanisms" (Kenny *et al*, 2002). Thus, "it is possible that normal pregnancy is associated with the development of an EDHF-mediated vasodilatory mechanism that operates in parallel with the NO pathway in the myometrial vascular bed" (Kenny *et al*, 2002). "This vascular bed assumes a unique significance in

pregnancy, as the maintenance of adequate uterine blood flow is necessary for normal placental and fetal development". "The additional influence of EDHF-type pathways may afford some protection for the maintenance of blood flow under conditions of reduced NO activity or production" (Kenny *et al*, 2002).

Normal pregnancy is associated with an increase in uterine blood flow (Magness *et al*, 1996). It has been known for fifty years that blood flow to the uterine tissue is augmented by oestrogen (Borell *et al*, 1953). Much has been learned about mechanisms contributing to these changes in blood flow from studies performed in experimental animals. For example, observations of nonneuronal vasodilatory effects of ACh in uterine arteries and the time course for modulation of this response by pregnancy and oestrogen preceded discovery of endothelium-derived relaxing factors (Bell, 1973). These first observations provided some indications as to both an immediate, nongenomic and longer-term modulation of vascular responses by sex steroid hormones. Subsequently, NO was identified as one of the endothelium-derived factors modulated by oestrogen in systemic blood vessels as well as blood vessels of the reproductive system (Magness *et al*, 1997; Nelson *et al*, 1998; Weiner *et al*, 1989b). Nelson *et al*, (2000), extended these observations by investigating expression of isoforms of NO synthase in uterine arteries from women after normal pregnancies compared with those from multipara, nonpregnant women. Results of this study confirmed observations in experimental animals: NOS expression increases in uterine arteries during pregnancy and NOS immunostaining increases in uterine arteries during the follicular compared with luteal phase of the menstrual cycle in nonpregnant women.

Studies involving human tissue are needed to confirm observations in experimental animals, because each type of study carries certain limitations. Results from animal studies reflect "the combined genetic and environmental influences that have resulted from evolutionary survival of a particular species in an environmental niche" (Miller, 2000). Antibodies and molecular probes developed for one species may not be right for another and, when used without careful controls, may have a negative effect on results and, therefore, conclusions.

Conversely, obtaining human tissue is difficult, technically and ethically. When the material is surgical waste, the following queries must be considered: the underlying condition or disease that required the need for surgery, the age, ethnicity and gender of the patient, medications for preexisting conditions and anesthesia. Handling and storage of tissue are also important issues, and the investigators should add in preliminary studies to define storage limitations.

Though it is essential to confirm observations from animal studies in human tissue, it is even more important that such studies provide new insights into mechanisms or disease processes that have not been made in animal studies. For example, the aforementioned study by Nelson *et al*, (2000), did that by confirming increased expression and activity of endothelial but not inducible NOS in uterine arteries during pregnancy. In addition, results of this study provided new insights into the distribution of neuronal NOS in the adventia of uterine arteries.

Investigators in clinical departments with access to human tissue are in the position to design experiments that go beyond corroborating observations from studies in experimental animals. Such studies could provide new information necessary to improve the health of women.

1.5.2.4 Maternal effects of acute and long-term NOS inhibition

Several studies have been conducted to investigate the impact of nitric oxide synthesis inhibition on the blood pressure and glomerular haemodynamic adaptations to pregnancy in the rat. Acute administration of L-NAME or N^{ω}-nitro-L-arginine to conscious rats produces a dose-response increase in MAP and reverses the vascular refractoriness to vasoconstrictors in pregnant rats (Molnar and Hertelendy, 1992; Nathan *et al*, 1995). Acute (4 hours) NOS inhibition has been shown to reverse the systemic and renal vasodilation response to pregnancy in rats (Danielson and Conrad, 1995).

Chronic administration of NOS inhibitors to pregnant rats results in a dose-dependent increase in systemic blood pressure (Baylis and Engels, 1992; Molnar *et al*, 1994), thus verifying the major role of NO in the maintenance of normal vascular tone during pregnancy. Marked thrombocytopenia and proteinuria, and reduced plasma volume expansion have been observed after NOS inhibition (Baylis and Engels, 1992; Molnar *et al*, 1994; Salas *et al*, 1995).

Chronic NOS blockade also influences maternal levels of other vasoactive agents. Pregnant rats treated with N^{ω}-nitro-L-arginine exhibited a reduced urinary excretion of thromboxane B_2 (TxB_2), without significant changes in 6-keto-prostaglandin F1α (6-keto-PGF1α) excretion (Salas *et al*, 1997). *In vitro* studies have shown that NO produced by endothelial cells increased the production of 6-keto-PGF1α and TxB_2, through activation of prostaglandin H synthase, and that L-NAME significantly reduced prostaglandin production (Davidge *et al*, 1995). Whether this mechanism is involved in the above observations remains to be clarified.

As reported in acute experiments, chronic blockade of NO production caused systemic hypertension, increased renal vascular resistance (RVR) and reduced renal plasma flow (RPF) in late pregnant Sprague-Dawley rats (Deng *et al*, 1996). In the same study, preglomerular and efferent arterioral resistance were increased in cortical nephrons, and there was a striking rise in glomerular capillary blood pressure. The

pressor and glomerular haemodynamic responses to NO blockade were similar in virgin and pregnant rats. Chronic NO blockade led to a significant reduction in the urinary NOx excretion in all groups. A recent study found that chronic (7 days) NOS inhibition reversed the glomerular hyperfiltration and renal vasodilatation in midterm pregnant rats independent of prostaglandin inhibition (Cadnapaphornchai *et al*, 2001).

These data suggest that chronic NO deficiency leads to derangement of the haemodynamic adaptations of pregnancy. Both acute and chronic responses are reduced when L-arginine, the substrate for NOS, is administered in high doses, confirming that these changes are specific to NO inhibition.

1.5.3 Endothelium-Derived Hyperpolarizing Factor (EDHF)

The vasodilating effect of ACh is thought to be mediated by different endothelial factors. It is well established that NO and PGI_2 play a major role in mediating this ACh-induced dilation. The fact that a distinct dilation remains even after combined inhibition of NOS and COX suggests a role for a third factor, the so-called endothelium-derived hyperpolarizing factor (EDHF) (Garland *et al*, 1995). EDHF is considered to elicit hyperpolarization and relaxation by opening potassium channels in the smooth muscle, since high K^+ concentrations (25-40 mM) abolished (Chen and Suzuki, 1989) and calcium-activated potassium channel inhibitors attenuated, the residual relaxation. "The hallmark of an EDHF-mediated response" (Busse *et al*, 2002) is the blockade of its release by a combination of apamin (a specific inhibitor of Ca^{2+}-sensitive K^+ channels of small conductance) plus charybdotoxin (a nonselective inhibitor of large conductance and intermediate conductance Ca^{2+}-sensitive K^+ channels, and of some voltage-dependent K^+ channels) (Edwards *et al*, 1998). In addition there is substantial support for the notion that the endothelium-dependent smooth muscle hyperpolarization is partially inhibited by barium (Ba^{2+}; at a concentration that selectively inhibits K_{IR} channels) and ouabain (an inhibitor of Na^+/K^+ ATPase), and is abolished by the application of

both inhibitors together in the rat hepatic and mesenteric arteries (Edwards *et al*, 1998). The maintenance of the endothelial cell hyperpolarization despite the abolition of smooth muscle hyperpolarization in the presence of barium plus ouabain indicates that these inhibitors act on the smooth muscle (Edwards *et al*, 1998). The ouabain sensitivity of the EDHF response suggests that the type of Na^+/K^+ ATPase involved is likely to contain a type 2 or 3 α-subunit (Edwards *et al*, 1999). It is important to note that submicromolar concentrations of ouabain selectively target the Na^+/K^+ ATPase and inhibit EDHF-mediated responses without affecting the resting membrane potential or gap junctional communication (Harris *et al*, 2000).

The importance of EDHF as an endothelial-derived vasorelaxant appears to be inversely related to vessel diameter, with the greatest activity being found in the resistance vasculature (Shimokawa *et al*, 1996), suggesting that this unidentified factor is likely to play a essential role in the regulation of local blood flow within the resistance vasculature, in tissue perfusion and in systemic blood pressure.

The identity of the hyperpolarizing factor remains disputable but it is thought that in mesenteric arteries arachidonoylethanolamide (anandamide) may be an EDHF (Randall *et al*, 1996). In rat mesentery, the nitric oxide and prostanoid independent relaxations were inhibited by the cannabinoid receptor antagonist SR141716A. In contrast, Plane *et al*, (1997), did not show any inhibitory effect of SR141716A in rat mesentery. Kenny *et al*, (2002), reported that neither anandamide, nor its more metabolically stable analogue, methanandamide, evoked a vasodilator response in human myometrial arteries. SR141716A, at a concentration selective for cannabinoid receptors (1μM), did not attenuate the response to bradykinin in human myometrial arteries. Thus Kenny *et al*, (2002), suggested that an endogenous cannabinoid is unlikely to be an EDHF in the myometrial vascular bed.

Other candidates for EDHF include the cytochrome P450 metabolites of arachidonic acid, such as epoxyeicosatrienoic acids (EETs) (Hecker *et al*, 1994; Li and Campbell, 1997; Fisslthaler *et al*, 1999), endothelium-derived K^+ ions and as it has been recently identified, hydrogen peroxide (H_2O_2) (Matoba and Shimokawa, 2003). Electrical communication between endothelial and smooth muscle cells through gap junctions has also been suggested to be involved in endothelium-

dependent hyperpolarization (Dora *et al*, 1999; Edwards *et al*, 1999a; Kenny *et al*, 2002).

EETs are short-lived metabolites of arachidonic acid and are produced via the cytochrome P450 epoxygenase pathway. Evidence in favour of EDHF being an EET has been obtained using bovine (Hecker *et al*, 1994), porcine (Popp *et al*, 1996), canine (Widmann *et al,* 1998) and human coronary arteries (Quilley and McGiff, 2000). In these blood vessels, EDHF-mediated responses are blocked by inhibitors of cytochrome P450. Although classical cytochrome P450 inhibitors were nonspecific, new selective and structurally different compounds do hinder EDHF-mediated responses in some arteries (Imig *et al,* 2001). Additionally, in porcine coronary arteries, endothelial cells express several cytochrome P450 epoxygenases and in this artery as well as in hamster resistance arteries, endothelium-dependent hyperpolarization and relaxation can be repressed by antisense oligonucleotides against cytochrome P450 2C (Fisslthaler *et al*, 1999; Bolz *et al*, 2000; Busse *et al*, 2002). Furthermore, EDHF-mediated responses are augmented by agents that improve the endothelial expression of cytochrome P450 (Fisslthaler *et al*, 1999). Altogether, such data suggest that the activation of a cytochrome P450 epoxygenase is a prerequisite for the generation of EDHF-mediated relaxation in the porcine coronary artery.

EETs are released from isolated porcine and bovine coronary arteries as well as from cultured endothelial cells in response to stimulation with receptor-dependent or –independent agonists, and haemodynamic stimuli, such as pulsatile stretch (Fisslthaler *et al*, 1999; Gebremedhin *et al*, 1998; Popp *et al*, 1996; Popp *et al*, 1998). Under such conditions, EETs are endothelium-derived hyperpolarizing factors that elicit smooth muscle relaxation by opening large conductance calcium-sensitive K^+ channels, rather than intermediate conductance and small conductance calcium-sensitive K^+ channels (the channels activated in EDHF responses), suggesting that a diffusible EET is not likely to account for the majority of EDHF-mediated responses. One possible explanation for the experimental observations could be that hyperpolarization of endothelial cells might be partially regulated by activation of the cytochrome P450. EETs might modulate endothelial Ca^{2+} influx in

response to Ca^{2+} store diminution and might facilitate the activation of endothelial K^+ channels by increasing their sensitivity to Ca^{2+} (Baron *et al*, 1997). Furthermore, EETs generated by the cytochrome P450 (CYP) 2C enzyme control interendothelial gap junctional communication (Popp *et al*, 2002). Functional gap junctional communication between vascular cells has been implicated in ascending dilatation and the cytochrome P-450 (CYP) inhibitor-sensitive and NO- and prostacyclin-independent dilatation of many vascular beds (Popp *et al*, 2002). In CYP 2C-expressing porcine coronary endothelial cells, bradykinin, which enhances EET formation, elicited a biphasic effect on the electrical coupling and transfer of Lucifer yellow between endothelial cells, (Popp *et al*, 2002). The initial phase was sensitive to the inhibitor of CYP 2C9 and to the inhibitors of protein kinase A (PKA) and could be mimicked by forskolin and caged cAMP as well as by the activators of PKA (Popp *et al*, 2002). Gap junction uncoupling in bradykinin-stimulated porcine coronary endothelial cells was prevented by inhibiting the activation of extracellular signal-regulated kinase (ERK)1/2. In human endothelial cells, which express little CYP 2C, bradykinin elicited only an ERK1/2-mediated inhibition of intercellular communication. The CYP 2C9 product, 11,12-EET, also exerted a dual effect on the electrical and dye coupling of human endothelial cells, which was sensitive to PKA inhibition (Popp *et al*, 2002). These studies by Popp and co-workers (2002) have shown that an agonist-activated CYP-dependent pathway as well as 11,12-EET can positively control interendothelial gap junctional communication, most probably via the activation of PKA, an effect that is reduced by the subsequent activation of ERK1/2 (Popp *et al*, 2002). So, in coronary arteries, EETs and other products of cytochrome P450 could be considered as second messengers required for the initiation and transmission of the endothelial cell hyperpolarization, and, as a result, for the EDHF-mediated hyperpolarization and relaxation of vascular smooth muscle cells.

Edwards *et al*, (1998), suggested that EDHF is K^+ released from potassium channels on the vascular endothelium which then acts on inwardly rectifying potassium channels (K_{IR}) and Na^+/K^+ ATPases on the smooth muscle cells to induce hyperpolarization. However, the suggestion that EDHF might simply be

endothelium-derived K^+ has been refuted (Andersson *et al*, 2000; Lacy *et al*, 2000; Doughty *et al*, 2000). Andersson *et al*, (2000), reported that neither the Na^+/K^+ ATPase inhibitor ouabain nor the K_{IR} blocker Ba^{2+} alone, or in combination, inhibited EDHF relaxations in the rat hepatic artery. In the rat mesenteric artery, $BaCl_2$ (50 μM) significantly inhibited the maximal relaxant response to K^+ in the presence of an intact endothelium in 5.5 mM potassium PSS, but had no effect on relaxation of de-endothelialized preparations in 1.5 mM potassium PSS (Lacy *et al*, 2000). Furthermore, ouabain (0.1 mM) abolished the relaxant response to potassium in 1.5 mM potassium PSS, but only partly inhibited the maximal relaxant response to acetylcholine in 5.5 mM potassium PSS. Thus Lacy and co-workers (2000), suggested that the response to K^+ is clearly different to that from ACh, indicating that K^+ does not mimic EDHF released by ACh in these arteries. Doughty *et al*, (2000), compared ACh-stimulated, EDHF-mediated dilatation/relaxation with raised K^+ in rat mesenteric arteries. Doughty *et al*, (2000), reported that Ba^{2+} (30 μM) did not affect dilatation to ACh, but abolished 40% of dilatations to raised K^+. If K^+ was lowered to 1.18 mM, restoring K^+ to 5.88 mM produced dilatation which was depressed by Ba^{2+} or ouabain (1 mM). Combined application of Ba^{2+} and ouabain abolished dilatation. In 1.18 mM K^+, dilatation to ACh was depressed by ouabain, but not by Ba^{2+}. Combined application of Ba^{2+} and ouabain depressed dilatation further. Doughty *et al*, (2000), showed that gap junction inhibitors (Gap-27 and 18-α-glycyrrhetinic acid) also depressed dilatation to ACh. The studies by Doughty and co-workers (2000), have provided data showing that K^+ may be a relaxing factor in mesenteric arteries, but its characteristics differ from EDHF. Gap junction inhibitors depress EDHF, implying an important role for myo-endothelial gap junctions.

K^+-induced relaxations and hyperpolarizations are not observed in some blood vessels that exhibit EDHF-mediated responses (Edwards *et al*, 1999a). These inconsistencies can be explained in many vessels. The theory of K^+ as an EDHF is based on electrophysiological experiments, while the criticisms were based on experiments measuring vascular tone. Experiments in which tension and membrane potential were measured at the same time showed that the ability of K^+ to act as an EDHF decreases as the level of agonist-dependent stimulation increases (Dora and

Garland, 2001; Busse *et al*, 2002). The depolarisation produced by the agonist increases the $[Ca^{2+}]_I$ that stimulates K^+ efflux from smooth muscle. This efflux might form a "K^+ cloud" that surrounds the myocytes and prevents any effect of additionally added K^+, or any component of the EDHF response (Busse *et al*, 2002). Under those circumstances, the contribution of gap junctions to the EDHF phenomenon takes over.

In human myometrial pregnant arteries, no vasodilator response was seen in increasing the extracellular K^+ concentration over the range 1-15 mM (Kenny *et al*, 2002), thus suggesting that K^+ is unlikely to be an EDHF in these vessels.

Recently, it has been suggested that direct intercellular communication via gap junctions, by providing electrical continuity between neighbouring cells, may play a central role in mediating endothelium-dependent relaxations ascribed to an EDHF (Chaytor *et al*, 1998; Dora *et al*, 1999; Edwards *et al*, 1999a). Gap junctions form intercellular channels through which ions and small molecules (less than approx. 1 kDa), can diffuse (Christ and Barr, 2000). Each channel is formed from two hemichannels, one donated from each of the adjacent cells. Each hemichannel is constructed from six connexin proteins, and channels may be constructed from two hemichannels that contain the same set of six connexin proteins (homotypic), or two different sets of connexin subtypes (heterotypic) (Brink *et al*, 2000).

Studies using rabbit conduit arteries have provided evidence that EDHF-type responses, evoked by ACh, may not be entirely passive because relaxation is linked with endothelium-dependent elevations in smooth muscle cAMP levels, and therefore attenuated by blocking adenylyl cyclase or protein kinase A (Taylor *et al*, 2001). Furthermore, EDHF-type relaxations are potentiated by inhibition of cAMP hydrolysis with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), while remaining susceptible to the combination of apamin plus charybdotoxin (Taylor *et al*, 2001). As mentioned earlier, sensitivity to these peptide toxins, is a hallmark of the EDHF phenomenon and thought to reflect the opening of apamin-sensitive small conductance K^+ channels (SK_{Ca}) and charybdotoxin-sensitive large and intermediate conductance K^+ channels (BK_{Ca} and IK_{Ca}) located on endothelial cells (Edwards and Weston, 2001). Consistent with a role for gap junctions, EDHF-

type relaxations and cAMP accumulation evoked by ACh, are inhibited by synthetic connexin-mimetic peptides, that interrupt intercellular communication in a connexin-specific fashion and by 18-α-glycyrrhetinic acid, that disrupts gap junction plaques at points of cell–cell contact (Chaytor *et al*, 2002; Taylor *et al*, 2001; Griffith and Taylor, 1999).

Because there is evidence that elevations in cAMP levels may rapidly enhance gap junctional communication (Gladwell and Jefferys, 2001), Griffith *et al*, (2002), have tested the hypothesis that this nucleotide underpins EDHF-type relaxations in conduit arteries, by facilitating the electrotonic spread of hyperpolarizing current from the endothelium into and through the media. In the study by Griffith *et al*, (2002), mechanical responses were correlated with measurements of smooth muscle cAMP levels and membrane potential, and modulation of gap junctional permeability by cAMP was evaluated by a dye transfer technique, whereby the entire endothelial monolayer was loaded with the cell permeant tracer calcein. This group have shown that EDHF-type relaxations to ACh in rabbit iliac arteries in the presence of cAMP phosphodiesterase inhibitor IBMX, or the cell permeant cAMP analogue 8-bromo-cAMP, became sustained with their maxima potentiated approximately 2-fold, rather than transient in nature. This group also showed that relaxation was associated with transient approximately 1.5-fold elevations in smooth muscle cAMP levels, with both mechanical and nucleotide responses being abolished by interrupting gap junctional communication with the connexin-mimetic peptide Gap 27 and by endothelial denudation. Gap 27 has previously been shown to inhibit EDHF-type responses through a receptor-independent mechanism (Dora *et al*, 1999). After selective loading of the endothelium with calcein, direct transfer of dye from the endothelium to the media was enhanced by IBMX or 8-bromo-cAMP, but not by 8-bromo-cGMP, whereas Gap 27 promoted sequestration within the intima. The findings by Griffith and coworkers (2002), support the hypothesis that 'endothelial hyperpolarization underpins the EDHF phenomenon, with cAMP governing subsequent electrotonic

signaling via both myoendothelial and homocellular smooth muscle gap junctions' (Griffith *et al*, 2002).

Most recently, Dora *et al*, (2003), provided evidence for the existence of functional myoendothelial gap junctions in the same, defined branches of BALB/C mouse mesenteric arteries which show robust EDHF-mediated smooth muscle relaxation. Cyclopiazonic acid was used to stimulate EDHF in arteries mounted under isometric conditions and constricted with phenylephrine (Dora *et al*, 2003). The relaxation to cyclopiazonic acid was endothelium dependent, associated with the opening of Ca^{2+}-activated K^+ channels, and only in part due to the release of nitric oxide. In the presence of the NO synthase inhibitor, L-NAME (100 μM), the relaxation to cyclopiazonic acid could be almost completely inhibited with the gap junction uncoupler, carbenoxolone (100 μM). Inhibition of the synthesis of prostaglandins, or metabolites of arachidonic acid had no effect under the same conditions, and small rises in exogenous K^+ failed to induce consistent, or marked smooth muscle relaxation, arguing against a role for these molecules and ions as EDHF. Serial section electron microscopy revealed a high incidence of myoendothelial gap junctions, which was correlated with heterocellular dye coupling. Taken together, these functional and morphological data by Dora and co-workers (2003), suggest that the EDHF response, in a defined mouse resistance artery, may be explained by extensive heterocellular coupling through myoendothelial gap junctions, enabling spread of hyperpolarizing current (Dora *et al*, 2003).

In human myometrial pregnant arteries, Kenny *et al*, (2002), has demonstrated a potential role for gap junctional communication in mediating EDHF-type relaxations. This group has shown that responses to bradykinin were unchanged by combined inhibition of COX and NOS in myometrial arteries isolated from normal pregnant women (Kenny *et al*, 2002; Kenny *et al*, 2002a). The added presence of clotrimazole (50 μM), attenuated, but did not abolish vasodilator responses to bradykinin (Kenny *et al*, 2002). Increasing extracellular K^+ over the range of 1-15 mM did not elicit relaxation, nor did the cannabinoids, anandamide

and methanandamide. Kenny *et al* (2002) showed that the non-nitric oxide and non-prostanoid mediated relaxation to bradykinin was virtually abolished in these vessels by the gap junction inhibitors 18-α glycyrrhetininc acid (100 μM), carbenoxolone (100 μM) and palmitoleic acid (50 μM). SR141716A at a high concentration (10 μM), also abolished the response to bradykinin (Kenny *et al*, 2002). Taken together, these data by Kenny and co-workers (2002), suggest that gap junctional communication is involved in the NO, prostanoid-independent vasodilator response to bradykinin in myometrial small arteries in normal pregnancy.

During pregnancy EDHF may contribute to the vascular adaptation. EDHF has been suggested to play an important role in the enhanced ACh-induced dilation of mesenteric arteries of pregnant rats (Gerber *et al*, 1998). Also, studies on the uterine vascular beds of pregnant rats have suggested that EDHF release is activated by a delayed rectifier type of voltage sensitive potassium channel (Fulep *et al*, 2001).

Most recently, the use of an electron spin resonance (ESR) method, gave evidence that H_2O_2 is an EDHF in porcine coronary microvessels (Matoba *et al*, 2003a). In the study by Matoba *et al*, (2003a), isometric tension and membrane-potential recordings showed that bradykinin and substance P caused EDHF-mediated relaxations and hyperpolarizations of porcine coronary microvessels in the presence of indomethacin and N^{ω}-nitro-L-arginine. The involvement of H_2O_2 to the EDHF-mediated responses was demonstrated by the inhibitory effect of catalase and by the relaxing and hyperpolarizing effects of exogenous H_2O_2 (Matoba *et al*, 2003a). Endothelial production of H_2O_2 was quantified in bradykinin- or substance P-stimulated intact blood vessels by ESR spectroscopy (Matoba *et al*, 2003a). Tiron, a superoxide scavenger that facilitates H_2O_2formation, enhanced bradykinin-induced production of H_2O_2, as well as the EDHF-mediated relaxations and hyperpolarizations. By contrast, cytochrome P450 inhibitors (sulfaphenazole or 17-octadecynoic acid), or a gap junction inhibitor (18α-glycyrrhetinic acid), failed to inhibit the EDHF-mediated relaxations (Matoba *et al*, 2003a).

The identification of EDHF, and selective activators or inhibitors of its biological activity, is likely to have a main impact on our understanding of cardiovascular homeostasis and may prove as important as the discovery of NO and PGI_2.

1.6 Pressor responses in pregnancy

Reduced pressor responses to vasoconstrictor agonists noradrenaline (NA), phenylephrine (PE) (Parent *et al*, 1990), arginine vasopressin and angiotensin II (Paller 1984; Molnar and Hertelendy, 1992) is a long known and well described feature of pregnancy. Reduced responses to PE in late pregnancy have been reported in the small mesenteric arteries of rats (Davidge and McLaughlin, 1992; D'Angelo and Osol, 1993). Uterine artery (but not carotid artery) response and sensitivity to NA, adrenaline and PE have been shown to be significantly reduced in pregnant guinea pigs, as compared to nonpregnant animals (Weiner *et al*, 1989). In a recent study, Hermsteiner and coworkers (2001) studied the response of isolated rat uterine radial and third-order mesenteric arteries to PE, Ang II, and endothelin-1 before and during pregnancy and found a decrease of vasoconstrictor sensitivity in the uterine circulation during early pregnancy. In mesenteric vessels, sensitivity to PE and Ang II was unchanged in early, but reduced in late pregnancy.

The decreased vasopressor responses and vascular reactivity to vasoconstrictor agonists during pregnancy have been attributed, in part, to increased synthesis/release of NO (Baylis and Engels, 1992; Conrad *et al*, 1993) and perhaps other vasodilatory substances such as PGI_2 and EDHF (Gerber *et al*, 1998; Fulep *et al*, 2001) by various maternal cells including vascular endothelial cells. The reduced vascular reactivity to Ang II and NA in pregnancy has been reported to be restored to nonpregnant levels by NOS inhibitors in the isolated hindlimb (Ahokas and Sibai, 1992) and the *in situ* perfused mesentery preparation (Chu and Beilin, 1993) of late pregnant rats, respectively, indicating that the attenuation of the vasoconstrictor response in pregnancy is associated with heightened NO production.

1.7. Flow-mediated responses

Endothelial cells are subjected to a frictional force induced by the flow of blood through a vessel known as 'shear stress'. Shearing forces acting on the luminal surface of vascular endothelium regulate the release of endothelium-derived vasoactive agents. Flow-mediated dilatation involves the release of NO and to a lesser extent PGI_2 (Rubanyi *et al*, 1986; Hecker *et al*, 1993; Learmont and Poston, 1996). Flow-mediated release of NO in response to shear stress has been shown in cultured endothelial cells (Noris *et al*, 1995), isolated arteries (Cooke *et al*, 1991; Koller *et al*, 1994) and *in vivo* in Doppler ultrasound studies (Meredith *et al*, 1996). Mechanisms for the mechanotransduction of the flow stimulus or frictional force at the endothelial surface may involve integrins, G proteins, tyrosine kinases and mitogen-activated protein kinases (Malek and Izumo, 1994). By this mechanism, increased flow velocity stimulates calcium release and increased cNOS activity.

Small resistance vessels may be more sensitive to flow changes than large arteries. Hull *et al*, (1986) showed that a 10-fold increase in flow caused a 9% increase in diameter in the femoral artery but also a larger 15% increase in the smaller, downstream saphenous artery. Oestrogen (Cockell and Poston, 1997) and pregnancy can enhance the reactivity of resistance vessels to flow/shear stress.

1.8 L-Arginine, the substrate for generation of NO

L-arginine is the exclusive physiological substrate for NOS. The NOS enzyme is stereospecific for L-arginine, as D-arginine is not a substrate (Palmer and Moncada, 1989). Half-saturating L-arginine concentrations (K_m) have been reported as 2.9 μmol/l for $_{ec}$NOS (Pollock *et al*, 1991). Experimentally, inhibition of NO generation is achieved using analogues of arginine that have been modified at the guanidino terminus (Rees *et al*, 1990). One such compound, N^G-monomethyl-L-arginine (L-NMMA), was the first known inhibitor of NO synthases, and has become the standard pharmacological inhibitor for use *in vitro* and *in vivo*. The NOS-catalysed oxidation of arginine to give citrulline and NO is dependent on multiple cofactors (see section 1.5.2.1 and figure 1.5.) which can be rate-limiting for the reaction. It has been reported that supplementation with tetrahydrobiopterin improved endothelium-dependent arterial relaxation in the aorta of the insulin-resistant rat (Shinozaki *et al*, 2000). N^{ω}-hydroxy-L-arginine is an enzyme-bound intermediate in the reaction pathway (Stuehr *et al*, 1991).

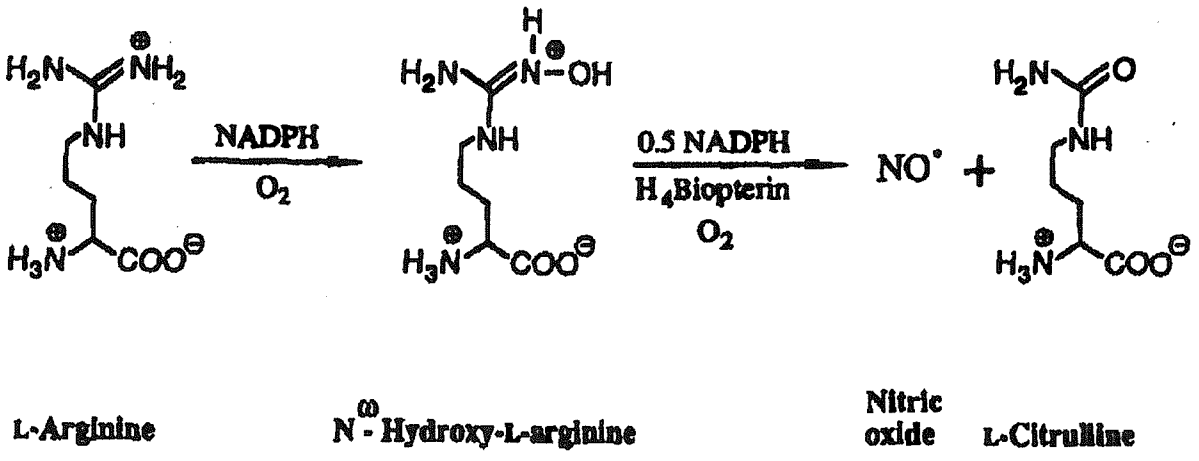


Figure 1.5.: Reaction pathway for NO formation from Arg oxidation by NOS.
The initial hydroxylation of L-arginine to generate N^{ω}-hydroxy-L-arginine is coupled to oxidation of 1 mol of NADPH. Generation of 'NO and L-citrulline from N^{ω}-hydroxy-L-arginine requires oxidation of 0.5 mol of NADPH and oxidation of BH_4 (Stuehr *et al*, 1991).

1.8.1. Importance of dietary L-arginine intake

Arginine is not an essential amino acid for healthy adult humans and rodents because it is synthesized in a collaboration between epithelial cells of the small intestine and the proximal tubular cells of the kidney (Morris, 2002). This pathway is known as the intestinal-renal axis. Briefly, citrulline is generated in the small intestine as one of the products of glutamine metabolism and released into the blood. Little or no citrulline is extracted from the blood as it passes through the liver, but citrulline is efficiently taken up by the proximal renal tubules, where it is converted to arginine which is released into the blood. Arginine has been classified as a conditionally essential or semi-essential amino acid owing to conditions in which endogenous synthesis is insufficient to meet the requirements for arginine (Barbul, 1986). Such conditions may involve reduced function of the kidney as a result of disease (Peters *et al*, 1999) or trauma as well as conditions involving elevated protein and amino acid catabolism, such as inflammation or wound healing.

Plasma L-arginine concentrations in humans and animals range from 95-250 μmol/L (Wu and Morris, 1998). In endothelial cells, however, the concentration of L-arginine has been estimated to be ~1 to 2 mmol/L (Hecker *et al*, 1990). L-arginine is actively transported into the endothelium by a y^+ transporter (a high affinity, Na^+-independent transporter of basic amino acids). Even in the absence of extracellular arginine, the endothelial cells can synthesize this amino acid from L-citrulline (Hecker *et al*, 1990). Although extracellular arginine is the major source of the Arg for endothelial NO synthesis, intracellular protein degradation or the Arg-citrulline cycle may provide Arg for supporting short-term basal NO production by endothelial cells when extracellular Arg is limited (Wu and Morris, 1998).

Dietary L-arginine supplementation has been shown to reverse endothelial dysfunction associated with hypercholesterolemia, hypertension, diabetes and atherosclerosis. Many studies have shown that supplementation with L-arginine reduced or completely reversed endothelial dysfunction in several vascular beds,

varying from arterioles to thoracic aorta, in hypercholesterolemic rabbits (Cooke *et al*, 1991; Maxwell and Cooke, 1998) and the diabetic rat (Ozcelikay *et al*, 2000). Increasing L-arginine intake is beneficial in experimental models of hypertensive renal disease characterized by increased blood pressure. Much research has shown that dietary L-arginine administration increased NO synthesis and prevented endothelial dysfunction in the salt-sensitive hypertensive Dahl/Rapp rat (Chen and Sanders, 1991).

Human studies have also consistently demonstrated the beneficial effects of Arg on improving endothelium dependent relaxation in hypercholesterolemic patients. Intravenous administration of L-arginine (but not D-arginine) improved the impaired endothelium-dependent vasodilatation to methacholine chloride of the forearm resistance vessels in the hypercholesterolemic patients (Creager *et al*, 1992). Oral Arg administration enhanced endothelium-dependent vasodilatation in patients with heart failure, and this beneficial effect was additive with exercise training (Hambrecht *et al*, 2000). In young men with coronary artery disease oral Arg enhanced brachial artery flow-mediated vasodilatation (Adams *et al*, 1997). In another study of patients with coronary artery disease, long-term (6 months) L-arginine supplementation decreased plasma endothelin-1 levels by 30% and increased coronary blood flow response to ACh by 150% (Lerman *et al*, 1998). Vascular endothelial dysfunction occurred in a patient with a natural deficiency of arginine (plasma arginine = 21 μM) owing to a defect in the basic amino acid transporter (Kamada *et al*, 2001).

Dietary arginine deficiency resulted in decreased plasma concentration, and decreased plasma L-arginine concentration has been reported in young rats fed a low protein diet (5% casein) compared with rats fed 20% casein (169 μM low protein; 207 μM control) (Wu *et al*, 1999). Feeding a 1% arginine diet to young rats increased plasma Arg concentration compared with rats fed a 0.3% arginine diet or an arginine-free diet (224 μM 1% Arg; 162 μM 0.3% Arg; 109 μM 0% Arg) (Wu *et al*, 1999). Studies *in vitro* have shown that increasing extracellular Arg concentrations from 0.1 to 10 mM dose-dependently increased NO production by cultured endothelial cells in the presence of physiological glutamine concentration (0.5-0.6 mM) (Arnal *et al*, 1995). In

the study by Wu *et al* (1999), dietary protein deficiency resulted in 52% decrease in whole body constitutive NO production by constitutive NOS and could not support maximal inducible NO synthesis by iNOS in young rats. A chronic reduction (52%) of NO synthesis by cNOS would have a detrimental effect on the circulatory system in protein or arginine-deficient subjects. In the hypercholesterolemic rabbit, 59% reduction in NO synthesis has been reported and was associated with severely impaired endothelial function and abnormal vascular structure (Bode-Böger *et al*, 1996). Overall, these data suggest that Arg supplementation can modulate in vivo NO production, but the mechanisms are far from clear. These data may also help to explain the cardiovascular dysfunction in Arg-deficient animals and humans.

1.8.2. The L-arginine paradox

The half-saturating L-arginine concentration (K_m) for $_{ec}$NOS has been reported as 2.9 μmol/l (Pollock *et al*, 1991). Plasma L-arginine concentrations in humans and animals range from 95-250 μmol/L (Wu and Morris, 1998). Freshly isolated endothelial cells have been found to contain up to 2 mmol/L L-arginine (Hecker *et al*, 1990). Considering this and a K_m of 2.9 μmol/l for L-arginine for $_{ec}$NOS, this enzyme should be saturated in endothelial cells and additional L-arginine should not enhance NO formation. It is surprising that intravenous or oral supplementation of Arg *in vivo* in humans increased endothelial NO production (Creager *et al*, 1992; Hambrecht *et al*, 2000). This phenomenon has been termed the L-arginine paradox and has found no adequate explanation so far. A possible explanation for this paradox is that in disease states, endothelial cell L-arginine may be low. Several groups have reported reduced plasma Arg levels in patients with major cardiovascular risk factors such as hypertension (Ignarro *et al*, 1999) and diabetes (Pieper, 1998) and with common cardiovascular disorders such as coronary and peripheral artery disease (Wu and Meininger, 2000) and

heart failure (Maxwell and Cooke, 1998) but it is improbable that Arg levels fall below the K_m for $_{ec}$NOS. A second explanation is that the functional K_m of the intracellular enzyme might be higher than that observed in broken cell preparations. There also may be sequestration or compartmentalization of Arg within the endothelial cell, so that variation of Arg concentration in the vicinity of the enzyme is relatively low. Given the high Arg concentration in many cells, including endothelial cells, it is unlikely that compartmental Arg levels at the site of $_{ec}$NOS is as low as 3 μM.

In endothelial cells the shear stress-dependent formation of NO is not affected by the lack of exogenous L-arginine, and the intracellular concentration of L-arginine in these cells decreases only by approximately 25% over several hours of superfusion with L-arginine-free physiological salt solution, whereas other amino acids (e.g. L-alanine, L-aspartate) are strongly depleted (Mitchell *et al*, 1990). These data demonstrate some intracellular formation of L-arginine. It has been reported that large changes in intracellular levels of L-arginine (from 0.48 to 10.3 mM) did not affect NO production (Arnal *et al*, 1995).

Other theories that have been proposed to explain the Arg paradox include colocalization of Arg transporter (CAT-1) and $_{ec}$NOS in membrane-associated caveolae, promotion of $_{ec}$NOS dimerization and competitive inhibition of $_{ec}$NOS by endogenous inhibitors. Two endogenous potent inhibitors of NOS-catalysed formation of NO are known: the methylated L-arginines, N^G,N^G-dimethyl-L-arginine (asymmetrical dimethylarginine, ADMA) and N^G-monomethyl-L-arginine (L-NMMA) (Leiper and Vallance, 1999). ADMA occurs in plasma of healthy humans at a concentration of ~ 0.1 μM (Vishwanathan *et al*, 2000). ADMA plasma levels have been shown to be elevated in various pathological conditions associated with endothelial dysfunction such as hypertension, atherosclerosis (Miyazaki *et al*, 1999) and hypercholesterolemia (Böger *et al*, 1998). Recently, Böger and colleagues (2001) reported that elevated ADMA levels may mediate the impaired endothelial function associated with hyperhomocyst(e)inaemia in humans. ADMA, symmetric dimethylarginine (SDMA) and L-NMMA compete with L-arginine transport via the y^+ transport system. Increased NO

production seen *in vivo* after Arg administration could result from an exchange of intracellular inhibitors against circulating Arg (Tsikas *et al*, 2000). The theory that $_{ec}$NOS is inhibited *in vitro* and *in vivo* in intact cells by compounds produced endogenously (ADMA), does not explain adequately the Arg paradox because plasma levels of ADMA in the healthy state are very low (~ 0.1 μM) compared with that of Arg (90-250 μM) and therefore is unlikely to antagonize Arg as a substrate for NOS.

L-arginine is also a substrate for a group of enzymes called arginases, which metabolize L-arginine to the non-protein amino acid L-ornithine and urea. While arginase I is constitutively present in endothelial cells, arginase II can be induced in endothelial cells by LPS and γ-interferon (Buga *et al*, 1996). Arginase may modulate NO production by limiting Arg as a substrate for NOS.

HYPOTHESES AND AIMS OF THESIS

Pregnancy is a state characterized by significant cardiovascular adaptive responses to meet the metabolic needs of the mother and fetus. Maternal blood volume and cardiac output are increased, while total vascular resistance and arterial pressure tend to decrease. Dietary protein restriction attenuates the cardiovascular response to pregnancy in rats and is linked with fetal growth restriction and raised blood pressure in the offspring.

The endogenous nitrovasodilator NO is considered to be one of the candidates contributing to the decreased peripheral resistance in pregnancy and may mediate the vasodilatation associated with increased protein feeding in rats. The purpose of the studies presented in this thesis was to investigate the hypotheses that *a maternal low protein diet (9% casein) (1) is associated with a decrease in maternal urinary concentrations of nitrite and nitrate (NOx) and cGMP in pregnant rats compared with pregnant rats fed a control protein diet (18% casein), (2) affects the mesenteric circulation of virgin female rats and pregnant rats through a change in the vasodilator response to acetylcholine, (3) is associated with a reduction in endothelial constitutive NOS ($_{ec}$NOS) and soluble guanylate cyclase (sGC) protein expression in vascular tissue from virgin and pregnant Wistar rats compared with virgin and pregnant rats fed a control protein diet (18% casein).* The specific aims of the studies presented in this thesis were as follows:

1) To examine the effects of maternal protein restriction (9% casein diet) on systemic NO production in pregnant rats by measurement of the urinary nitrate and nitrite (NOx) and cGMP excretion. These studies are presented in Chapter 2.

2) To investigate the influence of a low protein diet on vascular constrictor responses to the α_1 adrenoreceptor agonist PE, endothelium-dependent dilatation to ACh and endothelium-independent dilatation to SPN in isolated mesenteric arteries from virgin female rats and in pregnant rats (gestation day 18-19). The contribution of the endothelial-derived products -NO, prostanoids and EDHF- as mediators of ACh-induced vascular relaxation was also explored. The methodology is described in Chapter 3. Results in the virgin rats are given in Chapter 4 and in the pregnant rats in Chapter 5.

3) To clarify mechanisms underlying the reduced response to NO-mediated vasodilators in the 9% casein-fed virgin and pregnant rats by evaluating the expression of protein of the key enzymes of the NO/cGMP system: $_{ec}$NOS and sGC in vascular tissue from virgin and pregnant 18% and 9% casein-fed Wistar rats. These pilot studies are presented in Chapter 6.

CHAPTER 2
INVESTIGATION OF URINARY NITRIC OXIDE METABOLITES AND CYCLIC GUANOSINE MONOPHOSPHATE EXCRETION IN PREGNANT RATS

2.1 INTRODUCTION

2.1.1 Animal models of protein restriction

There has been increased interest in the effects of low protein diets administered to rats during pregnancy as a result of the "Barker" hypothesis (Barker, 1994). This hypothesis proposes that adaptations made by the human fetus to poor maternal nutrition program its subsequent development and increase its risks to of essential hypertension, type 2 diabetes and stroke in adult life (Barker *et al*, 1989, Law *et al*, 1993, Law *et al*, 1996, and Yiu *et al*, 1999). Rat models of low protein feeding are now widely used to investigate the mechanisms that link maternal nutrition in pregnancy with hypertension and abnormal glucose metabolism in the offspring. The 9% casein (low protein) diet used throughout the studies to be presented is based upon several years' experience (Langley-Evans *et al*, 1996) of this diet in Southampton University (using 18% casein the control). The effects of a low protein diet on maternal vascular function in rats has hitherto been restricted to the use of 6% casein (Rosso and Streeter, 1979; Lederman and Rosso, 1989).

In contemporary research, details of the low protein diets vary between laboratories and these may alter the programming effects. A direct comparison of two different diets in terms of their ability to programme cardiovascular disease in the offspring was undertaken by Langley-Evans (2000), using the diet used at Southampton and the Hope Farm diet used by Hales and colleagues in Cambridge (Hales *et al*, 1996) and others (Snoeck, *et al,* 1990, Ozanne *et al,* 1996). The Hope Farm diet

provided casein at 22% or 9% by weight (control or low protein diet respectively). The Southampton and Hope Farm diets differ, principally, in terms of the level of fat, the type of fat, the composition of the carbohydrate and the overall methionine content. The Southampton diet provides fat as corn oil and carbohydrate as a starch-sucrose mix (Langley-Evans *et al*, 1994). The Hope Farm diet provides fat as soya oil and carbohydrate as glucose (Lucas *et al*, 1996). The Hope Farm diet contains only half the fat of the Southampton diet. Soya oil provides three fold more α-linoleic acid (18 : 3) than corn oil. One explanation of the difference between the Hope Farm and Southampton low protein diets, in terms of their blood pressure programming effects, may be that the n-3 fatty acids of the soya oil in the Hope Farm 9% casein diet give rise to series 3 prostanoids which differ in their inflammatory and cardiovascular properties to the series 1 and 2 prostanoids generated from n-6 and n-9 fatty acids (Langley-Evans, 2000). The Hope Farm and Southampton 9% casein diets also differ in the overall methionine content. The Southampton 9% casein diet provides 0.5g methionine/ 100g diet compared to 0.08g/100g in the Hope Farm 9% casein diet, a sixfold difference. It was demonstrated that blood pressure was elevated in the offspring of rats fed the Southampton diet, whereas animals exposed to the Hope Farm diet *in utero* did not become hypertensive. Langley-Evans concluded that different low protein manipulations in rat pregnancy invoke different programming effects and that the balance of proteins with other nutrients may be a critical determinant of the long-term health effects of maternal undernutrition in pregnancy. The effects of subtle differences within the 9% casein diets have not been explored in the current studies; the most important difference with previous work being the use of 9% casein as opposed to 6% casein (Rosso and Streeter, 1979). 9% casein is regarded as a mild restriction of protein while 6% casein represents severe protein restriction.

2.1.2. Role of NO in the control of glomerular haemodynamics

During early pregnancy, in humans, rats and other mammals, marked vasodilatation of the kidney and other non-reproductive organs is one of the earliest maternal adaptations to occur during pregnancy. Effective renal plasma flow (ERPF), and glomerular filtration rate (GFR) rise from 30% to 80%, while total peripheral vascular resistance decreases and blood pressure declines modestly. The endogenous nitrovasodilator nitric oxide (NO), has been implicated in the maternal vasodilator response to normal pregnancy and may mediate the hyperfiltration and vasodilatation associated with increased protein feeding/amino acid infusion in rats.

Nitric oxide is a product of arginine metabolism (Palmer *et al*, 1989), it is produced by the vascular endothelium, plays a pivotal role in the regulation of vascular tone, renal blood flow, GFR, renin and angiotensin II (Ang II) generation and sodium excretion. NO, the major contributor to the biological action of EDRF, plays several functional roles in tubuloglomerular feedback regulation and the juxtaglomerular apparatus (Ito *et al*, 1996). The tubuloglomerular feedback or TGF, is the mechanism that operates within the juxtaglomerular apparatus of each nephron and regulates single-nephron GFR. The juxtaglomerular apparatus or JGA, plays a crucial role in the control of renal haemodynamics and renin release (Schnermann and Briggs, 1999) and displays an arrangement of the glomerular afferent and efferent arterioles and the macula densa (a plaque of specialised tubular epithelial cells). Four main mechanisms are thought to operate at the JGA. First, it is speculated that the afferent arteriole somehow senses changes in the luminal pressure, with increased pressure inhibiting renin release. The mechanism that controls renin release is called the baroreceptor. Second, the macula densa senses changes in NaCl concentration in the lumen with decreased NaCl concentration increasing renin release from the juxtaglomerular cells of the afferent and efferent arterioles, which are the major storage sites for renin. Renin released from these cells then functions as an enzyme to increase the formation of angiotensin I which is converted to Ang II. The latter constricts the efferent arterioles, thereby increasing

glomerular hydrostatic pressure and returning GFR to normal. The macula densa, which plays a crucial role in TGF, has the highest levels of NOS in the normal kidney (Wilcox *et al*, 1992). The third mechanism that operates at the JGA is the sympathetic nervous system and fourth is a variety of autacoid hormones involving Ang II, NO, prostaglandins (PGs) and adenosine. The mechanism that contributes to the maintenance of renal blood flow and GFR at a constant level entails the TGF and the myogenic response. The myogenic response exists in the afferent arterioles, which constrict in response to increased arterial pressure. Such constriction under constant luminal flow, would increase shear stress on the endothelium, resulting in enhanced production and release of NO, which in turn may participate in regulating vascular resistance. It has been shown that in isolated microperfused rabbit afferent arterioles, pressure-induced constriction was weaker in the presence of luminal flow (Juncos *et al*, 1995).

2.1.3 Effects of protein and amino acids on renal function

Long-term ingestion of increased levels of dietary protein or short-term intravenous infusion of amino acids, causes an increase in both renal and splanchnic blood flow, an increase in GFR, in both humans (Wada *et al*, 1991, Castellino *et al*, 1986) and animals (Premen, 1988). Ingestion of proteins has been shown to result in the release of a cascade of hormones and vasoactive substances including dopamine (El Sayed, *et al*, 1991), insulin-like growth factor I (IGF-I), angiotensin II (Ang-II) and nitric oxide. Nitric oxide has also been demonstrated to mediate the renal hyperfiltration and vasodilatation associated with acute amino acid infusions and changes in habitual protein intake (King, 1995). Tolins and Raij (1991) demonstrated that the renal vasodilatation and hyperfiltration observed in rats during short term intravenous infusion of a mixed amino acid solution was prevented by a NOS inhibitor but not by the unrelated vasoconstrictor phenylephrine. King *et al*, (1991) similarly demonstrated that

acute and chronic blockade of nitric oxide synthesis diminishes the renal haemodynamic response to protein in rats.

Important changes in nitric oxide metabolism and action occur in early pregnancy. Therefore, the effect of a low protein diet on these factors would be of interest as they are likely to affect the maternal vascular adaptation to pregnancy.

2.1.4 Hypotheses and Aims

In view of the role of NO on the renal haemodynamic effects of a high dietary protein intake (hyperfiltration and vasodilatation), the hypothesis proposes that *maternal urinary concentrations of NOx and cGMP will be lower in pregnant rats fed a low protein diet (9% casein) than in pregnant rats fed a control protein diet (18% casein).*

At the outset of my investigations samples of urine were available from 2 sets of rat experiments (described below), carried out by members of the Institute of Nutrition in Southampton University. The laboratory care of the animals was undertaken by others but information concerning the weight gain of the maternal rats and the urine volumes collected from them over 24 hour periods was provided. The studies described in this chapter are therefore pilot in nature and subject to certain constraints which are mentioned in the discussion.

The aim of the first study was to examine the effects of maternal protein restriction on maternal growth and urinary excretion of NOx during rat pregnancy. In order to establish how urinary concentrations of nitrite and nitrate (NOx) change with advancing gestation in pregnant rats fed either 18% casein or 9% casein, longitudinal measurements were made on gestational days 1, 3, 7, 13 and 18 in serial urine samples obtained from 11 rats. The urine samples were analysed for creatinine and NOx.

The aim of the second study was to measure the urinary excretion rate of the second messenger of NO, cGMP, before and at 13 days of gestation. In addition, the assessment of NO biosynthesis was undertaken in the second study (as in the first study) by measuring the relatively stable metabolites of NO, nitrate and nitrite (NOx) as index of its production.

2.2 MATERIALS AND METHODS

2.2.1 Animal protocols

All experimental work was carried out under license from the British Home Office and was in compliance with the 1986 Animal Act. All animals used were Wistar rats bred in the Biomedical Research Facility of the University of Southampton. Rats were kept in wire mesh cages at a temperature of 22^0C on a 12/12-hour light/dark cycle with free access to food and water at all times. Prior to the experimental diets, the non-pregnant animals received a standard non-purified laboratory chow diet *ad libitum* (CRMX, Special Diet Services, Cambridge, UK) (Appendix 1). On attaining body weights of 200-250 g, virgin female Wistar rats were naturally mated overnight and the morning on which vaginal plugs were identified on the cage floor was considered as day one of pregnancy and the dietary regimes were started at this time point. Mating occurred within one to four days of housing with males. Plug positive females were then separated randomly into two groups and transferred to single cages and fed *ad libitum* either a 18% casein (control) or a 9% casein (low protein) diet throughout subsequent pregnancy (Langley-Evans and Jackson, 1994). Animals were monitored serially for evidence of weight gain consistent with pregnancy. Rats were kept in metabolic cages throughout the experiments.

2.2.2 Dietary details

Casein was used as the protein source for the diets. The experimental diets were purified. The diets were isocaloric containing either 180 g casein/kg diet or 90 g casein /kg diet as the protein source. All experimental diets were made in the University animal facility. The reduction in energy attributable to the lower amount of protein in the 9% casein diet, was replaced with increased carbohydrate content (cornstarch and sucrose) of the diets to maintain the balance of energy but all other nutrients (minerals, vitamins, fat, fibre) were present in equal amounts (Langley-Evans, *et al*, 1996). Both diets were supplemented with DL-methionine to avoid sulphur deficiency, as casein is a poor source of methionine and cysteine. The composition of purified control and low

protein diets is shown in Appendix 2. All components were mixed and bound together with water. The mixture was rolled into small balls and oven dried at 60^0C for 24 hours. The diets were then stored in a cold room at -10^0C for up to three months.

Study 1.

Serial twenty-four hour urine collections on gestational days 1, 3, 7, 13, and 18, were obtained from 5 pregnant rats fed an 18% casein diet and 6 pregnant rats fed a 9% casein diet, yielding longitudinal data on changes in urine concentrations of nitrites/nitrates (NOx) and creatinine.

Study 2.

A longitudinal study was performed to examine concentrations of maternal urinary nitrite and nitrate (NOx), cGMP and creatinine before pregnancy and at 13 days of gestation in rats fed on diets containing either 18% (5 rats) or 9% casein (6 rats).

Because assessment of urinary nitrite and nitrate (NOx) for Studies 1 and 2 was made separately, and because these studies examined different sets of rats, these studies have been presented separately. Pregnancy was confirmed afterwards by allowing the animals to deliver.

2.2.3 Urine collections

Rats were individually housed in metabolic cages. Water and food were provided *ad libitum*. Twenty four hour urine collections were made from the rats using metabolic cages. These are plastic cages, which are designed so that food and water intake and urine and faeces output can be determined. The urine can be collected in a small container at the bottom of the cage. Food and water intake, as well as urinary flow rate were measured by gravimetric technique. Urine samples were sampled in sterile plastic containers and centrifuged at 1000 g for 15 minutes at room temperature to obtain a cell-free solution, acidified using 6 M HCl, to preserve them, and stored at -80^0C until analysis.

2.2.4 Measurement of NO release

Urinary concentrations of nitrite and nitrate (NOx) were measured in all 22 subjects by the reduction of nitrate to nitrite, the latter determined by the Griess reaction, which produces a colorimetric product measured at 540 nm.

2.2.5 Principle of the Griess reaction

Nitric oxide (NO), an unstable molecule, is synthesised from L-arginine by at least three isoforms of the Nitric Oxide Synthase (NOS) and reacts in biological systems with molecular oxygen (O_2), superoxide (O_2^-), and transition metals to form higher nitrogen oxides (NOx), peroxynitrite ($OONO^-$) and metal-nitrosyl adducts (M-NO) respectively.

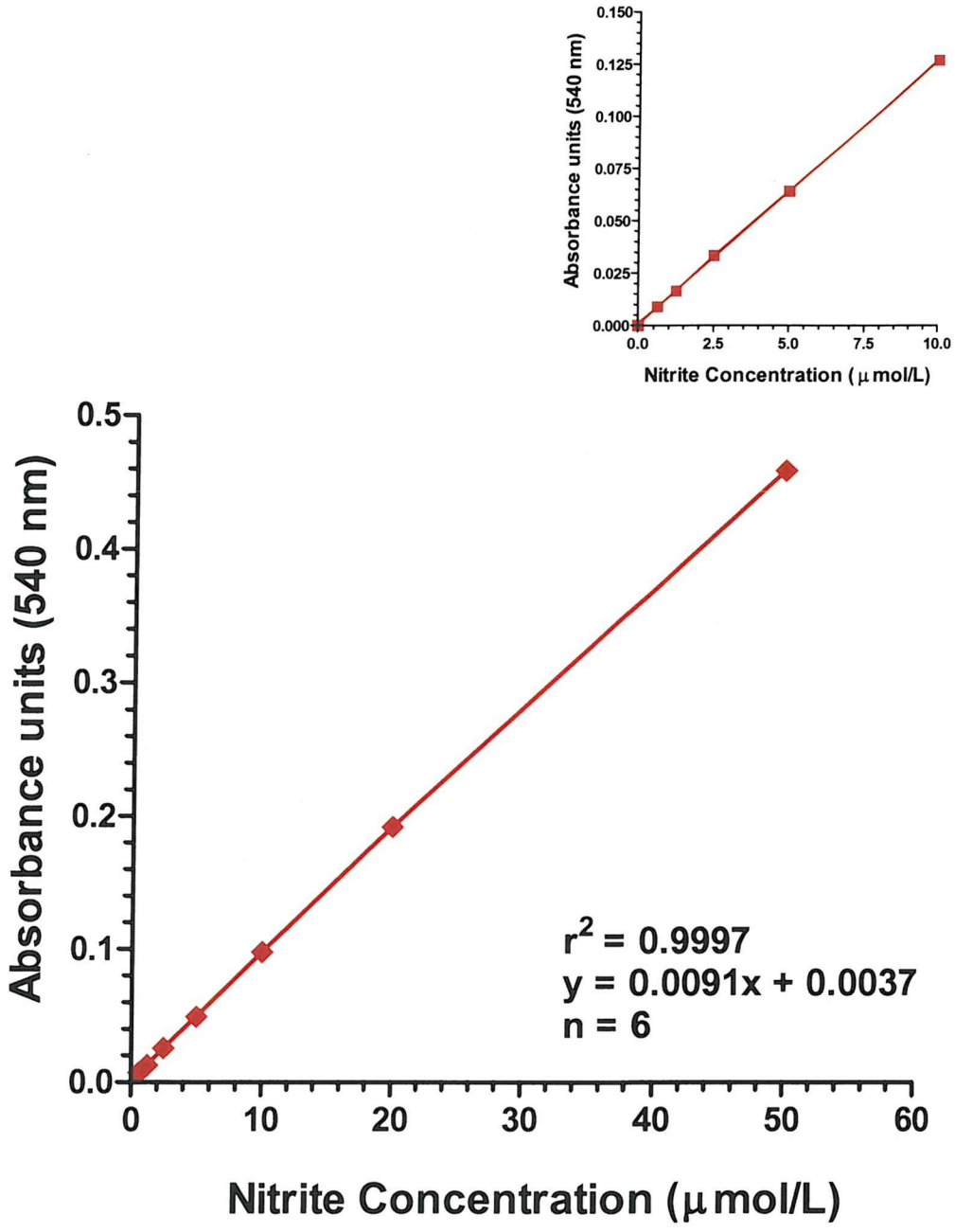
In aqueous solutions nitric oxide is rapidly converted to nitrite (NO_2^-) and nitrate (NO_3^-) which can be quantified by various methods. Nitric oxide production can be determined by measuring the accumulation of these decomposition products by a two-step procedure. The most frequently used method to measure the stable end products in biological fluids such as sera and urine, is based on a purple azo dye that was found by Griess to recognize NO_2^-. Nitrite can be quantitated spectrophotometrically immediately by the Griess reaction. However, the technique has its limitations regarding both sensitivity and its inability to detect NO_3^-. NO_3^- can be measured by the Griess reaction, following reduction back to nitrite before total nitrite (representing both NO_2^- and NO_3^-) can be determined. The reduction can be achieved by using bacterial nitrate reductase. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction. The Griess reaction is based on the two-step diazotisation reaction in which acidified nitrite produces a nitrosating agent which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo derivative which absorbs light at 540 nm (Miles *et al*, 1996).

2.2.6 Determination of Nitrite Based on the Griess reaction

According to various reports, the detection limit for nitrite is between 1.0 and 5.0 μM in biological fluids. (Gilliam *et al*, 1993).

In the present study, nitrite was measured spectrophotometrically by the Griess reaction, as adapted for the 96-well plate reader. Briefly, 100 μl of nitrite-containing standard or sample, was incubated with 100 μl Griess reagent [sulfanilamide, H_3PO_4, and *N*-(1-naphthyl)-ethylendiamine (NED)] (Sigma, cat No G-4410) for 10 min at room temperature in a 96-well plate to form a purple azo dye and absorbance was determined at a wavelength of 543 nm with a microplate reader. After reading at 543 nm, the urine blank was subtracted to yield urinary nitrite. Nitrite levels were calculated from NO_2^- standard curves (0-50 μM) generated in distilled water (Figure 2.1.) (sodium nitrite Sigma, Poole, UK, Cat No S-2252).

Figure 2.1. Mean Standard Curve for the detection of nitrite by the Griess reaction.



Nitrite standards (0.6-50 μM) were prepared in distilled H_2O. One hundred microlitres of sample was combined with 100 μl of Griess reagent [sulfanylamide H_3PO_4, and *N*-(1-naphthyl)-ethylendiamine (NED)], to form a purple azo dye complex measured at a wavelength of 540 nm. The inset shows the lower part of the same curve at a higher magnification. Data are expressed as means ± SE (n=6). Standard error typically 0.0004 - 0.01.

2.2.7 Reduction of nitrate to nitrite

For nitrate assays, each urine sample was diluted 40X with distilled water. After dilution, samples were ultrafiltered through a 10,000 Molecular Weight cutoff filter (Ultrafree-MC Milipore, UK) to eliminate high-molecular weight particles. Twenty units of nitrate reductase (Boehringer Mannheim Cat No 981249) were reconstituted in 2 ml distilled water, aliquoted and stored at -20°C. The assay was performed in a flat-bottomed 96-well microtitre plate (Corning). A 50 µl aliquot of standard or diluted urine sample (Table 2.1) was incubated with 50 µl of a mixture of 10 U/ml *Aspergillus* purified nitrate reductase (Boehringer Mannheim, Germany, Cat No 981249), 1 mM reduced β-nicotinamide dinucleotide phosphate (NADPH) (Sigma, Poole, Dorset UK, Cat No N-6505), 100 µM flavine adenine dinucleotide (FAD) (Boehringer Mannheim, Germany, Cat No 1102346), in a total volume of 100 µl in each well and giving final concentrations of 0.2 U/ml *Aspergillus* nitrate reductase, 5µM FAD and 0.1 mM NADPH. The solutions of sodium nitrate (Sigma, Poole, Dorset, UK, Cat No S-8170) were in distilled water (0-500 µM) (Figure 2.2).

The plate was incubated at 37° C for 1 h. During that time, quantitative reduction of nitrate to nitrite was achieved. NADPH, at concentrations necessary for catalysing nitrate reduction, interferes with the subsequent detection of nitrite by the Griess reaction. To alleviate this problem, 50µl of a mixture of lactate dehydrogenase (1500 U/ml) (Boehringer Mannheim Cat No 127230) and 100 mM sodium pyruvate (Boehringer Mannheim Cat No 128147) were added to each well to oxidise any unreacted NADPH and giving final concentrations 14 U/ml lactate dehydrogenase, and 10 mM sodium pyruvate. The total volume of 150 µl was then incubated for an additional 10 min at 37° C. 150 µl of Griess reagent were then added to each well of the 96-well plate. After a 10-min incubation at room temperature, total nitrite levels (representing NO_2^- + NO_3^-) were determined at 540 nm. The amount of NO_3^- was calculated by subtracting from total NO_2^- the amount of NO_2^- determined before reducing nitrate to nitrite. Many bacteria can reduce nitrate to nitrite and nitrogen and nitrite is the endpoint of determination of nitrate. Because I was not responsible for the

urine collections, urine could have been left outside the refrigerator. Many of my urine collections demonstrated detectable nitrite indicating formation of nitrite by bacteria. These urine specimens were excluded from the study.

2.2.8 Precision and range of assay

The intra-assay coefficients of variation, were 7% and 12% for urine samples of mean concentrations 7.3 μmol/L and 14.6 μmol/L respectively (Table 2.2). The samples were assayed six times on one plate to assess intra-assay precision. The inter-assay coefficients of variation were 7% and 4% for urine samples of mean concentrations 7.6 μmol/L and 14.9 μmol/L respectively (Table 2.2). The samples were assayed in three separate assays to assess inter-assay precision.

2.2.9 Sensitivity

The detection limit for NO_2-, defined as the concentration of NO_2- two standard deviations from the zero standard, was 2.5 μM. The Griess assay was linear between 0 and 100 μM NO_2- or NO_3-.

Table 2.1. Pipetting scheme for the determination of nitrite and nitrate using the Griess reaction.

Step	Procedure	Reagent/sample	Volume (µl)	Concentration Stock	Concentration Final
1	Nitrate reduction	Sample	50		
		Nitrate Reductase (U/ml)	2	10	0.2
		FAD (µM)	5	100	5.0
		NADPH (mM)	10	1	0.1
		Water	33		
		Total	100		
		Incubate at 37°C, 60 min			
2	NADPH oxidation	As in 1	100.0		
		Water	33.6		
		LDH (U/ml)	1.4	1500	14
		Sodium pyruvate (mM)	15.0	100	10
		Total	150.0		
		Incubate at 37°C, 10 min; then cool to 4°C			
3	Diazotization reaction and Azo coupling	As in 2	150		
		Griess Reagent	150		
		Sulfanilamide (H_3PO_4)			
		Hydrochloric acid (HCl)			
		NEDA (mM)			
		Total	300		
		Incubate at room temperature, 10 min; read A_2 at 548 nm (540-nm filter)			

LDH, lactate dehydrogenase; NEDA, *N*-(1-napthyl)-ethylenediamine.

Nitrate Assay

Intra-assay Precision

Sample 1 (µmol/L)	Sample 2 (µmol/L)
7.5	15.0
7.5	17.5
7.5	15.0
7.5	13.8
6.3	12.5
7.5	13.7

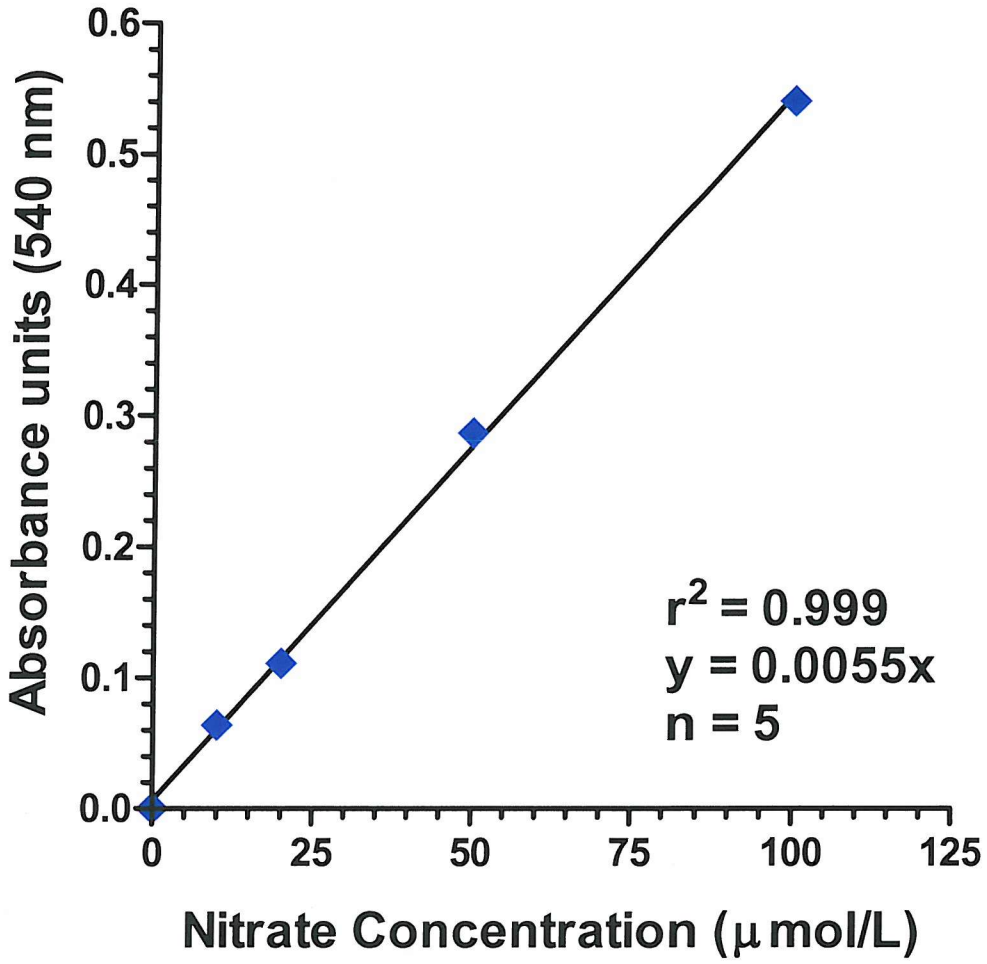
Inter-assay Precision

Sample 1 (µmol/L)	Sample 2 (µmol/L)
7.3	14.6
7.3	15.6
8.2	14.4

	Number of analyses	Mean Concentration (µmol/L) ± SD	Intra-assay Coefficient of variation (%)
Sample 1	6	7.3 ± 0.51	7
Sample 2	6	14.6 ± 1.71	12
	Number of analyses	**Mean Concentration (µmol/L) ± SD**	**Inter-assay Coefficient of variation (%)**
Sample 1	3	7.6 ± 0.51	7
Sample 2	3	14.9 ± 0.64	4

Table 2.2. Precision of Nitrate assay, intra- and inter- coefficient of variation. SD = standard deviation.

Figure 2.2. Mean Standard Curve for the detection of nitrate by the Griess reaction.



Various $NaNO_3$ concentrations were prepared in distilled water. Standards (50 µl) were incubated with 50 µl of a mixture of nitrate reductase (10 U/ml), NADPH (1 mM), and FAD (100 µM) and the nitrite levels were determined as described under Materials and Methods. Values are means ± SE of 5 standard assays. Standard error typically 0.01 - 0.06.

2.2.10 Validation of the Nitrate Assay

The conversion of nitrate to nitrite for each standard (n = 5 determinations per standard) was evaluated (Table 2.3).

Table 2.3. Validation of the nitrate assay.

NO_3^- Standard Concentration (µmol/L)	Average % Conversion	Range (%)
10	65.0	62.0 - 69.0
20	58.0	55.0 - 60.0
50	68.0	65.0 - 70.0

2.2.11 Measurement of the urinary excretion of cGMP during pregnancy in rats

The cGMP was measured by specific RIA. A commercially available ^{125}I-cGMP assay system was used to assay urinary cGMP concentrations (Biotrak cGMP RIA system code RPA525, Amersham International Plc., UK).

2.2.12 Principle of cGMP RIA

The assay utilises a competitive RIA technique. This depends on the competition between unlabelled cGMP present in the sample and ^{125}I-labelled cGMP for a fixed number of binding sites in a polyclonal rabbit anti-cGMP antiserum. An anti-rabbit antibody is then allowed to react with the anti-cGMP complex. As the anti-rabbit antibody is attached to magnetizable polymer particles, the rabbit antibody-bound cGMP fraction can be separated by centrifugation of the mixture followed by decantation of the supernatant. Measurement of the radioactivity in the pellet enables the amount of ^{125}I-labelled cGMP in the bound fraction to be calculated. The concentration of cGMP present in the sample under investigation can then be determined by interpolation from a standard curve.

2.2.13 Buffers and solutions

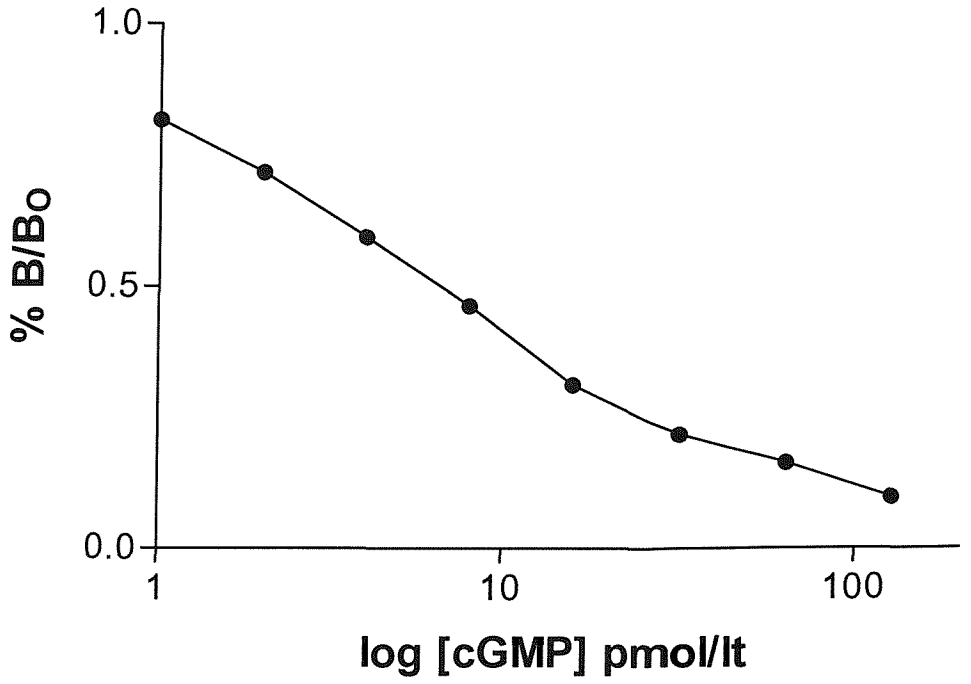
assay buffer: 0.05 M sodium acetate pH 5.8, containing 0.01% (w/v) sodium azide.
standards: 128 pmol/ml stock cyclic guanosine monophosphate non-acetylation standards were stored in 0.05 M acetate buffer pH 5.8 in 500 µl aliquots. Stock solution was double diluted before assay, in assay buffer to give 8 standards of concentrations 1-128 pmol/l.
antiserum: rabbit antiserum to cGMP diluted in 0.05 M acetate buffer containing 1% (w/v) bovine serum albumin and 0.01 % (w/v) sodium azide.
tracer: guanosine 3',5'-cyclic phosphoric acid 2'-O-succinyl 3-[^{125}I] iodotyrosine methyl ester (approximately 1.1 µCi/11 ml assay buffer).
second antibody: Amerlex-M donkey anti-rabbit.

2.2.14 Method of the assay

The assay was performed at room temperature in non-sterile round-bottomed polystyrene assay tubes (code 112201, Greiner Labortechnik, Gloucestershire, UK). Rat urine samples were diluted 1:1000. A 100 µl aliquot of standard or unknown urine sample was assayed in duplicate. 100 µl of rabbit anti-cGMP antibody suspension was dispensed into each tube. The mixture was vortexed thoroughly, and incubated for 1 hour at room temperature. Following the incubation 100 µl [^{125}I]-cGMP (1.1 µCi/11 ml assay buffer) was added to all assay samples, standards and unknowns. The mixture was vortexed thoroughly and the samples were incubated at 2-8°C overnight. On the following day, 500 µl of Amerlex -M second antibody were added to each sample, standard or unknown. All samples were mixed thoroughly and further incubated for 10 min at room temperature. The rack holding the assay tubes were then attached to a magnetic separator base and left for 15 min. The antibody bound fraction was separated by magnetic separation followed by decantation of the supernatant. Following the decantation of the supernatants, the tubes were drained for 5 min onto blotting

paper. The radioactivity present in each sample was determined by counting for at least 60 seconds in a gamma scintillation counter (NE 1608, Nuclear Enterprises, UK) and analysed by computer using the RIA-CALC software program (LKB-Wallac, Finland) The concentration of cGMP in unknown samples was then determined from the standard curve generated (Figure 2.3). The inter-assay coefficient of variation was 9%.

Figure 2.3: Standard curve of cyclic guanosine monophosphate RIA.



cGMP standards (1-128 pmol/lt) were prepared in 0.05 sodium acetate. The percent bound for each standard (% B/B_0) is plotted as a function of the log cGMP concentration. Data points at each concentration of cGMP standard are duplicates.

2.2.15 Creatinine assay

A commercially available creatinine kit was used to measure urinary creatinine concentrations (Sigma, Catalogue No 555-A, Poole, Dorset, UK) (Appendix 3).

2.2.16 Statistical analysis

Urinary NOx and cGMP were calculated as excretion rates /24 h, or expressed in relation to urinary creatinine concentration. Data are presented as median, 25 and 75 percentiles, as well as maximum and minimum values. Statistical analyses were carried out with Prism statistical software package (Graph Pad Software Inc., San Diego, CA, USA). With few data points, the distribution of means does not follow a Gaussian distribution, as indicated by testing for skewness. Skewness is a value that shows how skewed a curve is. The closer to zero the value for the skewness is, the more symmetrical is the distribution. With few data points, the formal test that detects how close a distribution is to normality, the Kolmogorov-Smirnov (KS) test, has little power to discriminate between Gaussian and nongaussian populations (Graph Pad Software Inc., San Diego, CA, USA). The KS statistic quantifies the discrepancy between the distribution of the data and an ideal Gaussian distribution -larger values denoting larger discrepancies. In the studies described in the present chapter, between-group comparisons were analysed nonparametrically by the Mann-Whitney *U* test. The Wilcoxon matched pairs test (nonparametric) was used for comparisons within each diet group. The relationship between $U_{NOx}V$ and $U_{cGMP}V$ levels was evaluated with scatter plots and a Spearman correlation coefficient. Statistical significance was accepted at the 0.05 level of probability.

2.3 RESULTS

2.3A Nitrite and nitrate (NOx) content of the diet.

The NOx content of the experimental diets (18% and 9% casein diets) and of the laboratory chow diet (CRMX, Special Diet Services, Cambridge, UK) were determined after water extraction, homogenisation, and precipitation of proteins with zinc sulfate (Conrad *et al*, 1993). The 18% casein diet ($n = 5$ diet extractions) contained 185.5 ± 20.4 nmol/g NOx. The 9% casein diet ($n = 5$ diet extractions) contained 157.3 ± 14.8 nmol/g NOx. There were no significant differences in NOx content between the two experimental diets ($P = 0.4$). Laboratory chow diet ($n = 5$ diet extractions) contained 359.4 ± 18.8 nmol/g NOx.

2.3.1 Longitudinal Study 1

2.3.1.1 Effect of feeding a low protein diet during pregnancy on maternal dietary food intake, dietary intake of nitrite and nitrate (NOx) and weight gain.

Five virgin female Wistar rats were mated and given free access to a control protein diet (18% casein) throughout gestation. A further six were fed a low protein diet (9% casein) throughout gestation. The food intake was similar in both groups (Table 2.4.).

TABLE 2.4.

Longitudinal changes in dietary food intake during pregnancy in two groups of rats fed either 18% casein or 9% casein.

Dietary Intake (g/24 hrs)

	18% Casein N = 5			9% Casein N = 6			*P* value
	Median	**25th percentile**	**75th percentile**	**Median**	**25th percentile**	**75th percentile**	
Day 1	20.7	19.6	20.8	21.4	20.0	22.9	0.60
Day 3	24.0	22.0	26.5	24.7	17.2	33.5	0.79
Day 7	20.1	18.0	24.0	24.1	19.2	28.0	0.32
Day 13	22.0	21.6	25.0	22.0	16.3	28.0	0.79
Day 18	24.0	22.0	24.0	22.0	19.0	27.5	0.53

Data are presented as median, 25 and 75 percentiles and the *P* value denotes the level of significance of the difference from the control (Mann Whitney *U* test).

Although in general the values of food intake were increased during pregnancy for both the control and low protein groups, this was not significant (Wilcoxon matched pairs test).

The nitrite and nitrate (NOx) intake was similar in both groups (Table 2.4A). Dietary intake of NOx was not significantly altered within groups during gestation (by Wilcoxon matched pairs test).

TABLE 2.4A

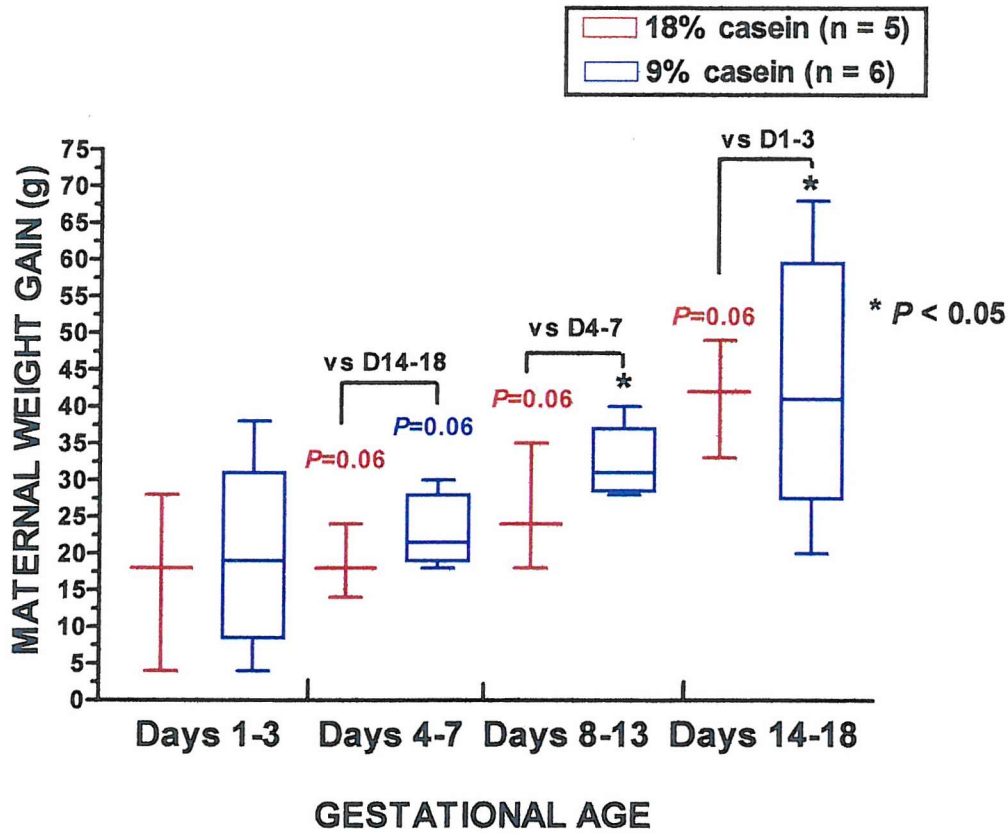
Longitudinal changes in dietary intake of nitrite and nitrate (NOx) during pregnancy in two groups of rats fed either 18% casein or 9% casein.

	Dietary Intake of NOx (μmol/24 hrs)						
	18% Casein N = 5			9% Casein N = 6			*P* value
	Median	25th percentile	75th percentile	Median	25th percentile	75th percentile	
Day 1	3.84	3.64	3.85	3.36	3.14	3.60	0.40
Day 3	4.45	4.08	4.92	3.88	3.20	4.64	0.40
Day 7	3.73	3.34	4.45	3.79	3.45	4.26	1.00
Day 13	4.08	4.01	4.64	3.54	3.03	4.29	0.24
Day 18	4.45	4.08	4.45	3.78	3.30	4.01	0.20

Data are presented as median, 25 and 75 percentiles, and the *P* value denotes the level of significance of the difference from the control (Mann Whitney *U* test).

There was an increase in the mean maternal weight gain in pregnancy within each group. This was significant in the 9% casein group (Figure 2.4). In the 18% casein diet-fed animals, a trend towards an increased weight gain was observed on days 8-13 compared with days 4-7, on days 14-18 compared with days 1-3 and 4-7 ($P = 0.06$) (Wilcoxon matched pairs test) (Figure 2.4).There was a tendency for the 9% casein-fed pregnant rats to gain weight more at each time point compared with 18% casein-fed. However, there were no significant differences in the mean maternal weight gain between the two groups (Mann-Whitney U test).

Figure 2.4. Longitudinal changes in maternal weight gain during gestation in two groups of rats fed either an 18% or a 9% casein diet.



Maternal weight gain in female Wistar rats fed either an 18% or a 9% casein diet during pregnancy. Horizontal line within the box depicts the median value. Top and bottom lines of each box represent the 75th and 25th percentiles, and "flags" at top and bottom show the maximum and the minimum values, respectively. There were no significant differences in the maternal weight gain between the two groups (Mann-Whitney U test). Maternal weight gain (median value) was significantly increased in the 9% casein group at days 1-3 compared with days 14-18 ($P = 0.03$) and at days 4-7 compared with days 8-13 ($P = 0.03$) (Wilcoxon matched pairs test).

The values of total body weight were not significantly different between the two groups throughout the period of gestation (Table 2.5). There were increases in the median values of maternal weight in pregnancy in both groups across all the time points examined (Figure 2.5).

TABLE 2.5.

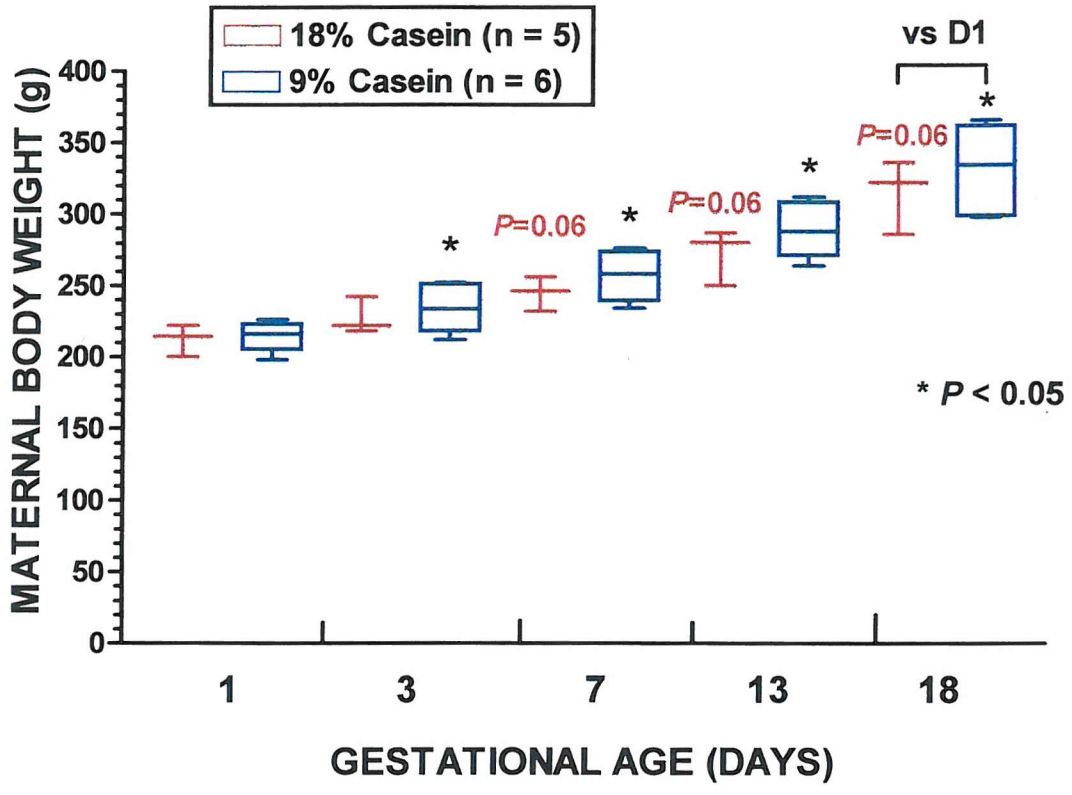
Longitudinal changes in maternal body weight during pregnancy in two groups of rats fed either an 18% or a 9% casein diet.

Maternal Body Weight (g)

	18% Casein N = 5			9% Casein N = 6			*P* value
	Median	**25th percentile**	**75th percentile**	**Median**	**25th percentile**	**75th percentile**	
Day 1	214.0	214.0	214.0	216.0	212.5	219.5	0.79
Day 3	222.0	218.0	234.0	233.5	224.3	248.0[a]	0.32
Day 7	246.0	240.0	252.0	258.0	244.5	271.5[a b]	0.32
Day 13	280.0	264.0	280.0	288.0	278.0	303.3[a b c]	0.32
Day 18	322.0	297.0	323.0	334.5	307.3	353.5[a b c d]	0.18

Data are presented as median, 25 and 75 percentiles, and the *P* value denotes the level of significance of the difference from the control. Data were analysed using the Mann-Whitney *U* test. Median values were not significantly different between the two groups. Data also show significant increase in the median values of maternal body weight in pregnancy within the 9% casein group as early as gestational day 3 (Wilcoxon matched pairs test). The within group significance; 9% casein group: [a]$P = 0.03$ *vs.* day 1. [b]$P = 0.03$ *vs.* day 3. [c]$P = 0.03$ *vs.* day 7. [d]$P = 0.03$ *vs.* day 13.

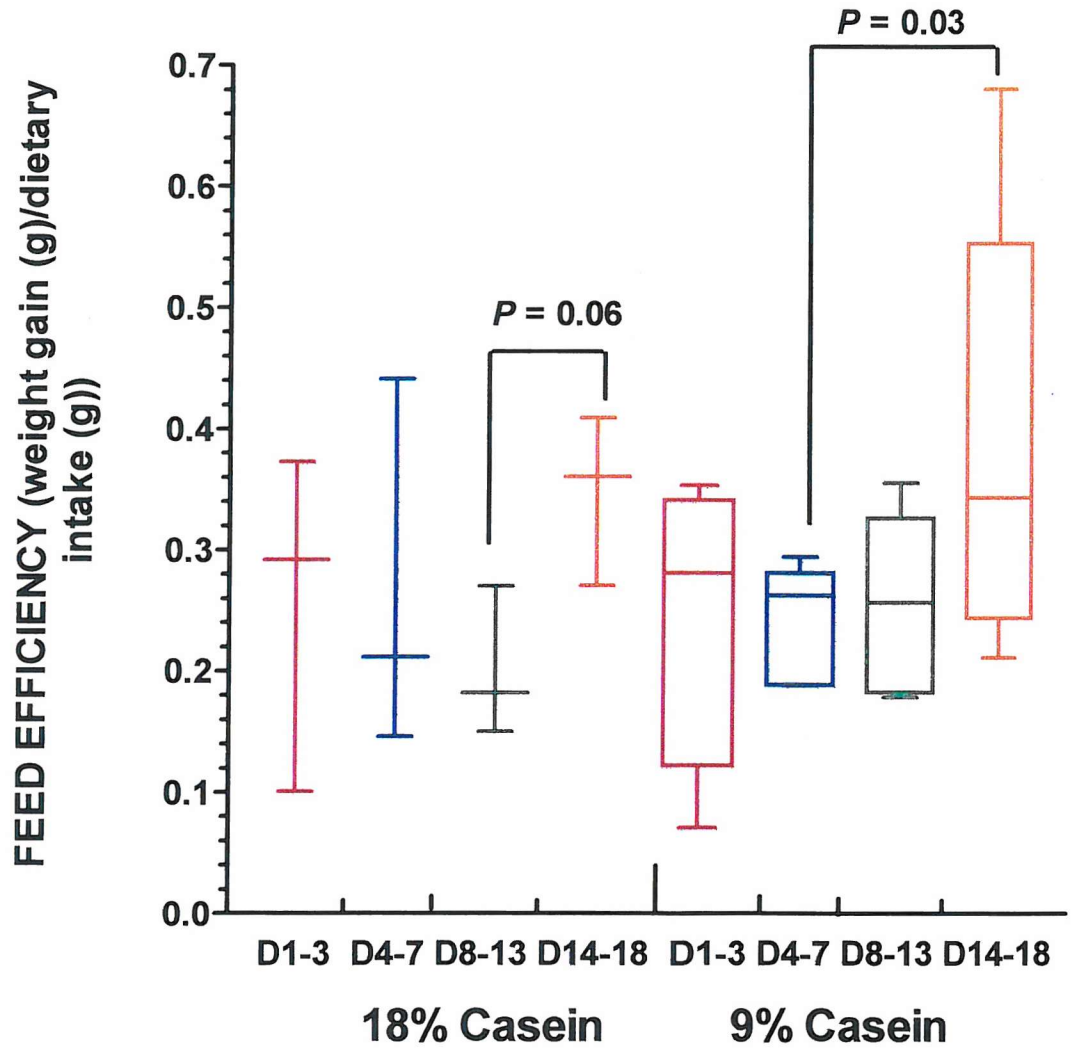
Figure 2.5. Longitudinal changes in maternal body weight during gestation in two groups of rats fed either an 18% or a 9% casein.



Horizontal line within the box depicts the median value. Top and bottom lines of each box represent the 75th and 25th percentiles, and "flags" at top and bottom show the maximum and the minimum values, respectively. In the 18% casein group, medians (horizontal lines), maximum and minimum values are shown for maternal body weight. There were no significant differences in the body weight between the groups (Mann-Whitney *U* test). Data show significant weight gains in pregnancy within the 9% casein group as early as gestational day 3 ($P = 0.03$, Wilcoxon matched pairs test). The within group significance is shown in the figure.

The values of feed efficiency (weight gain/food intake) were not significantly different between the two groups during pregnancy. Feed efficiency increased in the latter part of pregnancy in both groups (Figure 2.6).

Figure 2.6. Longitudinal changes in feed efficiency during pregnancy in two groups of rats fed either an 18% or a 9% casein diet.



Horizontal line within the box portrays the median value. Top and bottom lines of each box represent the 75th and 25th percentiles, and "flags" at top and bottom show the maximum and the minimum values, respectively. In the 18% casein group, medians (horizontal lines), maximum and minimum values are shown for feed efficiency. There were no significant differences in the median values of feed efficiency between the two groups (Mann-Whitney *U* test). Data also show an increase in the median values of feed efficiency in late pregnancy in both groups (Wilcoxon matched pairs test).

Longitudinal Study 1

2.3.1.2 Longitudinal measurements of urinary nitrite and nitrate (NOx)/urinary creatinine ratio.

Pregnancy induced a significant increase in urinary output on gestational day 3 in the 9% casein diet-fed animals ($P = 0.03$, by Wilcoxon matched pairs test) and a trend towards an increased output on day 7 ($P = 0.06$) (Table 2.6). A trend towards an increased output was observed on days 3 and 7 in the 18% casein diet-fed animals ($P = 0.06$) (Table 2.6). The urinary output was significantly reduced ($P = 0.03$) by day 18 in the 9% casein group (Table 2.6). There were no significant differences in urinary output between the groups other than on day 13 when the output was significantly greater in the 9% casein group (Table 2.6). The values of urinary creatinine are shown in Table 2.7. The urinary excretion of creatinine ($U_{CR}V$) was not significantly altered within groups during gestation (by Wilcoxon matched pairs test). There were significant differences in the urinary excretion of creatinine on gestational day 13 between the two groups, ($P = 0.03$, by Mann-Whitney *U* test) (Table 2.7).

Data on twenty four-hour urinary excretion rate of NOx in pregnant rats on the low protein diet showed no alterations in excretion from days 1 to 13, but a decrease on day 18 compared with 18% casein-fed pregnant rats (Fig. 2.7) (18% casein: median value=22.05, 25^{th} percentile = 14.42, 75^{th} percentile = 28.85; 9% casein: median value = 2.37, 25^{th} percentile = 1.37, 75^{th} percentile = 13.62; $P = 0.04$, by Mann-Whitney *U* test). Following adjustment for urinary creatinine concentration, the median value of urinary nitrite and nitrate (NOx) was also significantly greater in the 18% casein group on gestational day 18 compared with the 9% casein group (Fig. 2.8) (18% casein: median value = 3.73, 25^{th} percentile= 2.95, 75^{th} percentile = 4.55; 9% casein: median value = 0.36, 25^{th} percentile = 0.19, 75^{th} percentile = 1.97; $P = 0.019$, by Mann-Whitney *U* test).

TABLE 2.6.

Longitudinal changes in urinary output in female Wistar rats fed either an 18% or a 9% casein diet during pregnancy.

	Urinary Output (ml/24 h)						
	18% Casein $N = 5$			9% Casein $N = 6$			
	Median	25th percentile	75th percentile	Median	25th percentile	75th percentile	*P* value
Day 1	11.0	8.0	11.5	8.5	6.1	13.0	0.93
Day 3	22.4	21.0	32.0[a]	32.0	20.5	33.5[b]	0.66
Day 7	23.0	15.0	30.0[a]	22.0	19.3	25.5[c]	0.93
Day 13	15.0	15.0	19.0	29.6	28.0	32.0[c]	0.008
Day 18	14.5	6.5	21.3	15.0	12.5	16.8[d,e]	0.76

Data are presented as median, 25 and 75 percentiles; N = no of animals. There were significant differences in the urinary output on day 13 between the two groups (Mann-Whitney U test). Data [b] also shows significant increase in the urinary output in pregnancy for the 9% casein group on day 3 ([b] $P = 0.03$ *vs.* day 1; by Wilcoxon matched pairs test). 18% casein: [a] $P = 0.06$ *vs.* day 1. 9% casein: [c] $P = 0.06$ *vs.* day 1. [e] $P = 0.06$ *vs.* days 3, 13. The urinary output was significantly reduced ([d] $P = 0.03$) in the 9% casein group at day 18 compared with day 7.

TABLE 2.7.

Longitudinal changes in urinary excretion of creatinine ($U_{CR}V$) in female Wistar rats fed either an 18% or a 9% casein diet during pregnancy.

	Urinary excretion of creatinine (mg/24 h)						
	18% Casein N = 5			9% Casein N = 6			*P* value
	Median	25th percentile	75th percentile	Median	25th percentile	75th percentile	
Day 1	7.57	5.80	7.59	4.12	3.71	5.41	0.54
Day 3	6.52	6.03	7.32	6.46	5.70	8.03	1.00
Day 7	6.92	6.30	7.49	6.62	5.89	6.77	0.32
Day 13	5.11	4.32	5.78	7.64	7.06	8.84*	0.03
Day 18	5.22	4.84	5.83	6.73	6.02	7.22	0.06

Data are presented as median, 25 and 75 percentiles; *$P < 0.05$.

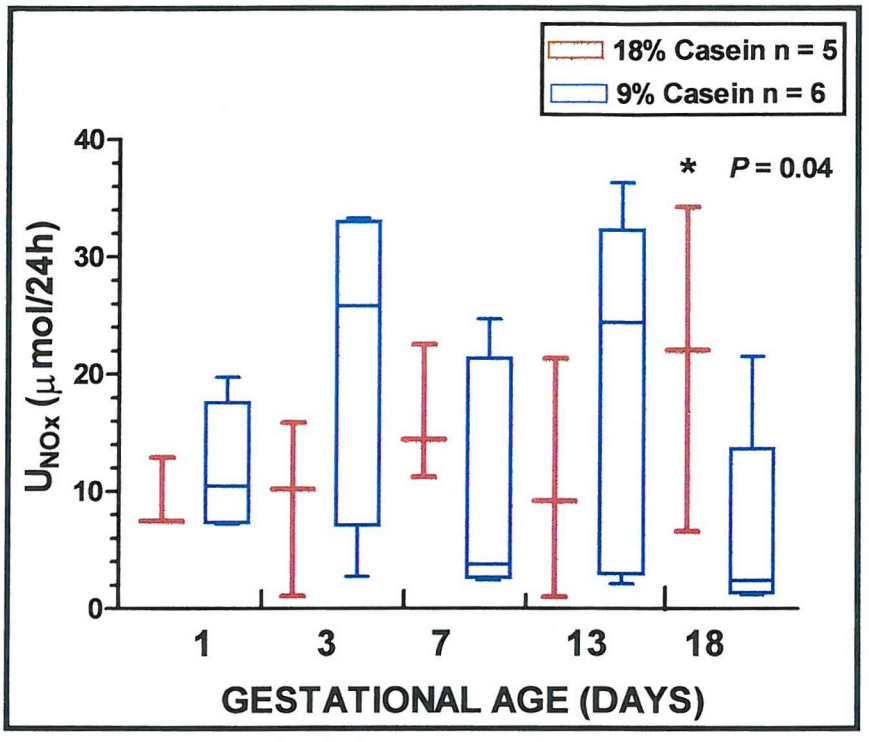


Figure 2.7. Longitudinal changes in 24-hour urinary excretion rate of nitrite and nitrate (U_{NOx}) in female Wistar rats fed either an 18% or a 9% casein diet during pregnancy. See legend to Fig. 2.6 for complete description of boxes, squares and lines.

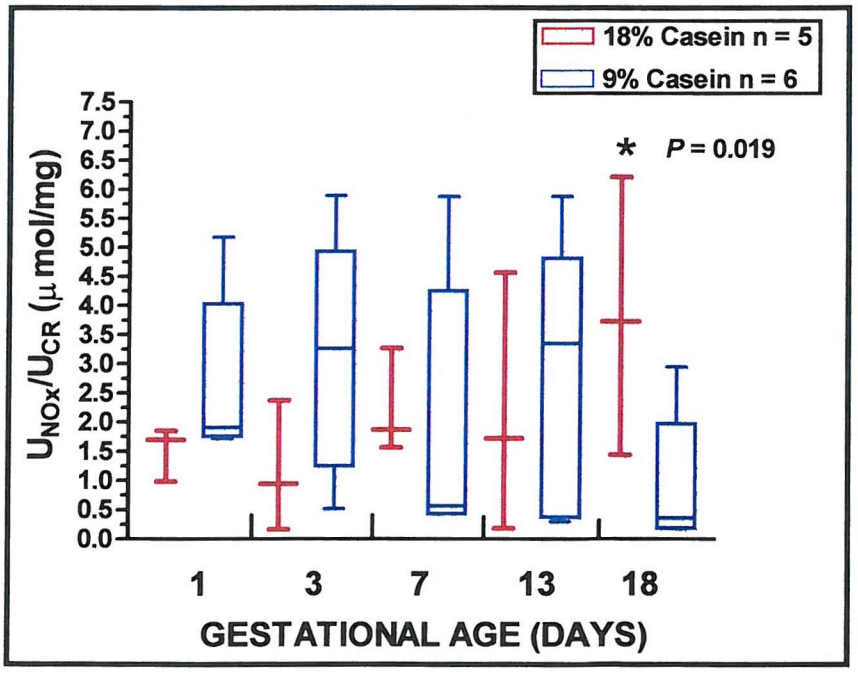


Figure 2.8. Longitudinal changes in urinary nitrite and nitrate (NOx)/urinary creatinine ratio (U_{NOx}/U_{CR}) in female Wistar rats fed either an 18% or a 9% casein diet during pregnancy. See legend to Fig. 2.6 for complete description of boxes, squares and lines.

2.3.2 Longitudinal Study 2

Serial urine samples before and at 13 days of gestation were obtained from five pregnant female Wistar rats fed on a diet containing 18% casein during pregnancy. A further six were fed a 9% casein diet until day 13 of gestation. This study provided longitudinal data on 24 h urinary excretion of nitrite and nitrate (NOx), cGMP and creatinine in pregnant and prepregnant rats fed either 18% or 9% casein during pregnancy. In addition, this study investigated the relationship between urinary cGMP and NOx concentrations in the first two weeks of rat pregnancy.

2.3.2.1 Longitudinal measurements of nitrite and nitrate (NOx), cGMP, and creatinine before pregnancy and at 13 days of gestation in rats fed on diets containing either 18% or 9% casein.

When expressed as micromoles per 24 hours, the urinary excretion of NOx ($U_{NOx}V$) was not significantly different between the two groups either before ($P = 0.6$, by Mann-Whitney test) (Table 2.8), or at 13 days of gestation ($P = 0.6$, by Mann Whitney test) (Table 2.8). A similar pattern was observed when the excretion rates were adjusted for urinary creatinine excretion. When expressed as micromoles per milligram creatinine, the urinary excretion of NOx (U_{NOx}/U_{CR}) was not significantly different between the two groups either before ($P = 0.6$ by Mann-Whitney test) (Table 2.8) or at 13 days of gestation ($P = 0.19$, by Mann-Whitney test) (Fig. 2.9) (Table 2.8).

When expressed as micromoles per 24 hours the urinary excretion of NOx was elevated at day 13 in both diet groups compared with the prepregnant level but not significantly ($U_{NOx}V$, 18% casein pregnant *vs* 18% casein prepregnant, $P= 0.2$), (Table 2.8). ($U_{NOx}V$, 9% casein pregnant *vs* 9% casein prepregnant $P = 0.6$, by Wilcoxon matched pairs test). When expressed as micromoles per milligram creatinine, the urinary excretion of NOx was elevated at day 13 in the 18% casein group only compared with the prepregnant level but not significantly ($P = 0.1$).

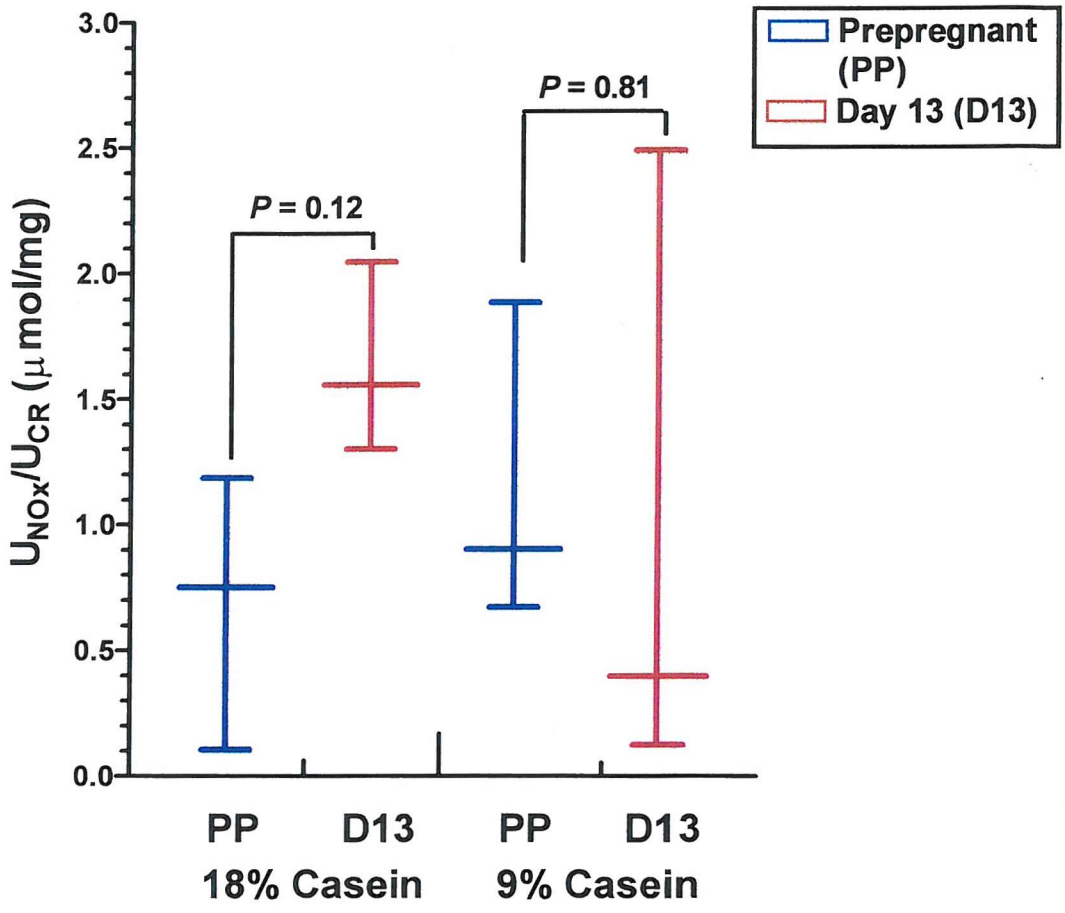
TABLE 2.8.

LONGITUDINAL STUDY 2.: Laboratory data for rats in the study.

	18% Casein Prepregnant		9% Casein Prepregnant		18% Casein Pregnant		9% Casein Pregnant	
	Median	Percentiles 25th 75th	Median	Percentiles 25th 75th	Median	Percentiles 25th 75th	Median	Percentiles 25th 75th
$U_{NOx}V$ (µmol/24h)	5.13	1.58 6.78 (5)	5.97	5.14 7.55 (6)	11.95	8.30 15.24 (5)	6.66	1.50 14.20 (6)
U_{NOx}/U_{CR} (µmol/mg creatinine)	0.76	0.20 0.98 (5)	0.90	0.69 1.01 (5)	1.56	1.32 1.86 (5)	0.40	0.22 1.05 (5)
$U_{cGMP}V$ (µmol/24h)	0.017	0.010 0.029 (5)	0.017	0.017 0.020 (6)	0.034	0.020 0.035 (5)	0.025	0.012 0.030 (6)
U_{cGMP}/U_{CR} (µmol/mg creatinine)	0.0022	0.0019 0.0028 (5)	0.0023	0.0021 0.0027 (5)	0.0042	0.0037 0.0050 (5)	0.0033	0.0023 0.0042 (5)

Data are presented as median, 25 and 75 percentiles; number of rats is stated in parentheses.

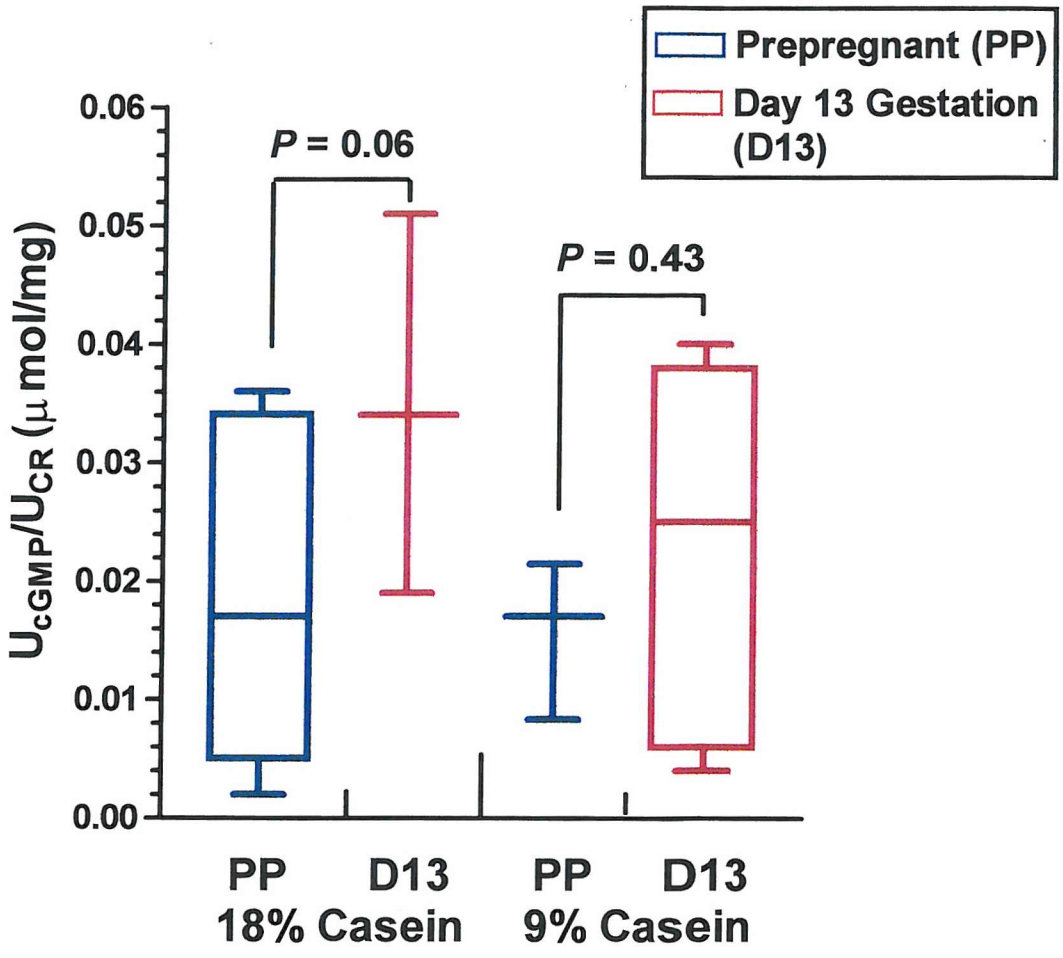
Figure 2.9. **Longitudinal changes in urinary nitrite and nitrate (NOx)/urinary creatinine ratio (U_{NOx}/U_{CR}) before pregnancy and at 13 d of gestation in rats fed on diets containing either 18% (n = 5) or 9% casein (n = 5).**



Medians, maximum and minimum values are shown for the urinary excretion of nitrite and nitrate (NOx)/urinary creatinine ratio (U_{NOx}/U_{Cr}). There were no significant differences in the median values of U_{NOx}/U_{Cr} between the two groups (Mann-Whitney U test). U_{NOx}/U_{Cr} was not different within groups before and at 13 d of gestation (by Wilcoxon matched pairs test).

When expressed as μmol/24h, the median value of $U_{cGMP}V$ was not significantly different between the two groups either before ($P = 0.9$) or at 13 days of gestation ($P = 0.6$). $U_{cGMP}V$ was not significantly different within groups before and at day 13; 18% casein: $P = 0.1$; 9% casein: $P = 1.0$ (by Wilcoxon matched pairs test). When expressed as μmol/mg creatinine, the urinary excretion of cGMP was not significantly different between the two groups either before ($P = 0.9$ by Mann-Whitney test) or at 13 days of gestation ($P = 0.3$) (Table 2.8) (Figure 2.10).

Figure 2.10. Longitudinal changes in urinary cGMP/urinary creatinine ratio (U_{cGMP}/U_{CR}) before pregnancy and at 13 d of gestation in rats fed either an 18% ($n = 5$) or a 9% casein ($n = 5$).

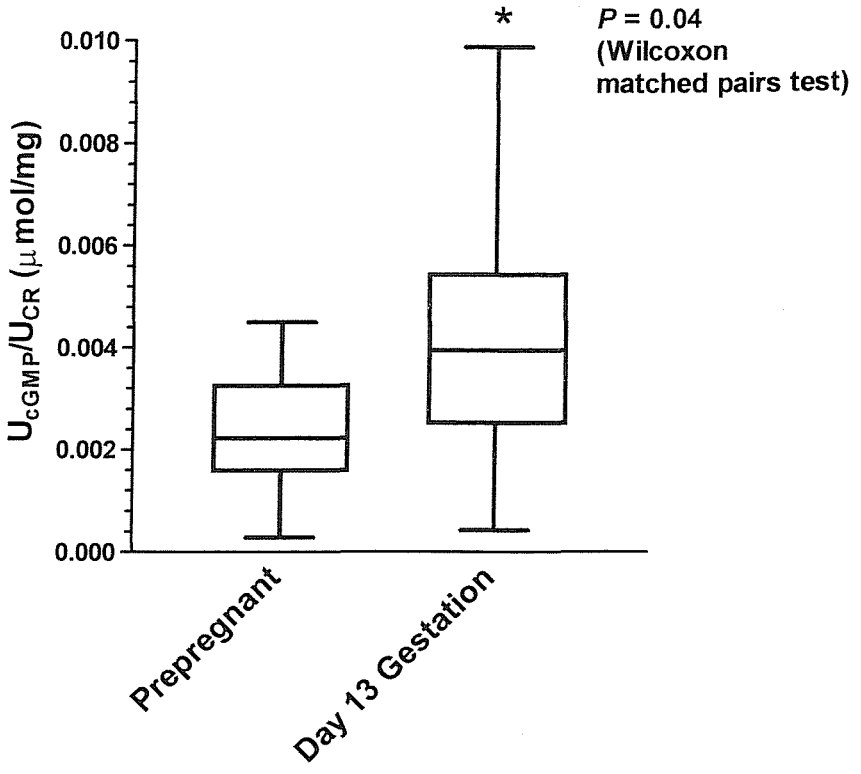


U_{cGMP}/U_{Cr} was not different within groups before or at 13 d of gestation (by Wilcoxon matched pairs test). See legend to Fig. 2.6 for complete description of boxes and lines.

The urinary excretion of cGMP expressed either as micromoles per 24 h or micromoles per mg creatinine, was elevated at day 13 of pregnancy compared with the prepregnant level in both diet groups (Table 2.8). Pregnant rats on the 18% casein had higher urinary cGMP/U_{CR} ratio than rats on the 9% casein (Fig. 2.10). There were no changes in urinary excretion of creatinine ($U_{CR}V$) between the two groups before (18% casein, $U_{CR}V$: median value = 6.97, 25th percentile = 5.55, 75th percentile = 7.60, *n* 6, *vs* 9% casein, median value = 6.90, 25th percentile = 5.05, 75th percentile = 8.40 mg/24h creatinine, *n* 5; *P* = 0.9 by Mann-Whitney test) or at 13 days of gestation (18% casein, $U_{CR}V$: median value = 6.80, 25th percentile = 5.16, 75th percentile = 7.74, *n* 5, *vs* 9% casein, median value = 6.20, 25th percentile = 4.14, 75th percentile = 9.96, mg/24h creatinine, *n* 6; *P* = 0.9).

When the two diet groups were combined, gravid rats of 13 gestational days demonstrated higher levels of urinary NOx when compared with prepregnant values, but it did not reach statistical significance ($U_{NOx}V$, day 13 gestation: median value = 9.84, 25th percentile = 1.83, 75th percentile = 15.57, *n* 11, *vs* prepregnant: median value = 5.87, 25th percentile = 3.24, 75th percentile = 8.29 μmol/24h, *n* 10; *P* = 0.2, by Wilcoxon matched pairs test). The increment in NOx excretion above prepregnant values was approximately 90%. U_{NOx}/U_{CR} was also increased at day 13 when compared with prepregnant values, when the two diet groups were combined (U_{NOx}/U_{CR}, prepregnant: median value = 0.83, 25th percentile = 0.43, 75th percentile = 1.10, *n* 10, *vs* day 13 gestation: median value = 1.30, 25th percentile = 0.31, 75th percentile = 1.92 μmol/mg creatinine, *n* 9; *P* = 0.3, by Wilcoxon matched pairs test). Urinary excretion rate of cGMP was also increased at day 13 when compared with prepregnant values when the two diet groups were combined, but again it did not reach statistical significance ($U_{cGMP}V$, day 13 gestation: median value = 0.017, 25th percentile = 0.012, 75th percentile = 0.027 *n* 11 *vs* prepregnant: median value = 0.026, 25th percentile = 0.020, 75th percentile = 0.038; *P* = 0.1, by Wilcoxon matched pairs test). The overall increment in cGMP excretion above prepregnant values was approximately 60%. A similar pattern was observed when the cGMP excretion rates were expressed in relation to urinary creatinine concentration (Fig. 2.11).

Figure 2.11. Longitudinal changes in urinary cGMP/urinary creatinine ratio (U_{cGMP}/U_{CR}) in 10 rats before and at 13 d of gestation when the 18% and 9% casein groups were combined.



Horizontal line within the box portrays the median value. Top and bottom lines of each box represent the 75th and 25th percentiles, and "flags" at top and bottom show the maximum and the minimum values, respectively. Median values for the urinary cGMP/urinary creatinine ratio were significantly greater on gestational day 13 compared with prepregnant values (P = 0.04 by Wilcoxon matched pairs test).

Furthermore, as shown in Fig. 2.12, linear regression analyses demonstrated a significant positive correlation between the urinary excretion rates of NOx and cGMP when the 18% and 9% casein groups were combined (r=0.63; P<0.01; Fig. 2.12). Urinary NOx excretion rates showed a significant correlation to cGMP excretion in the 9% casein group at day 13 of gestation only. (r=0.886; P<0.05; Table 2.8).

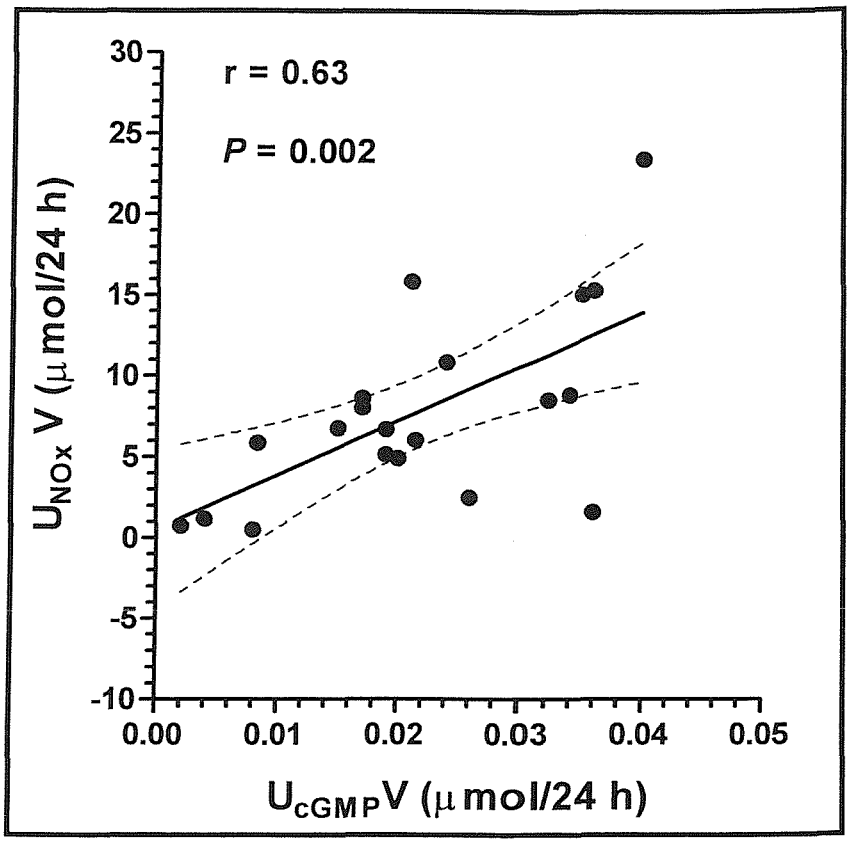


Figure 2.12. The relationship between urinary excretion of cGMP ($U_{cGMP}V$) and NOx ($U_{NOx}V$) in 20 rats before and at d 13 of pregnancy when the 18% and 9% casein groups were combined. Regression line is shown. A significant relationship was observed. (n = 20, Spearman r = 0.63, P = 0.002).

TABLE 2.9.

Correlation between the urinary excretion of NOx and cGMP in female Wistar rats fed on diets containing either 18% or 9% casein.

	Urinary NOx (µmol/24h) *vs* urinary cGMP (µmol/24h)	
	18% CASEIN (n = 5)	**9% CASEIN (n = 6)**
Prepregnant		
R	0.300	-0.154
P	0.683	0.783
Gestational Day 13		
R	0.400	0.886
P	0.750	0.033*
ALL (n = 10)		
	Prepregnant	**Gestational Day 13**
R	0.055	0.733
P	0.892	0.020*

R, Spearman's correlation coefficient for simple regression; P, two-tailed level of significance; *P<0.05.

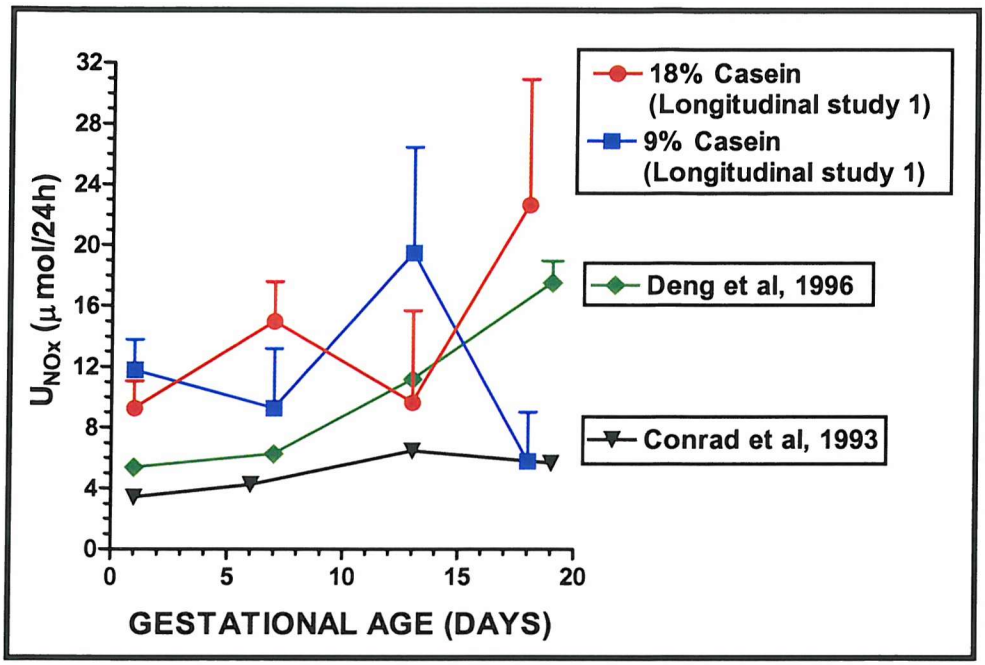


Figure 2.13. Urine excretory rates of NOx during pregnancy. Data obtained from study 1 are compared with published data from Conrad *et al*, FASEB J. 7: 566-71; 1993 and Deng *et al*, Kidney International, 50: 1132-1138; 1996.

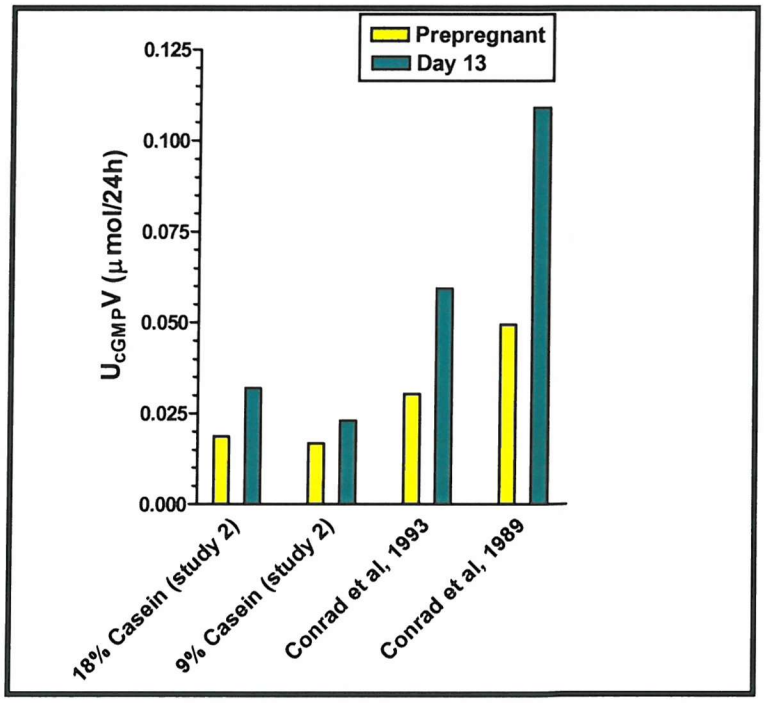


Figure 2.14. Urinary excretion rates of cGMP before pregnancy and at 13 days of gestation. Data obtained from longitudinal study 2 are compared with data from two published works from Conrad and colleagues: 1. *Am. J. Physiol.* 257: R847-R853; 1989 and 2. *FASEB J.* 7: 566-71; 1993.

2.4 DISCUSSION

In the current study, the effects of a low (9% casein) and normal (18% casein) dietary protein intake on the urinary excretion rates of biologic markers of activity of the endogenous nitric oxide system were investigated in pregnant rats.

STUDY 1.

a) Effect of feeding a low protein diet during pregnancy on maternal dietary food intake, dietary intake of nitrite and nitrate (NOx) and weight gain.

The maternal food intakes were not significantly different on the two diets (Table 2.4) which is consistent with a study by Rees *et al* (1999), in which a 9% casein diet identical to that used in the present thesis did not significantly effect maternal food intake during a 19-day experimental period. There was therefore no evidence to suggest that the rats on a mild dietary restriction increased their food intake to compensate for the decrease in protein. The intake of protein remained at approximately 75% of the requirements for non-pregnant rat (Clarke *et al*, 1978). Reduced food intake has been observed when pregnant rats are fed a 6% casein diet during gestation (Lederman and Rosso, 1989).

In the present study, the average dietary intake of NOx by 18% casein-fed pregnant rats on day 18 was 4.48 ± 0.34 μmol/24 h, which might have accounted for an average hourly urinary NOx excretion of 187 ± 14.3 nmol/h. However, urinary excretion of NOx was 21.22 ± 6.0 μmol/24 h on day 18, that is, a value higher than expected from NOx intake. The average dietary NOx intake by pregnant rats on the 9% casein diet on day 18 was 3.72 ± 0.27 μmol/24 h, which might have accounted for an average hourly urinary NOx excretion of 155.1 ± 10.0 nmol/h. Despite the evidence that the mean dietary intake of NOx was 3.72 ± 0.27 μmol/24 h, urinary excretion of NOx was 5.78 ± 3.21 μmol/24 h on day 18 in rats on the 9% casein, a value greater than observed from NOx intake. These data agree with earlier findings by Green *et al* (1981a, 1981b) who reported that dietary NOx ingestion does not account for all of the NOx excreted into the urine in rats and in the man. It has been concluded that a major part of net

urinary NOx excretion is derived from endogenously synthesised NO (Moncada *et al*, 1991). There is evidence that NO synthesised from the terminal guanidino nitrogen of L-Arg is a precursor for nitrite and nitrate in cultured cells (Schmidt *et al*, 1988) as well as in human subjects (Leaf *et al*, 1989). In a study by Böger *et al* (1996a), urinary NOx excretion decreased by 86% after oral bolus administration of L-NAME, which emphasizes the relative contribution of endogenous NO to urinary NOx excretion rates.

The low protein diet administration (9% casein) has no effect on litter size (Rees *et al*, 1999; Rees *et al*, 2000) which is consistent with later studies in this thesis (Chapter 5). Previous studies have shown that litter size is not significantly altered in food-restricted (50%) or low protein fed (6% casein) pregnant rats compared with rats fed 25% casein *ad libitum* (Rosso and Kava, 1980; Lederman and Rosso, 1989). Others have reported a 40% reduction in litter size in rats given a 6% casein diet (Rees *et al*, 1999).

During rat pregnancy, the needs for the elaboration of maternal tissues, net tissue deposition and fetal and placental growth represent additional demands for amino acids. The increase in the rates of protein synthesis during pregnancy, where net deposition is taking place, may be accounted for almost entirely by the high rates of synthesis associated with fetal growth. Nitrogen metabolism in the rat during pregnancy appears to follow a biphasic course. The first 14 days (anabolic phase) are characterised by a marked decrease in amino acid catabolism (Naismith, 1973) and the rat builds up a reserve of protein, and it was by day 7 of gestation that the dams fed the 9% casein diet increased their food intake compared with day 1 (Table 2.4). During the third week (days 15-21) when rapid growth of the fetus takes place, the protein reserves are utilized. This catabolic phase occurs irrespective of the protein intake of the dam and is under hormonal control (Naismith, 1969).

The present data show that the maternal body weight gain (Fig. 2.4) and maternal total body weight (Table 2.5, Fig. 2.5) did not differ between the two groups. Total body weight increased an average of almost 100g in 18% casein-fed

control rats over gestational days 1-18. There was a maternal weight gain of almost 117g by day 18 in the low protein-fed group, an increase of approximately 54% of the initial body weight. Feeding a 9% casein diet during gestation had no effect on maternal net weight at day 19 or 21 compared to pregnant rats fed 18% casein (Rees *et al*, 1999). Rees and coworkers (1999), showed a similar significant increase in net weight gain above weight at mating in dams of the Rowett Hooded strain fed on a low protein diet identical to that used in this thesis and fetal weight had increased at day 19 in the protein-restricted pregnant dams but fell on day of delivery. A later study in this thesis (Chapter 5) showed a decrease in the growth of the fetuses in pregnant rats fed 9% casein at day 18. Naismith (1966, 1969), demonstrated that a diet deficient in protein caused a decrease in maternal carcass weight during the last week of gestation, which is a period of exponential growth and greatest demand for nutrients by the fetus and hypothesized that utilization of maternal nutrient stores moderates the effect of malnutrition (Naismith and Morgan, 1975). Anderson *et al* (1980), showed that an inadequate maternal diet, due to a low food intake (50% restricted), given during the catabolic phase only (days 15-21), or throughout pregnancy, caused increased net maternal weight loss, and suggested that the dam is able to mobilize nutrients stored (body weight gained) during days 1-14 of gestation (anabolic phase). However, mobilization of these nutrients did not significantly increase fetal growth, supporting the hypothesis that the dam is able to compartmentalize available nutrients during malnutrition and prevent serious depletion of these stores by the fetus during the last stages of gestation (Anderson *et al,* 1980). It has been shown that pregnant rats fed 9% casein throughout gestation had growth-restricted fetuses (Langley and Jackson, 1994; Rees *et al*, 1999; Rees *et al*, 2000). In rats fed a protein deficient diet throughout gestation, fetal growth restriction became evident at day 14 (Rosso, 1977a). The dam still had a significant amount of extra weight suggesting that the shortage of protein affects fetal growth before the maternal stores are depleted. However, the most important evidence of maternal compartmentalization of nutrients was the observation that placental nutrient transfer was reduced near term in rats fed a protein-deficient diet from day 5 of gestation. In these studies it was found that the transfer of α-amino isobutyric acid

and both glucose and α-methyl-glucopyranoside to the fetus was significantly reduced in the malnourished animals (Rosso, 1977b; 1977c).

Besides reducing the transfer of nutrients into the fetus, there are also indications that the malnourished dam maintains an increased feed efficiency (Rosso, 1977c). Feed efficiency (weight gained/food eaten) was increased in late pregnancy in both 18% and 9% casein groups (Fig. 2.6). This may be due to the 'programming' of the pattern of growth by early undernutrition (Harding and Johnston, 1995). Increased feed efficiency has previously been reported in the last half of pregnancy in both restricted and well-fed rats (Lederman and Rosso, 1980); to some extent, this probably helps the restricted mother maintain her energy and protein stores.

A number of enzymes involved in amino acid catabolism, including alanine amino-transferase show prominently reduced activity in the latter half of pregnancy (Naismith and Fears, 1971). The activity of alanine amino-transferase in the livers of pregnant rats on a high-protein diet (250 g casein/kg) in late pregnancy (days 15-21) was reduced to one-third of the value for the non-pregnant control (Naismith and Morgan, 1975). In contrast, the activity of the enzyme tryptophan pyrrolase which initiates the degradation of tryptophan to nicotinic acid, has been reported to rise in late pregnancy to a value three times over nonpregnant control values (Harding *et al*, 1961, Naismith and Morgan 1975). The latter study has also shown that the activities of alanine amino-transferase and tryptophan pyrrolase were significantly decreased by 30% and threefold respectively in pregnant rats fed a diet containing 80% less casein (50 g casein/kg) compared to the high-protein exposed rats (250 g casein/kg) during the last week of pregnancy.

STUDY 1.

b) Longitudinal measurements of urinary nitrite and nitrate (NOx)/urinary creatinine ratio.

In normal pregnancy, urinary excretion of NOx has been shown to increase progressively in the rat and reaches a maximum by late pregnancy (Deng *et al*, 1996; Fig. 2.13). In addition to a general increase in NO production, which contributes to the gestational vascular refractoriness and fall in blood pressure, increased NO production plays a role in the renal vasodilatation of pregnancy (Raij and Baylis, 1995). Chronic NO blockade during pregnancy leads to suppression of the normal peripheral and renal vasodilatation (Baylis and Engels, 1992). Therefore, increased NO production may be responsible for the peripheral and renal vasodilatation in the normal pregnant rat. Recent work by Cadnapaphornchai and colleagues (2001) has shown elevated plasma NOx levels at early and midpregnancy (from days 7-14) in the rat compared with non-pregnant values. These data for excretion of nitrite/nitrate are consistent with a previous study where urinary NOx excretion increased throughout pregnancy with a 3.4-fold increase present at day 19 (12.2±0.7 to 41.1±1.3 μmol/24 h) and returned to prepregnant values by day 4 postpartum (Alexander *et al*, 1999). In the same study, the observed increase in NOx excretion during pregnancy coincided with a decrease in serum L-arginine levels with the lowest levels observed by mid pregnancy (day 13), compared with prepregnant levels. Another prior report of pregnant rats on a normal diet has documented progressively increased levels of urinary metabolites nitrate and nitrite (NOx) during pregnancy (Conrad *et al*, 1993). In the studies by Conrad *et al* (1993), in addition to 24-hour $U_{NOx}V$, increases in plasma NOx concentration were also observed during gestation that were closely correlated with increases in cGMP, the second messenger of NO. This latter study found increased urinary nitrate excretion (26% increase

compared with prepregnant values) in pregnant rats as early as gestational day 6, around the time of implantation (Conrad *et al*, 1993, Fig. 2.13).

The results of the first study showed no alterations in urinary NOx excretion from days 1-13 of pregnancy in the rats exposed to the 9% casein diet but on day 18, a significantly reduced urinary NOx excretion was noted on the low protein diet both in the absolute excretion (Fig. 2.7) and following adjustment for urinary creatinine concentration (Fig. 2.8). Because both the 18% and 9% casein groups had similar food intakes, it is unlikely that differences in dietary nitrate intake could explain these results. Although these data suggest that the activity of the endogenous vascular NO system may have been reduced on the 9% casein diet, the urinary NOx reflects whole body NO production and it is uncertain how much of that reflects vascular NO production. Furthermore, NO metabolism in the kidney itself may also be important; it has been reported that the amount of renal endothelial NOS ($_{ec}$NOS) decreases by 39% in late pregnant rats, whereas the renal inducible NOS (iNOS) and neuronal NOS (nNOS) increase by 31 and 25% respectively (Alexander *et al*, 1999). These factors mean that the data obtained in this pilot study must be interpreted with caution.

Finally, there was an important difference in urine NOx levels between the present work and Conrad's work (Conrad *et al*, 1993). The urine NOx levels at any given day of gestation were at least 3-fold greater in the present study. This difference in urine NOx levels between the two studies could be due to methodological weaknesses. First, the dietary intake of nitrate can dramatically affect the urinary excretion of NOx and more reliable measurements of nitrate, and hence NO biosynthesis, may be obtained when dietary intake of nitrate is reduced and controlled. Such dietary measures were implemented in the current work as well as Conrad's work. Second, 24-h urine collections may not have been reliable and therefore would not represent the steady state. Third, the variation between the present study and Conrad's work could be due to differences in the NOx assays used. Direct measurement of NO is difficult, especially *in vivo*. NO decomposes rapidly in biologic solutions into NO_2 and NO_3. These stable decomposition products serve as reliable markers of NO production in vitro and in

vivo. Changes in renal excretory function may influence urinary excretion rates of NOx and this may interfere with the usefulness of the urinary NO_2 and NO_3 as index parameters for NO formation. Correction of urinary NOx concentrations by urinary creatinine concentration eliminates the dependency on renal excretory function (Böger *et al*, 1996).

STUDY 2.

Previous reports on plasma level or urinary excretion of NOx during normal human pregnancy are controversial, with some showing an increase in either the plasma or urinary nitrate (or nitrite) level (Myatt *et al*, 1992, Nobunaga *et al*, 1996) and others no change (Smarason *et al*, 1997). In a recent study, Conrad and coworkers (1999) reported increases in cGMP production during the first trimester in women on a reduced NOx diet. In contrast, whole body nitric oxide production as estimated by the plasma level and urinary excretion of NOx was either unchanged or reduced during early pregnancy when both the increase in cGMP and active maternal vasodilatation are marked. These findings suggest that unlike the rat, in which NO mediates vasodilatation of the renal and perhaps other vascular beds during pregnancy (Sladek *et al*, 1997), another vasodilator such as carbon monoxide (Durante and Schafer, 1998) or C-type natriuretic factor (Espiner *et al*, 1995), may mediate increased cGMP production during human pregnancy.

Conrad and Vernier (1989), have found that the metabolic production rate of cGMP was increased by twofold in conscious gravid rats, as determined by the constant-infusion technique using [^{3}H]cGMP. Because cGMP is filtered at the glomerulus, increased plasma levels, resulting from enhanced production of cGMP during pregnancy, would raise the filtered load, thereby potentially augmenting urinary excretion rate. When the amount of cGMP excreted into the urine was factored by urinary creatinine, renal excretion of cGMP was also increased, thus gestational increments of GFR do not explain augmented urinary excretion rate.

In the second study, the effects of low (9% casein) and normal (18% casein) dietary protein intake on urinary excretion of NOx and cGMP were investigated before pregnancy and at day 13 of gestation. At day 13 of gestation, urinary NO_2 and NO_3 and cGMP excretion were reduced but not significantly in rats receiving a low protein diet as compared with controls, either in terms of absolute excretion rate or when corrected for creatinine concentration. Urinary excretion of cGMP increased during pregnancy in both groups but did not reach statistical significance possibly because an insufficient number of animals were examined. When the two dietary treatment groups were combined, U_{cGMP}/U_{CR} rose to significantly exceed nonpregnant values at day 13, although only by a small degree (P=0.04) (Fig. 2.11). In the absence of plasma levels, it is not possible from the study described in the present chapter to determine with certainty the source of this cGMP. It is not known to what extent cGMP produced by the renal microcirculation enters the urinary space.

In conclusion, assessment of whole body NO production by measurement of 24 h nitrate/nitrite or by measurement of urinary cGMP levels did not consistently support the hypothesis that NO production was reduced on the 9% casein diet.

To overcome the shortcomings of investigating NO production through measurements of urinary NOx, the direct effects of a 9% casein diet on the mesenteric vascular function were studied in arteries from virgin and pregnant rats. The responses of the vessels to vasoactive agents, the effects of which are known to be mediated by, or are independent of NO, were investigated using small vessel myography. The studies are presented in Chapters 3, 4 and 5. If the 9% casein diet alters the vascular reactivity of the blood vessels, this would provide evidence for mechanisms that might contribute to the impairment of the maternal cardiovascular adaptation to pregnancy in the malnourished dams. The search for the cellular and vascular mechanisms underlying the reduction in the expansion of plasma volume in protein-restricted pregnant rats should help us to understand better the physiological response to malnutrition in pregnant women.

CHAPTER 3
INVESTIGATION OF VASCULAR FUNCTION USING MYOGRAPHY

3.1 INTRODUCTION

The vascular system is composed of a number of vascular segments coupled in series and in parallel. The segments coupled in series consist of large elastic arteries, different orders of large and small arteries, arterioles, capillaries, venules and veins (see Chapter 1). The primary role of the precapillary vessels within the vascular system is to transport blood to the tissues. This role is achieved through the ability of the cardiovascular system to adjust the resistance of each vessel by controlling its lumen diameter. The vessels that contribute most to the precapillary resistance are known as resistance arteries (Christensen and Mulvany, 1993). They include prearteriolar vessels with diameters less than 500 micrometers. The resistance vessels exert their function through the resistance which they present to the blood flow. Small arteries also play an important role in the determination of peripheral vascular resistance (Mulvany, 1990). The introduction of the myograph allowing segments with diameters as small as 150-200 micrometers to be studied, has been a major methodological advancement (Mulvany and Halpern, 1977) and has made possible the functional *in vitro* study of small arteries.

3.1.1 In vivo techniques

There are several techniques which have been used in humans to assess vascular reactivity *in vivo*. Most of the available non-invasive methods that have been utilised to assess NO bioactivity in human peripheral vessels evaluate either basal or agonist-induced changes in vascular resistance, or changes in the diameter of the vessel in response to changes in blood flow. A well-established technique that has been

employed to study mechanisms of vasomotor function is venous occlusion plethysmography, which when applied to the peripheral vasculature, detects changes in the limb volume over time and can be used to assess limb blood flow and venous capacitance (Hokanson *et al*, 1975). Venous occlusion plethysmography has been used to evaluate the effect of endothelium-dependent and endothelium-independent vasodilators on limb resistance vessels. Drugs are delivered into the limb via a cannula which is placed in an artery proximal to the area of interest, most often the brachial artery for infusion of drugs and if vascular resistance is to be calculated, measurement of blood pressure is also involved. Forearm blood flow is assessed during the drug infusions by venous occlusion plethysmography. A variety of endothelium dependent vasodilators can be administered via the brachial artery, including the cholinergic agonists acetylcholine, substance P and bradykinin. Using this technique, the first demonstration in humans that an abnormality of endothelium-dependent vasorelaxation occurs in essential hypertension was obtained (Linder *et al*, 1990, Panza *et al*, 1990, Calver *et al*, 1992).

Doppler velocimetry measures flow velocity. Recent studies have indicated that in combination with intravascular ultrasound imaging to measure vascular cross-sectional area, Doppler velocimetry gives an estimate of volumetric blood flow. Other techniques such as high-resolution vascular ultrasound use transducers that transmit high-frequency sound waves, typically in the range of 7.5-10 MHZ, into the tissue. A two-dimensional image of the blood vessel is produced when a series of sound waves is transmitted and the reflected echoes are reformatted into a single display. High-resolution ultrasound permits precise calculation of brachial artery diameter, to give a direct measure of vasodilatation. Several groups have utilised this method because it is convenient and non-invasive. Its limitation is that it provides data only on flow-mediated vasodilatation which is only one aspect of endothelium-dependent vasodilatation. Newer imaging techniques include magnetic resonance imaging (MRI) and are rapidly becoming a preferred non-invasive method of imaging the cardiovascular system. MRI uses radio waves and a strong magnetic field, rather than x-rays, to create detailed images of blood vessels without the use of contrast media

necessary in x-ray angiography. Laser Doppler flowmetry (LDF) permits the measurement of the flux of red blood cells, defined as the number of red blood cells times their velocity, as an indication of blood flow in the microcirculation. LDF has been used to measure microcirculatory blood flow in many tissues, including neural, muscle, skin, bone and intestine.

3.1.2 In vitro techniques

The first apparatus, or myograph, for the determination of contractility of small arteries to agonist stimulation was developed by Bevan and Osher (1972). They showed it was possible to mount segments of 200-μm vessels as ring preparations on two fine wires, with the wires being clamped at each end, to ensure that the responses were isometric. Subsequently, a more stable myograph was developed using tungsten 30- to 40- μm mounting wires (Mulvany and Halpern, 1977) that allowed the investigation of 100-μm vessels. The technique was characterised by being relatively atraumatic, with the measured responses exceeding the estimated in vivo responses. The Mulvany myograph was used for the studies presented in this thesis.

Pressure myography has been used to test pressure-diameter relations of cannulated small arteries. Video measurements are done of external and internal diameters through a widow in the base of the myograph with continuous display of microscope image, vessel dimensions, flow, force, and temperature on monitor. Such myographs are clearly a more physiological arrangement than the wire myograph and potentially even less traumatic. Pressure myography also allows smaller vessels to be examined (down to 12 micrometers pressurised diameter). Moreover, the pressure myographs allow investigation of the vasoconstrictor response to raised intraluminal pressure, which the geometry of the wire myograph clearly precludes. The less sophisticated wire myographs, have advantages regarding the investigation of specific mechanisms where vessels can be maintained under isometric conditions.

3.2 Myographic protocols used in this study

3.2.1 Mesenteric resistance artery preparation

The arteries used in this thesis were all from female Wistar adult rats. The rat mesentery was prepared as previously described (Gerber *et al*, 1999). Rats were killed by inhalation of a rising concentration of CO_2 followed by cervical dislocation. The abdomen was then opened and approximately 10 cm of the small intestine together with its feeding vasculature, including part of the superior mesenteric artery, was quickly excised and placed in ice-cold physiological salt solution (PSS; see *Drugs and solutions*, section 3.5) and transported directly to the laboratory. All dissection was performed in dissection dishes filled with Sylgard (R) 184 silicone elastomer gel (Industrial Silicones and Lubricants Ltd., Stourbridge, UK) to hold the fixing pins. The mesenteric arcade was spread out in the dissection dish containing cold PSS and secured with butterfly needles through the gut wall. Care was taken to avoid longitudinal stretch. Mesenteric arteries were dissected from the biopsy using a light microscope (magnification x 40). Care was taken to preserve viability by repeatedly replacing the dissection medium with fresh cold solution. Small mesenteric arteries were exposed by carefully dissecting fat and connective tissue overlying them, using small Vanna scissors (straight 3 mm cutting edge; Product number 0103102, John Weiss and Son Ltd., Milton Keynes, UK) and fine dissecting forceps (Watchmaker's needlepoint; Product number 0101372, Weiss). The artery and vein were distinguished and the vein dissected away from the artery. The artery was then cleaned of remaining connective tissue by cutting the fine membrane connecting the connective tissue to the artery; care taken to avoid any direct contact between the dissection equipment and the artery to be investigated. Isolated artery segments of about 10 mm in length were divided into approximately three equal pieces.

For the experiments described in Chapters 4 and 5, arteries were selected and isolated on the basis of their branching order from the superior mesenteric artery. In

order to obtain arteries of approximately equal diameters in control and low protein fed animals the third order mesenteric arteries were routinely dissected. Third order mesenteric arteries were those starting from the third branch point and were used throughout those experiments. The average arterial internal diameter was approximately 300 μm and was not different between groups in these experiments. Vessels were stored at 4° C in PSS and mounted within one hour.

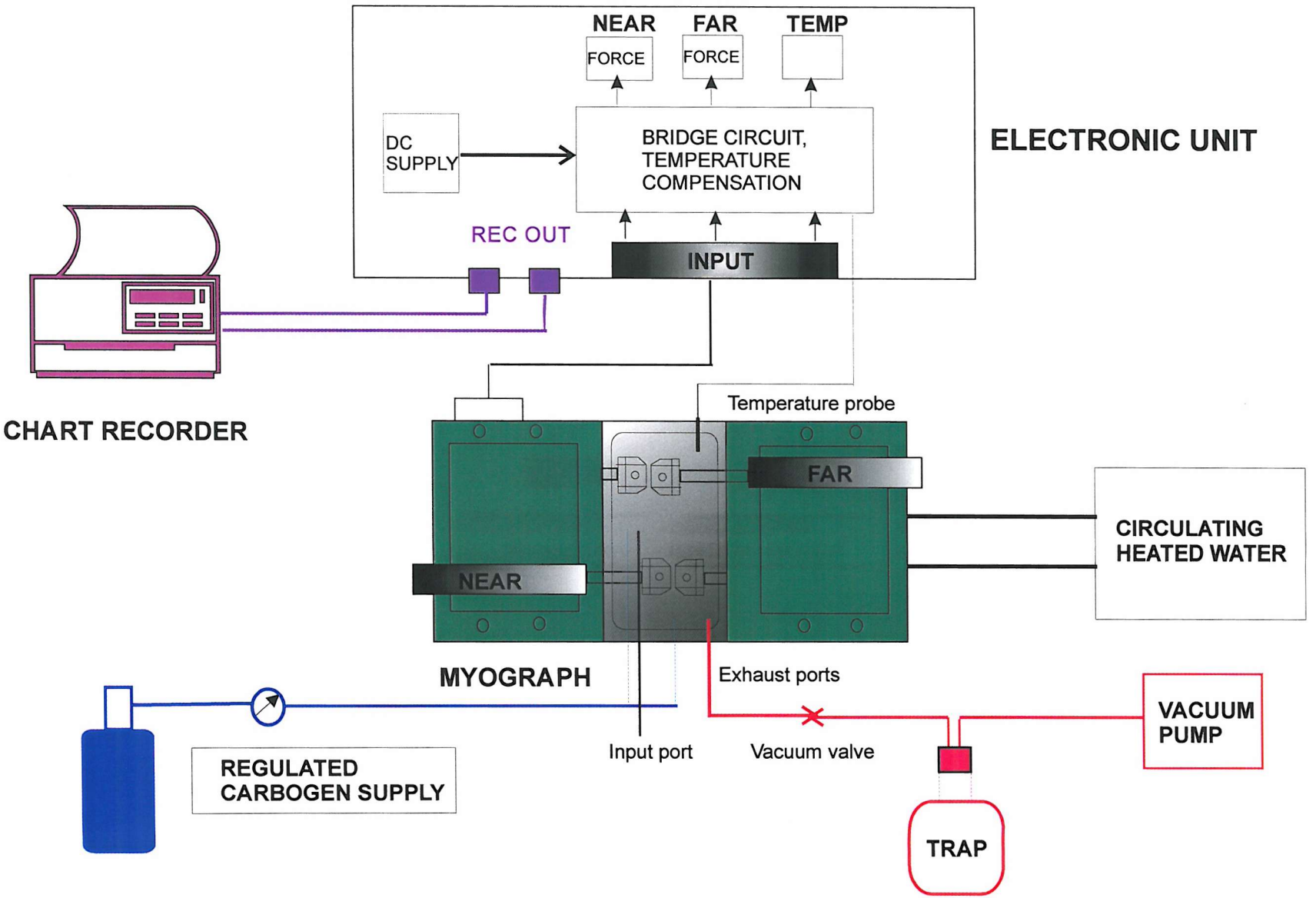
3.2.2 The wire myograph

The Mulvany-Halpern small vessel wire myograph (model 400A, J.P. Trading, DK-8000 Aarhus, Denmark) allows examination of small vessels (internal diameter 0.1-3mm) under isometric conditions, in which the circumference of the vessels is kept constant. In the isometric system, the muscle contracts against a force transducer without decreasing the muscle length. This system (Fig. 3.1) consists of a specialised stainless steel vessel bath chamber, the myograph, with inlet and outlet ports in the chamber cover for rapid draining and gas supply and an optical window underneath that permits morphological or fluorescence measurements. The myograph used contained two sets of specimen supports enabling the simultaneous examination of two arteries. The vessels were each mounted on a pair of jaws; one of the two jaws was connected to a force transducer to measure tension development, the other to a micrometer to permit adjustment of the vessel circumference. The angle of the jaws can be adjusted at the micrometer side by controlling the four Allan screws located on the corners of the uppermost plate of the peripheral blocks. The arteries were held in place by two tungsten wires 40 μm in diameter passed through the lumen. The chamber has a capacity of 15 ml, though 10 ml of liquid is adequate to cover the vessels. Each arterial segment was mounted as a ring preparation and was suspended within the chamber. Solution was delivered to the vessel by a funnel connected to an input port situated on one side. Solution was removed from the chamber by bilateral exhaust ports, connected by tubing to the trap of a vacuum pump (Fig. 3.1).

There were two further input ports situated on the chamber with a low set aperture. This was to allow continuous aeration (with 95% O_2 and 5% CO_2) of the solution in the chamber to maintain a pH of 7.4 and also to ensure circulation of the solution within the vessel bath chamber (Fig. 3.1).

As mentioned before, the bath chamber was located in the middle of a large stainless steel block through which thermostatically heated water from a waterbath was circulated (Fig. 3.1). This block acted as a thermal sink and limited slight changes in the temperature of the chamber. When the vessels had been mounted, a perspex lid was placed over the chamber in order to decrease heat loss. A temperature probe was inserted into the chamber so that its tip was in contact with the solution inside. This was connected to the electronics unit and the temperature of the solution in the vessel chamber of the myograph was monitored constantly and maintained at 36.6-37°C. Test solutions were heated and gassed with 95% oxygen in a waterbath in order to ensure a temperature of 37°C at the moment of delivery into the vessel chamber.

Figure 3.1.: Arrangement of the myograph and associated equipment.


NEAR
FAR
TEMP
FORCE
FORCE
DC SUPPLY
BRIDGE CIRCUIT, TEMPERATURE COMPENSATION
ELECTRONIC UNIT
REC OUT
INPUT
CHART RECORDER
Temperature probe
FAR
NEAR
CIRCULATING HEATED WATER
MYOGRAPH
Exhaust ports
VACUUM PUMP
REGULATED CARBOGEN SUPPLY
Input port
Vacuum valve
TRAP

3.2.3 Force calibration

The myograph force transducers were calibrated before carrying out each set of experiments and after transport of equipment or replacement of force transducers. The force transducers were calibrated using a uniquely devised calibrating balance obtained together with the myograph from J.P. Trading, Aarhus, Denmark. The principle of the force calibration procedure is that when a load W is placed on the appropriate load pan, the force transducer is subjected to a force $F_{transducer}$ equal to:

$$F_{transducer} = W \bullet g \bullet (\text{pan arm} / \text{transducer arm length})$$

where *g* is the gravitational acceleration (9.81 $m \bullet s^{-2}$ = 9.81 mN/gram) and pan arm/ transducer arm length is the 'arm ratio'. In the standard calibrating balance, pan arm = 2 cm, and transducer arm = 4 cm. Thus arm ratio = 2/4 = 0.5.

The myograph chamber was filled with liquid (water or PSS) and heated to 37°C. A wire was attached to the force transducer mounting jaw. The calibrating balance was placed on the myograph such that placement of a weight on the relevant load pan loaded the wire in the same manner as a vessel. Loads of 0, 1, 2, 5 g were placed on the load pan and the wall force readings were recorded on the force calibration record sheet (Appendix 6). This was repeated four times. A plot of wall force readings against loads W was constructed and the slope of regression line was determined. The coefficient of determination r^2 was also noted. The calibration factor, alpha, was calculated by:

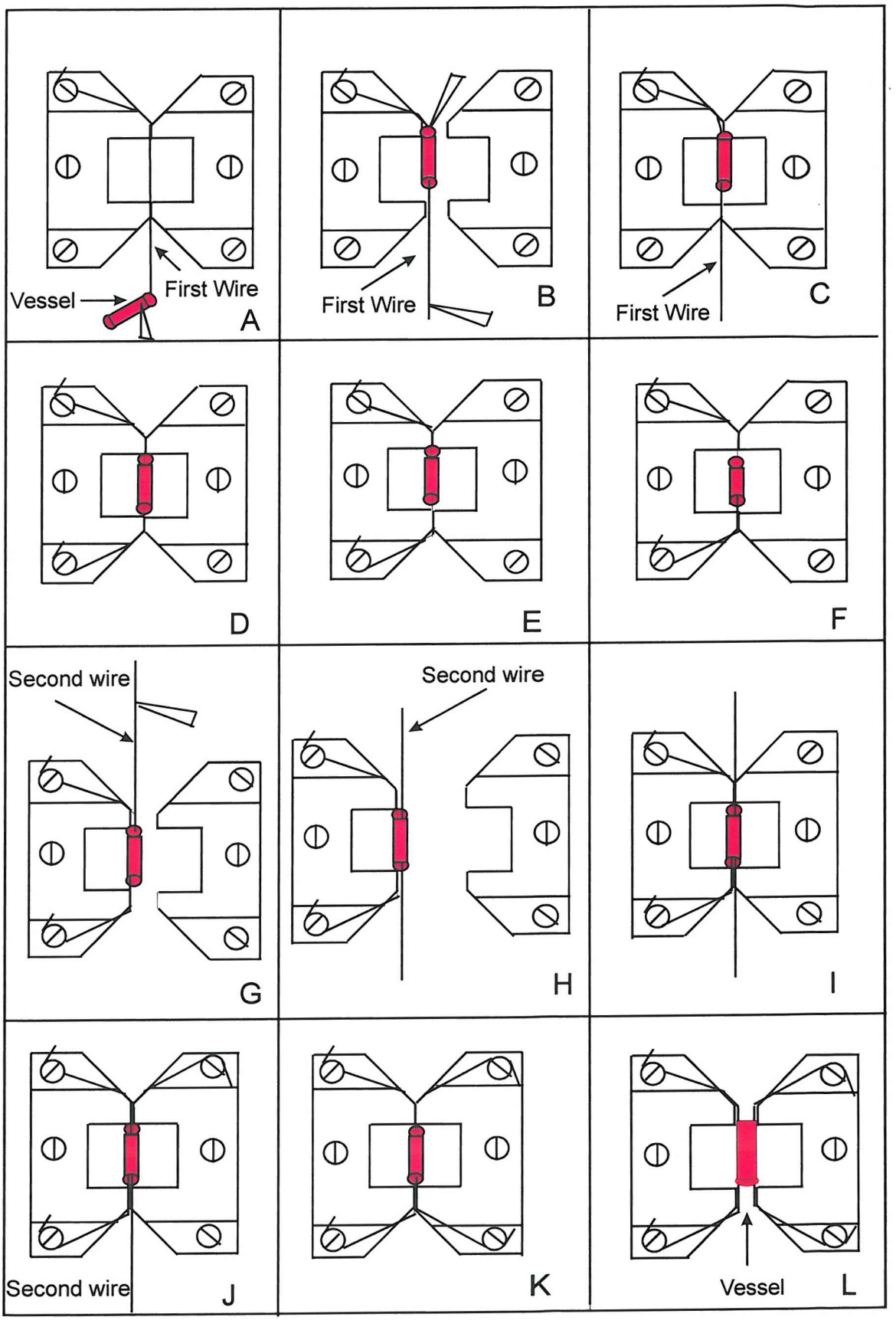
$$\text{alpha} = \frac{1}{b} \bullet 9.81 \bullet \text{arm ratio}$$

where *b* denotes the slope of regression line (units: reading/ gram) and the arm ratio = 0.5. The calibration factor alpha was then entered into the basic normalisation programme (Appendix 5).

3.2.4 Vessel mounting procedure

After dissection, isolated arterial segments with a length of 3 mm were transferred to the chamber of a small vessel wire myograph (model 400A, J.P. Trading, DK-8000 Aarhus, Denmark) and mounted onto two lengths of fine tungsten wire (40 μm diameter) thus securing the vessels via these wires to the two myograph jaws, which are in turn attached to the force transducer. The myograph chamber was filled with 10 ml cold PSS (for composition see *Drugs and solutions*) and equilibrated with room air. A length of wire approximately 2 cm long was placed between the jaws. The jaws were pressed together to clamp the wire, which was secured under the top left screw in a clockwise direction. The first vessel was threaded onto the free near end of the first wire [Fig. 3.2 (A)]. The jaws opened and the vessel was pulled up along the wire, such that the furthest edge of the vessel was clamped at the top when the jaws were rejoined [Fig. 3.2 (B) and (C)]. Care was taken to avoid stretching the vessel if the end of the wire caught on the vessel wall. The near end of the artery should lie about 0.1 mm inside the jaw gap and therefore should not be touched by the jaws thus allowing the artery to move freely in the longitudinal direction [Fig. 3.2 (C)]. The near end of the wire was then secured under the near left screw, again in a clockwise direction, so that tightening the screw also tightens the wire [Fig. 3.2 (D)]. The jaws were then reopened [Fig. 3.2 (G)] and a second wire of similar length was threaded into the far end of the lumen, care taken not to scrape the inner endothelial cell layer. When the wire had fully passed through the lumen, it was positioned with the vessel at the centre [Fig. 3.2 (H)]. The jaws were then closed to secure the second wire [Fig.3.2 (I)]. The second wire was then secured at both ends by the right hand screws [Fig. 3.2 (J) and (K)] and the wires were adjusted to make them parallel and in the same level [Fig. 3.2 (L)]. The jaws were moved hardly apart, so that the vessel was very slightly stretched [Fig. 3.2 (L)]. This procedure was then repeated for the second vessel. The length of both vessels was measured using an ocular micrometer. Thermostatically heated water from a waterbath circulated through a large stainless steel block surrounding the myograph chamber. The myograph chamber contained 10 ml of PSS gassed with 95% O_2 / 5% CO_2 (pH = 7.4). When the temperature in the chamber reached 37°C the vessels were set to their normalised lumen diameter (see *normalisation procedure*).

Figure 3.2.: Mounting procedure (see section 3.2.4 for details).


Vessel
First Wire
A
First Wire
B
First Wire
C
D
E
F
Second wire
G
Second wire
H
I
Second wire
J
K
Vessel
L

3.2.5 Recording output from the myograph

The output from each of the two transducers and the temperature probe was delivered to the electronics unit (obtained together with the myograph from J.P. Trading, Aarhus, Denmark, Fig. 3.1). This unit provided a stabilised power supply for the force transducers and converted the output from the transducers to a voltage appropriate for a two channel chart recorder (Servogor, Model 102). The electronics unit also comprised: LED visual display for each of the two transducers and for the temperature probe, a hold circuit enabling the figures on the visual display unit to be frozen, and a pair of potentiometers for each transducer, required for temperature compensation.

3.2.6 Normalisation of internal circumference

3.2.6.1 Principle of the procedure

The amplitude of active tension development in all muscle types is dependent upon the muscle length as a result of different degrees of overlap of the myosin and actin filaments at different lengths. When a muscle is at its normal *resting length*, it contracts approximately with maximum force of contraction. If the muscle is stretched to greater than normal length before contraction, a large amount of *resting tension* develops in the muscle even before contraction takes place; this tension results from the elastic forces of connective tissue, sarcolemma, blood vessels, nerves and so forth. However, the increase in tension that occurs during contraction, called *active tension* decreases as the muscle is stretched beyond its normal length. This is the active tension-muscle length relation of all muscle types.

The aim of the normalisation procedure was to determine the internal circumference, denoted $L_{100,}$ which the artery would have had if resting *in vivo* under a passive transmural pressure of 100 mmHg. Because the active response of a vessel

depends on the degree of stretch, according to the active tension-internal circumference relation, one should therefore stretch the vessel at a circumference at which it exerts maximum isometric tension. To accomplish this the vessel, once equilibrated at 37°C for 30 min, was stretched sequentially (approximately five times) until an effective pressure (P_i) of approximately 13.3 kPa (100 mmHg) was reached (Fig. 3.3). At each step the wall force readings and the micrometer reading were noted. The micrometer reading gives a measure of internal diameter which is a function of internal circumference (μm) and the wall force reading is divided by the wall length to give wall tension (mN/mm). The effective pressure (P_i) produced at each step was then determined from these parameters using the Laplace equation:

$$\text{Effective pressure } (P_i) = \frac{\text{Wall Tension}}{\frac{\text{Internal Circumference}}{(2 \bullet \pi)}}$$

After the fifth stretch, the lumen diameter was obtained accurately from the passive characteristics of the vessel by finding the intersection of the isobaric line for 100 mmHg, and the passive length-tension relation, which can be fitted by an exponential curve as follows:

$$\text{Resting Wall Tension} = A \bullet e^{\beta D}$$

where A is a constant and β denotes the stiffness factor and D the diameter of the vessel. The isobar has an equation:

$$\text{Resting Wall Tension} = (100 \text{ mmHg}) \bullet \frac{IC}{2\pi}$$

The point where the isobar crosses the curve gives the internal circumference L_{100}. The internal circumference of the artery was then set at 90% of this value ($L_1 = 0.9 \bullet L_{100}$). It has been shown that mesenteric small arteries of adult normotensive rats develop their maximum active tension at approximately this circumference (Mulvany and Halpern, 1977; Aalkjaer and Mulvany, 1981).

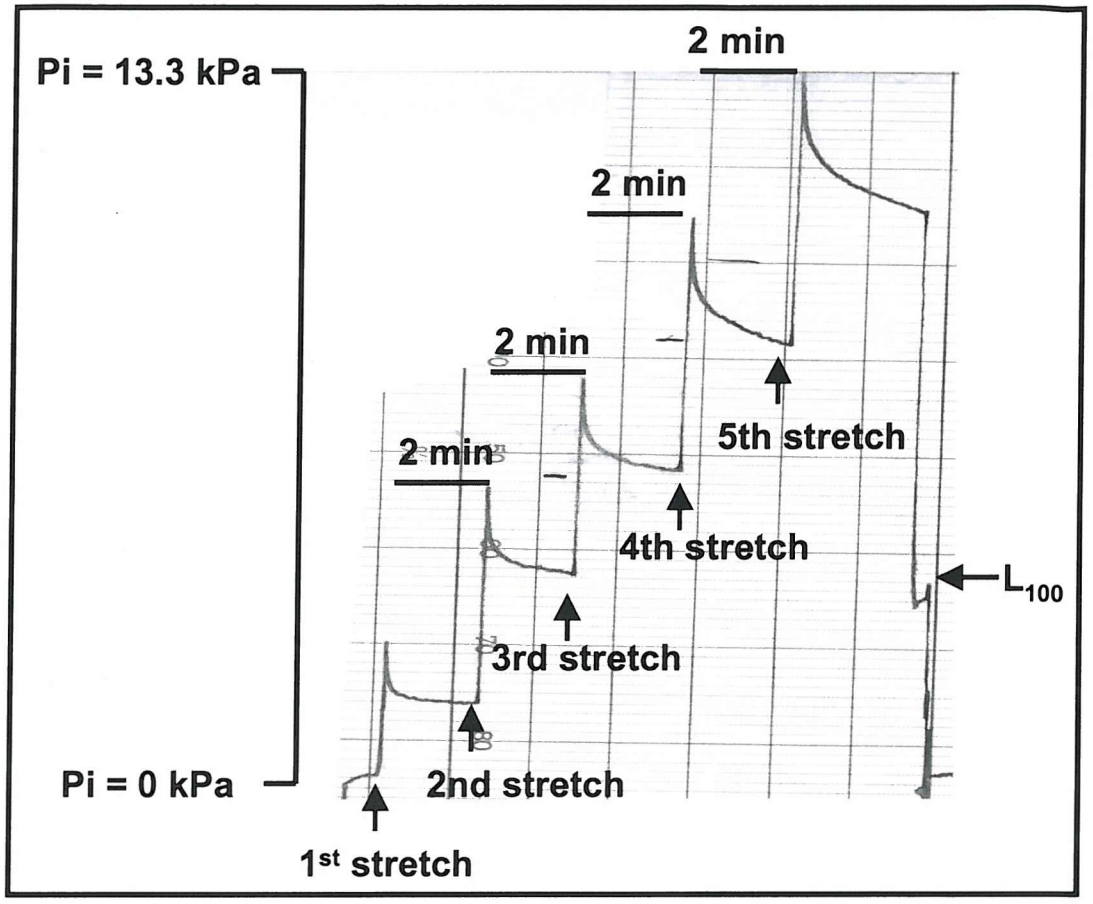


Figure 3.3.: Normalization of internal circumference.

A disadvantage of this approach may be that in vivo, the transmural pressure at the site of the vessel is not necessarily 100 mmHg. In such case using 13.3 KPa (100 mmHg) as intersection point may not be appropriate.

3.2.6.2 The normalisation procedure

The far end and near end of the vessel segment (a_1 and a_2 respectively) were measured in ocular divisions using an Olympus measuring eye-piece mounted in the binocular dissection microscope. These measurements were noted on the experiment record sheet (Appendix 4). Data was entered into a specially written BASIC programme (Appendix 5) run on an IBM PS/2 personal computer.

The wires in the myograph were then set so that they just touched each other. This was evident from a sudden negative displacement on the chart recorder. At this position of the wires the micrometer reading (X_0) was noted. Using the micrometer, the specimen support heads were then moved slightly apart, and at this point the transducer output (Y_0), corresponded to zero wall force, was noted on the experiment record sheet. Y_0 corresponded to the transducer output at X_0. These variables were entered into the programme where alpha (α) and delta (δ) are constants relating to the calibration of the transducer and the magnification of the microscope respectively.

A passive length-tension curve was then constructed by stretching the vessel and calculating the internal circumference and the effective pressure (P_i) from the micrometer reading and wall force reading, respectively. The vessels were stretched using the micrometer so that the output on the chart recorder rose approximately 1 mN. Due to 'stress relaxation' the force then fell and one minute after stretching the vessel, the micrometer reading (X_1) and the corresponding wall force reading (Y_1) were recorded. P_i was calculated instantly. The vessels were subsequently stretched on four more occasions until their calculated P_i exceeded 13.3 KPa. At that point, the programme fitted the length-tension curve to an exponential curve and calculated the three following variables:

I) r^2 : The regression coefficient for the fit of the collected data points of internal circumference and wall tension to an exponential curve. If this coefficient did not equal or exceed 0.99 then the normalisation protocol was repeated.

II) l_{100} : The value for the normalised internal diameter which corresponded to a transmural pressure of 100 mmHg. $l_{100} = L_1/\pi$ where $L_1 = 0.9 \bullet L_{100}$ (i.e. L_{100} is the internal circumference which the artery would have had *in situ* when subjected to a transmural pressure of 100 mm Hg).

III) X_1 : The micrometer setting for the internal circumference which corresponded to an internal diameter of 90% of the normalised lumen diameter l_{100}.

Following normalisation, the micrometer of each artery was adjusted to set the arteries to an internal circumference $L_1 = 0.9 \bullet L_{100}$ where they develop maximal active tension. Arteries were then washed with PSS and allowed to equilibrate for 15 minutes after normalisation before the beginning of experimentation.

3.3 Stimulation and vessel viability protocol (Fig. 3.4).

When both arteries were set to their normalised diameter, a standardized 'run up' procedure of four sequential contractions was performed to test their responsiveness and stability. All arteries were first contracted with 5 μM noradrenaline (NA) in 125 mM potassium solution (KPSS, made by equimolar substitution of NaCl with KCl in PSS) (NAK) for 2 min (Fig. 3.4). A second contraction was produced with 5 μM noradrenaline in PSS (NA). A third contraction was produced by a maximally depolarising K^+ physiological salt solution (PSS containing 125 mM KCl). The vessels were contracted on one further occasion with 5 μM NA in potassium-substituted PSS (125 mM, NAK). The preparations were equilibrated in PSS and then they were subjected to NA or high potassium-induced contractions by changing the solution from PSS to the NA (5 μM), or high K^+ solution containing 125 mM K^+, for 2 min. All solutions were warmed in the waterbath to 37°C before adding them to the chamber. Following each contraction the arteries were washed four times with warm PSS and rested for 10 min between contractions.

3.3.1 Criterion for rejection of results

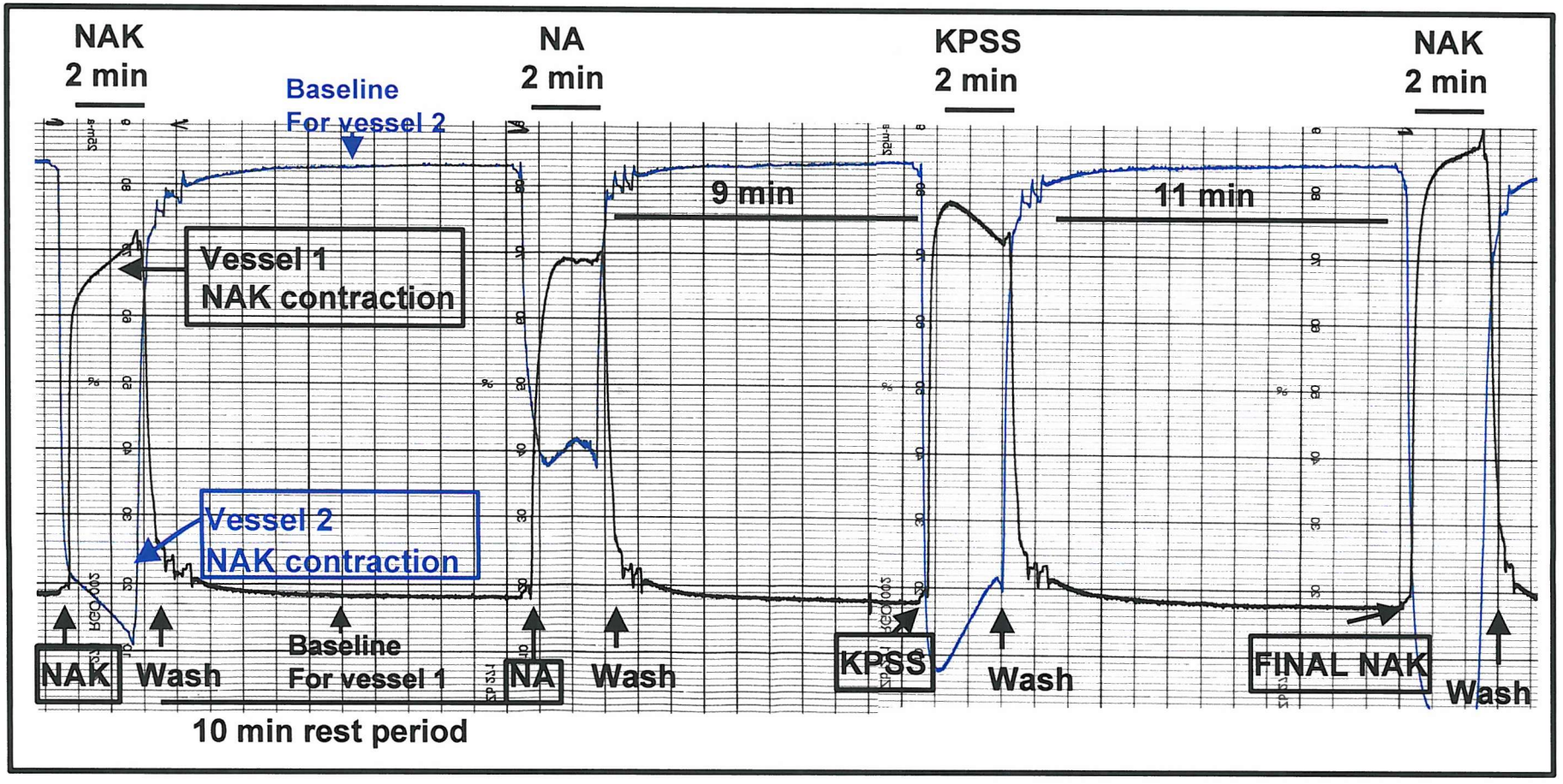
The effective pressure produced by the arteries on the final application of NAK was used as an index to assess vessel viability. Arteries failing to produce maximum active tension equivalent to an effective pressure of 100 mmHg (13.3 KPa) on the last NAK were excluded from the study. Viable vessels were then subjected to concentration response curves according to the protocol required (see *Experimental protocol*, section 3.4).

3.3.2 Functional endothelium test

The integrity of the endothelium was assessed by precontraction to phenylephrine (3-5 μM) and addition of acetylcholine (1 μM). The endothelium was regarded as intact in arteries in which acetylcholine reversed phenylephrine-induced tone by more than 90%.

Figure 3.4.: Representative trace showing the standardized 'run up' procedure of four sequential contractions performed to test vessel responsiveness and viability.

Vessel 1 and vessel 2 were first contracted with 5 μM noradrenaline (NA) in 125 mM potassium solution (NAK) for 2 min. A second contraction was produced with 5 μM NA in PSS. A third contraction was produced by a maximally depolarising KPSS containing 125 mM KCl. The vessels were contracted on one further occasion with 5 μM NA in K^+-substituted PSS (NAK). The preparations were equilibrated in PSS and then they were subjected to NA or high K^+-induced contractions by changing the solution from PSS to the NA (5 μM), or high K^+ solution containing 125 mM K^+, for 2 min.


NAK
2 min
Baseline
For vessel 2
NA
2 min
KPSS
2 min
NAK
2 min
9 min
11 min
Vessel 1
NAK contraction
Vessel 2
NAK contraction
NAK
Wash
Baseline
For vessel 1
NA
Wash
KPSS
Wash
FINAL NAK
Wash
10 min rest period

3.4 Experimental protocol (Fig.3.5)

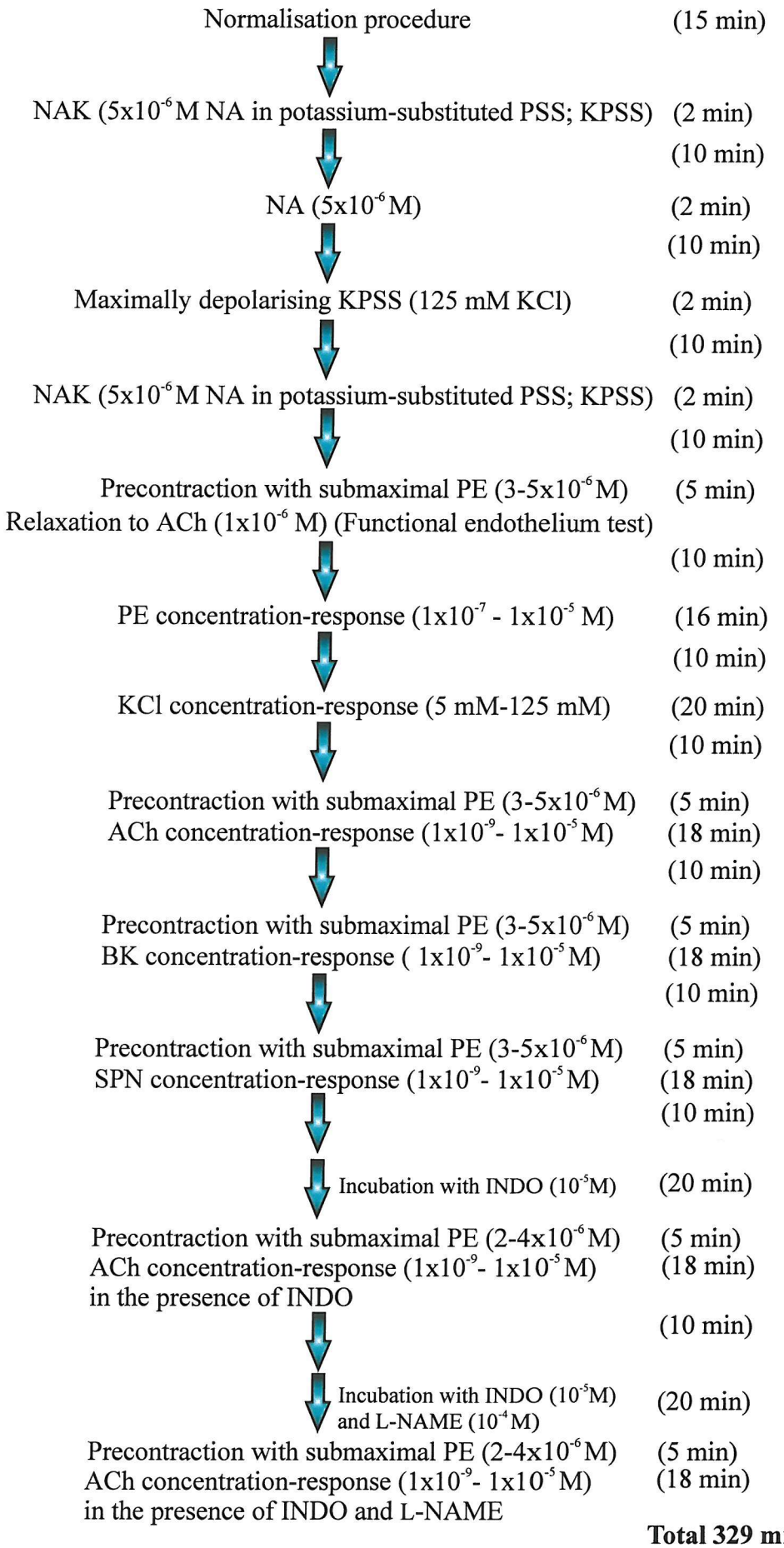
After the routine 'run up' procedure, cumulative concentration response curves, at increments of 2 min duration, were constructed to phenylephrine (PE; $1x10^{-7}$-$1x10^{-5}$ M). Arteries were then washed four times with warm PSS and allowed to equilibrate for 10 min before continuation of the protocol.

Following washout and equilibration (10 min), a dose response curve to potassium chloride was constructed (KCl: 5mM - 125 mM). The myograph chamber was then washed four times with PSS and a 10 min recovery period allowed before the arteries were precontracted with a submaximal (achieving a contraction level of 80% of maximal contraction to KPSS) concentration of PE (5 μM). Once the PE-induced precontraction had reached a plateau, a concentration-response curve was constructed to cumulative doses of acetylcholine (ACh: $1x10^{-9}$ - $1x10^{-5}$ M) at 2 min intervals to determine whether differences existed in endothelium-dependent dilatation between the control and low protein fed groups. A similar protocol was used to assess responsiveness of the vascular smooth muscle to exogenous nitric oxide. Endothelium-independent relaxation to spermine NONOate (SPN: $1x10^{-9}$ - $1x10^{-5}$ M) was assessed in arteries submaximally precontracted with 5 μM PE. The reproducibility of the relaxation to ACh and SPN was tested by obtaining a second concentration-response curve under similar conditions in most experiments.

To determine the relative contributions of prostaglandins and/or NO to the ACh-induced relaxation the arteries were incubated in the appropriate solution for at least 20 minutes before addition of phenylephrine. The ACh response was repeated firstly after incubation of the arteries for 20 minutes with indomethacin (INDO, 10 μM) before addition of PE and secondly following incubation for 20 minutes with both indomethacin (10 μM) and N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM). Arteries were preconstricted with 2-4 μM PE, the concentration being adjusted in order to evoke similar preconstrictor tone to that observed without inhibitors.

Fig. 3.5. : Experimental protocol.

NA noradrenaline, PE phenylephrine, KCl potassium chloride, ACh acetylcholine, BK bradykinin, SPN spermine NONOate, INDO indomethacin, L-NAME N^{ω}-nitro-L-arginine methyl ester.


Normalisation procedure (15 min)
NAK (5x10-6 M NA in potassium-substituted PSS; KPSS) (2 min)
(10 min)
NA (5x10-6 M) (2 min)
(10 min)
Maximally depolarising KPSS (125 mM KCl) (2 min)
(10 min)
NAK (5x10-6 M NA in potassium-substituted PSS; KPSS) (2 min)
(10 min)
Precontraction with submaximal PE (3-5x10-6 M) (5 min)
Relaxation to ACh (1x10-6 M) (Functional endothelium test)
(10 min)
PE concentration-response (1x10-7 - 1x10-5 M) (16 min)
(10 min)
KCl concentration-response (5 mM-125 mM) (20 min)
(10 min)
Precontraction with submaximal PE (3-5x10-6 M) (5 min)
ACh concentration-response (1x10-9- 1x10-5 M) (18 min)
(10 min)
Precontraction with submaximal PE (3-5x10-6 M) (5 min)
BK concentration-response (1x10-9- 1x10-5 M) (18 min)
(10 min)
Precontraction with submaximal PE (3-5x10-6 M) (5 min)
SPN concentration-response (1x10-9- 1x10-5 M) (18 min)
(10 min)
Incubation with INDO (10-5M) (20 min)
Precontraction with submaximal PE (2-4x10-6 M) (5 min)
ACh concentration-response (1x10-9- 1x10-5 M) (18 min)
in the presence of INDO
(10 min)
Incubation with INDO (10-5M) and L-NAME (10-4 M) (20 min)
Precontraction with submaximal PE (2-4x10-6 M) (5 min)
ACh concentration-response (1x10-9- 1x10-5 M) (18 min)
in the presence of INDO and L-NAME
Total 329 min

3.5 Drugs and solutions

During the experimental procedures arteries were dissected, mounted and continuously held in physiological salt solution (PSS). The constituents of the PSS (expressed as mmol/l) were as follows: NaCl 119 (Catalogue number 102415K), KCl 4.7 (Cat No 101985M), $CaCl_2$ 2.5 (Cat No 190464K), $MgSO_4 \cdot 7H_2O$ 1.17 (Cat No 101514Y), $NaHCO_3$ 25 (Cat No 102475W), KH_2PO_4 1.16 (Cat No 102034B), ethylenediamine-tetra-acetic acid disodium salt (EDTA) 0.026 (Cat No 28024), D-glucose 6.0 (Cat No 101174Y). All these chemicals were of Analar grade and were purchased from BDH Chemicals (Poole, Dorset, UK). In high potassium solution (KPSS) NaCl was replaced by KCl on an equimolar basis, giving a final concentration of 125 mM K^+. Solutions were equilibrated with 5% CO_2 in O_2 and had a pH of 7.4-7.5. Drugs were applied extraluminally to the myograph chamber; all concentrations are expressed as final molar concentrations in myograph bath. L-phenylephrine hydrochloride (alpha1-adrenergic agonist, Cat No P-6126), acetylcholine chloride (Cat No A-6625), indomethacin (Cat No I-7378), bradykinin (Cat No B-3259), 8-bromoguanosine-3':5'-cyclic monophosphate-8-Br-cGMP (Cat No B-1381) and N-omega-nitro-L-arginine methyl ester (L-NAME; irreversible inhibitor of constitutive nitric oxide synthase and a reversible inhibitor of inducible nitric oxide synthase, Cat No N-5501) were all purchased from Sigma Chemicals (Poole, Dorset, UK). Spermine NONOate (SPER/NO, (Z)-1-{N-[3-Aminopropyl]-N-[4-(3 amino propyl ammonio) butyl] -amino}-diazen-1-ium-1,2-diolate], Cat No 430-013-M005, was obtained from Alexis Corporation (Nottingham, UK). Noradrenaline bitartrate was purchased from Sanofi Winthrop Laboratories (Guildford, Surrey, UK). Chemicals were prepared as stock solutions dissolved in PSS except indomethacin which was prepared as a 1 mM stock solution in phosphate buffer. The constituents of the phosphate buffer (expressed as mmol/l) were as follows: KH_2PO_4 20 (Cat No 102034B, BDH Chemicals, Poole, UK); $NaH_2PO_4 \cdot 2H_2O$ 120 (Cat No 301324Q, BDH Chemicals, Poole, UK). The pH of the phosphate buffer was balanced to 7.8.

3.6 Analysis of data and statistics

All data in the figures and text are expressed as the means (± SEM) and *n* represents the number of animals used. Mechanical responses of the vessels were measured as force and expressed as active wall tension (ΔT) which is the increase in force above baseline (ΔF), divided by twice the segment length. For vascular protocols, in order to account for differences in artery diameter and therefore in tension development, concentration responses to PE and KCl were expressed as a percentage of the contractile response to KPSS (125 mM KCl)-induced tension. Relaxations were expressed as a percentage of the initial precontraction to PE. Constrictor responses to PE and relaxation responses to ACh were assessed by the concentration of a drug required to provoke a response halfway between the baseline and maximum response (EC_{50}; pEC_{50} = -log EC_{50}), the maximum contraction and maximum relaxation (expressed as a percentage of the maximum active contraction to phenylephrine for a given dose of dilator). Concentration-response curves were analysed by nonlinear regression analysis using the curve fitting programme Graphpad Prism (Graphpad Software Inc., San Diego, CA, USA). Each regression line was fitted to a sigmoid equation:

$$Y = \text{Bottom} + (\text{Top-Bottom}) / (1 + 10^{\text{LogEC50-X *HillSlope}})$$

where X is the logarithm of agonist concentration, Y is the response caused by the agonist, bottom is the response effected by the agonist at the bottom plateau of the curve, top is the response produced by the agonist at the top plateau and Log EC_{50} is the logarithm of agonist concentration required to affect 50% of the maximum response. Two arteries were used from each animal and means were calculated. Data for pEC_{50} are presented as the mean ± SEM of the individual pEC_{50}s. Differences between the means were analysed with Student's unpaired two-tailed *t*-test, as appropriate, or by ANOVA. Paired two-tailed *t*-tests were used to compare sequential concentration-response curves in the presence and absence of inhibitors. Means were considered significantly different at $P<0.05$.

CHAPTER 4

THE EFFECTS OF DIETARY PROTEIN RESTRICTION ON VASCULAR REACTIVITY IN ISOLATED MESENTERIC RESISTANCE ARTERIES FROM VIRGIN FEMALE RATS.

4.1 INTRODUCTION

Animal and human diets contain a mixture of nutrients and factors that are beneficial to health. An overabundance or lack of dietary nutrients causes a wide variety of chronic diseases and long-lasting health impairment. For example, high dietary intake of saturated fatty acids has been implicated in the development of cardiovascular disease (Henning *et al*, 1994). The endothelium is crucial to key arterial functions, including the regulation of vascular tone (Drexler and Hornig, 1999). Endothelial cells produce substances that modulate vascular tone, the most important of which is nitric oxide (NO) which acts on the underlying vascular smooth muscle (Mombouli and Vanhoutte, 1999). In the nonpregnant rat, endogenously produced NO in vascular endothelial cells tonically contributes to control of blood pressure and renal function (Moncada *et al*, 1991, Raij and Baylis, 1995). Impaired endothelium-dependent NO-mediated vasodilatation has been reported both in humans and in animal models of hypertension (Ignarro *et al*, 1999), diabetes mellitus (Pieper, 1998) and hypercholesterolemia and decreased bioavailability of NO is thought to be involved in these defects.

A dietary deficiency of protein, vitamin A, or iron impairs immune function (Stephensen, 2001). Feeding a 5% casein diet to young rats results in 18% and 52% decrease in plasma L-arginine concentration and whole body constitutive NO production by constitutive NOS and cannot support maximal inducible NO synthesis

by iNOS (Wu *et al*, 1999). Similarly, feeding a low protein (6% casein) to adult rats decreases both systemic and renal NO production, compared with a high protein diet (50%) (Tolins *et al*, 1995).

As a prelude to the experiments on pregnant rats, the experiments described in the present chapter were aimed to elucidate the effects of a low protein diet on the vascular function of mesenteric arteries from virgin female rats. The responses of the vessels to vasoactive agents, the effects of which are known to be mediated by, or are independent of NO, were investigated. Vascular constrictor responses and endothelium-dependent and -independent vasodilatation were assessed in isolated arteries using a small vessel myograph. Serum 17β-oestradiol and plasma cholesterol and triglyceride concentrations were measured. The present study has been presented to the Physiological Society (Koumentaki *et al*, 2000).

Initial myography validation studies

4.2A Methods

Female nonpregnant Wistar rats, age matched and weighing 220-230 g, were randomly allocated to be mated or to serve as controls. Pregnant rats were studied at day 18 or 19 of a 22-day gestation, and age-matched nonpregnant animals were used as controls. The aforementioned animals were utilised for initial experiments to decide the myography protocol for experimental procedure. The care and mating protocols for the animals and the procedure for the isolation of mesenteric arteries have been described in detail in sections 2.2.1 and 3.2, respectively. Nonpregnant and pregnant animals received a standard non-purified laboratory chow diet *ad libitum* (CRMX, Special Diet Services, Cambridge, UK) (Appendix 1). The rats were killed by CO_2 inhalation and cervical dislocation. The small intestine and intact mesentery were removed from the animal and placed in ice-cold physiological saline solution (PSS). Third branch mesenteric arteries were dissected free of connective tissue and fat using a light microscope and mounted on a wire myograph (Mulvany and Halpern, 1977) as a ring preparation (see section 3.2, Chapter 3) using two 40 μm tungsten wires. The myograph bath contained PSS at 37°C and was bubbled continuously with 5% carbon dioxide in oxygen to maintain a pH of 7.4. After 30 minutes equilibration at 37°C, the arteries were stretched sequentially (approximately five times) to achieve an internal circumference equivalent to 90% of that which they would have when relaxed *in vivo* under a transmural pressure of 13 kPa (100 mmHg) (section 3.2.6) (mean internal diameter nonpregnant: 296 ± 9 μm, *n* 18 arteries *vs* pregnant: 314 ± 12 μm, *n* 14 arteries, *P* not significant). Following normalisation, the arteries were washed with PSS and allowed to equilibrate for 15 minutes before the beginning of experimentation.

At the beginning of each experiment the arteries were first contracted with 5 μM noradrenaline (NA) in 125 mM potassium solution (KPSS, made by equimolar substitution of NaCl with KCl in PSS) (NAK) for 2 min). (See section 3.3: *stimulation and vessel viability protocol*). A second contraction was produced with 5 μM noradrenaline in PSS (NA). A third contraction was produced by a maximally depolarising K^+ physiological salt solution (PSS containing 125 mM KCl). The vessels were contracted on one further occasion with 5 μM NA in

potassium-substituted PSS (125 mM, NAK). Only if the artery produced maximum active tension equivalent to an effective pressure of 100 mmHg (13.3 KPa) on the last NAK was used for further experimentation. Subsequently, in order to confirm the presence of the endothelium, preparations precontracted with the α_1-adrenoreceptor agonist phenylephrine (3-5 μM) were challenged with acetylcholine (1 μM). The endothelium was regarded as undamaged in arteries in which ACh reversed phenylephrine-induced tone by more than 90%. After 10 minutes, a cumulative dose response curve for PE ($1x10^{-7}$-$1x10^{-5}$ M) was constructed by adding increasing concentrations of this compound at two min intervals. Arteries were then washed four times with PSS and after a 10-min equilibration period a concentration-response curve for KCl (5mM - 125 mM) was constructed. Arteries were then washed four times with PSS and after a 10-min equilibration period they were submaximally contracted to a stable plateau by adding PE (3-5 μM). A concentration response curves with acetylcholine ($1x10^{-9}$ -$1x10^{-5}$ M) was obtained by adding increasing concentrations of ACh at 2 min intervals. After washout for 10 min, arteries were submaximally contracted in response to PE (3-5 μM) and a concentration-response curve with spermine NONOate ($1x10^{-9}$ -$1x10^{-5}$ M) was obtained, followed by four washes, and a 10-min equilibration period. After the equilibration period, indomethacin (10 μM) was added to the bath for 20 min and the arteries were again contracted with submaximal PE in the continued presence of indomethacin. A second concentration-response curve to ACh was then determined in the continued presence of indomethacin, followed by four washes and a 10-min equilibration period. After washout for 10 min, indomethacin and the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) were added to the bath for 20 minutes and the arteries were again contracted with submaximal PE in the continued presence of indomethacin and L-NAME. A third concentration-effect curve to ACh was carried out. In order to determine whether phenylephrine contractions were sustained and reproducible throughout the duration of the experimental period, four sequential contractions to PE were performed in the absence of ACh. The reproducibility of ACh relaxation was determined by three dose response curves to ACh in the absence of the inhibitors.

4.2 MATERIALS AND METHODS

Effects of feeding an 18% and a 9% casein diet on vascular function in the small mesenteric arteries from virgin Wistar rats.

4.2.1 Study Animals

Virgin female Wistar rats were obtained from Charles River UK. The animals were housed in the Biomedical Research Facility (University of Southampton) on a 12-hour light/dark cycle at a temperature of 22 °C with free access to food and water at all times. Prior to the experimental diets, the non-pregnant animals received a standard non-purified laboratory chow diet *ad libitum* (CRMX, Special Diet Services, Cambridge, UK) (Appendix 1) with free access to plain tap water. The experimental protocol conformed with the 1986 Animal Act and had been approved by the British Home Office. On attaining body weights of 200-220 g, virgin rats were randomly divided into two dietary treatment groups and fed *ad libitum* either a 18% or a 9% casein diet for 18-19 days. The experimental diet composition is shown in Appendix 2. At the end of the feeding period, the rats were killed by CO_2 inhalation and cervical dislocation and blood was collected by cardiac puncture and dispensed into tubes containing either ethylenediamine-tetra-acetic acid (EDTA; 28 mmol/L) or no anticoagulant. After weighing the rat, the mesentery was removed and placed in physiological saline solution (PSS). The thoracic aorta were excised and immediately rinsed with phosphate buffered saline (PBS), collected into sterile tubes, snap frozen in liquid nitrogen and subsequently stored at -70 °C for measurements of NOS protein mass (Chapter 6). The blood was centrifuged, after standing at 4 °C for 30 minutes, (2500g, 15 min, 4°C) and the plasma and serum samples collected were frozen and stored at -20°C for later measurements of blood analytes [cholesterol, triacylglycerols (TGs) and 17β-oestradiol].

4.2.2. Evaluation of vascular function

Twenty-one virgin female Wistar rats were studied. The small intestine and intact mesentery were removed from the animal and placed in ice-cold physiological saline solution (PSS). Third branch mesenteric arteries were dissected free of

connective tissue and fat using a light microscope and mounted on two 40 μm diameter stainless steel wires on a small vessel myograph (Mulvany and Halpern, 1977) for the measurement of isometric tension (see section 3.2, Chapter 3).

To confirm viability of the arteries, four contractions (2 min duration) were performed to 5 μM NA, 125 mM KCl in PSS, or a combination of both. (see section 3.3: *stimulation and vessel viability protocol*). Arteries failing to produce a maximum active tension equivalent to an effective pressure of 100 mmHg (13.3 KPa) on the last NAK were excluded from the study. In this study, four arteries were rejected from each group.

After the routine run-up procedure, cumulative dose response curves, at increments of 2 min duration, were constructed to PE ($1x10^{-7}$-$1x10^{-5}$ M) (section 3.4). Following repeated washing and recovery (10 minutes) a dose response curve to KCl (5mM - 125 mM) was constructed. The myograph chamber was then washed four times with PSS and a 10 min recovery period allowed before the arteries were precontracted with a submaximal concentration of PE (3-5 μM) to achieve approximately 80% of the maximum response and relaxation induced by ACh ($1x10^{-9}$ - $1x10^{-5}$ M) was then assessed. A similar protocol was used to assess endothelium-independent relaxation, by evaluating relaxation to SPN ($1x10^{-9}$ -$1x10^{-5}$ M).

After washout for 10 minutes indomethacin (10 μM) was added to the bath for 20 minutes and the arteries were again precontracted with PE in the continued presence of indomethacin. A second concentration-effect curve to ACh was then determined in the continued presence of indomethacin. The effect of indomethacin alone on ACh relaxation was tested in arteries from 18% casein-fed rats only. After washout for 10 min, indomethacin and N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) were added to the bath for 20 minutes and a third concentration-effect curve to ACh was carried out. Arteries were preconstricted with 2-4 μM PE, the concentration being adjusted in order to evoke similar preconstrictor tone to that observed without inhibitors.

4.2.3. Determination of serum 17β-oestradiol concentration

Blood was obtained by cardiac puncture and was immediately cooled on ice (30 minutes) and centrifuged at 2500 g for 15 min at 4°C. Serum was then stored at -20°C until assayed. A commercially available radioimmunoassay (RIA) (Codes 12264/12268, Estradiol Maia, Serono Diagnostics, UK) was used for the quantitative measurement of 17β-oestradiol concentration in serum.

The assay procedure is based on the competitive binding principles of radioimmunoassay. Oestradiol (unlabelled antigen) present in the serum sample, standards and controls competes with oestradiol ^{125}I (labelled antigen) for a limited number of binding sites on a rabbit anti-oestradiol antiserum. A goat anti-rabbit antibody is then allowed to react with the anti-oestradiol complex. As the anti-rabbit antibody is covalently bound to magnetizable polymer particles, the rabbit antibody-bound oestradiol fraction can be separated by magnetic sedimentation followed by decantation of the supernatant. Measurement of the radioactivity in the pellet enables the amount of ^{125}I-labelled oestradiol in the bound fraction to be measured. The amount of oestradiol ^{125}I bound by the antibody is inversely proportional to the amount of oestradiol present in the sample, standard or control. The concentration of oestradiol present in the sample under investigation can then be determined by interpolation from a standard curve.

4.2.3.1. Method of the 17β-oestradiol assay

The assay was performed at room temperature in non-sterile round-bottomed polystyrene assay tubes (code 112201, Greiner Labortechnik, Gloucestershire, UK). A 50 µl aliquot of standard, control or unknown serum sample was assayed in duplicate. 100 µl of ^{125}I-labelled oestradiol tracer and then 100 µl of rabbit anti-oestradiol antibody suspension were dispensed into each tube. The mixture was vortexed thoroughly and the samples were incubated for 1 hour at 37°C in water-bath. 1 ml of Maia separation reagent (goat anti-rabbit gammaglobulin coupled to magnetic particles) was then added to each sample, standard or unknown. All samples were mixed thoroughly and further incubated for 10 min at room temperature. The rack holding the assay tubes were then attached to a magnetic

separator base and left for 10 min. The antibody bound fraction was separated by magnetic separation followed by decantation of the supernatant. Following the decantation of the supernatant, the tubes were drained for 5 minutes onto blotting paper. The radioactivity present in each sample was determined by counting for at least 60 seconds in a gamma scintillation counter (NE 1608, Nuclear Enterprises, UK) and analysed by computer using the RIA-CALC software program (LKB-Wallac, Finland) The concentration of 17β-oestradiol in unknown samples was then determined from the standard curve generated.

4.2.3.2. Precision and range of the 17β-oestradiol assay

The inter-assay coefficient of variation for the oestradiol assay was 5.6 %. The detection limit of the essay, defined as the concentration of oestradiol two standard deviations from the zero standard was 55 pmol/L. The range extended to 18350 pmol/L.

4.2.4. Determination of plasma Cholesterol and Triglyceride (TG) concentration

Plasma cholesterol and TG concentrations were measured at the Chemical Pathology Department, Southampton General Hospital. Samples were collected in EDTA tubes and kept at < 4 °C until the plasma was separated and frozen at -20 °C. EDTA plasma samples were then used for the measurement of cholesterol and triglyceride concentrations using an automated process (ADVIA 1650 Chemistry system, Bayer). In this process, cholesterol detection is based on an enzymatic method utilizing conversion by cholesterol esterase and cholesterol oxidase, followed by a Trinder end-point. TG detection is based on the Fossati three-step enzymatic reaction, also using a Trinder end-point. The procedure quantitates the total TGs, including mono- and di-glycerides and the free glycerol fractions.

4.2.5. Drugs

All general chemicals are described in the drugs and solutions section (Chapter 3). All drugs were added to the vessel chamber and final concentrations are reported.

4.2.6. Data calculation and statistical analysis

All values are given as the mean ± SEM. In all experiments with PE, KCl, ACh and SPN, and for the determination of the serum 17β-oestradiol and plasma cholesterol and TG data, n = number of animals from which vessels, blood samples and body weight were obtained. Contractions to PE and KCl were expressed either as absolute values (mN mm^{-1} artery length) or as a percentage of the response to KPSS (125 mM KCl). Relaxations to ACh and SPN were calculated as a percentage of the initial precontraction to phenylephrine. The maximum and half-maximum effective concentration (EC_{50}, expressed as -log mol/L = pEC_{50}) were calculated as the mean ± SEM of the individual values by nonlinear regression using the curve fitting programme 'Prism 3.0' (GraphPad Software Inc., San Diego, CA., USA) (see section 3.6). The effect of the diet on contractions of mesenteric arteries to PE and KCl and on relaxations in response to ACh and SPN, was tested by comparing the pEC_{50} values and maximum responses between the groups with use of the two-tailed Student's unpaired *t* test. Student's unpaired *t* test was chosen because data were consistent with a Gaussian population as indicated by testing for skewness. Skewness is a value that shows how skewed a curve is. The closer to zero the value for the skewness is, the more symmetrical is the distribution. A test was also used to detect how close a distribution is to normality. The Kolmogorov-Smirnov (KS) test was used to test for deviations from Gaussian distribution. Since the Gaussian distribution is also called the Normal distribution, the test is called a normality test. The Student's paired *t* test (parametric) was used for comparisons within each diet group. Dose-response analyses also employed two-way repeated-measures analysis of variance (ANOVA) (Prism 3.0, GraphPad Software Inc.). The effect of indomethacin and L-NAME on relaxation in response to ACh within each group was tested by two-way repeated-measures ANOVA followed by a Bonferroni *t* test. Statistical analysis of serum 17β-oestradiol and plasma cholesterol and TG data was carried out using the unpaired Student's *t* test. Comparisons were considered significant when $P < 0.05$ and are reported as trends when $0.05 < P < 0.10$.

4.3A RESULTS

Initial myography validation experiments

A cumulative dose response curve, at increments of 2 min duration, was constructed for PE ($1x10^{-7}$-$1x10^{-5}$ M)-induced contraction in a group of nonpregnant (n = 10) and a group of pregnant rats (n = 8) (Figure 4.1A). No difference in negative log concentration of PE required to produce 50% of the maximum response was detected between arteries of nonpregnant and pregnant rats (nonpregnant rats, pEC_{50} 5.59 ± 0.02, n = 10, *vs* pregnant rats 5.62 ± 0.03, n = 8; not significant, Figure 4.1A).

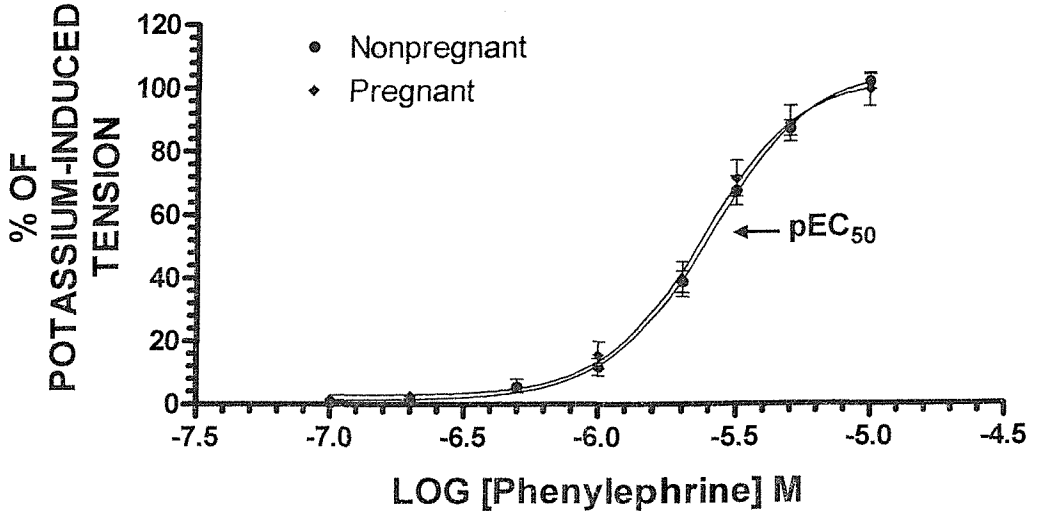


Figure 4.1A.: Concentration-effect curves for phenylephrine-induced contraction of mesenteric resistance arteries from nonpregnant (•, n = 10) and pregnant rats (♦, n = 8).

Constrictor responses to the cumulative addition of potassium chloride (KCl, 5mM - 125 mM), were established with some arteries from both nonpregnant (n = 4), and pregnant rats (n = 4) (Figure 4.2A), as a measure of receptor-independent contractile function of the vascular smooth muscle. Dose-response curves to KCl were determined 10 minutes after PE concentration-response curve was obtained and after repeated washing. Pregnancy had an effect on maximal contraction induced by KCl (Figure 4.2A). At 1.25 x 10^{-1} M, KCl-induced contraction was significantly less (P <0.01) in arteries from pregnant

animals (nonpregnant rats, maximal contraction 45.43 ± 2.26 %, $n = 4$, *vs* pregnant rats 36.40 ± 3.4 %, $n = 4$; $P < 0.01$, Figure 4.2A).

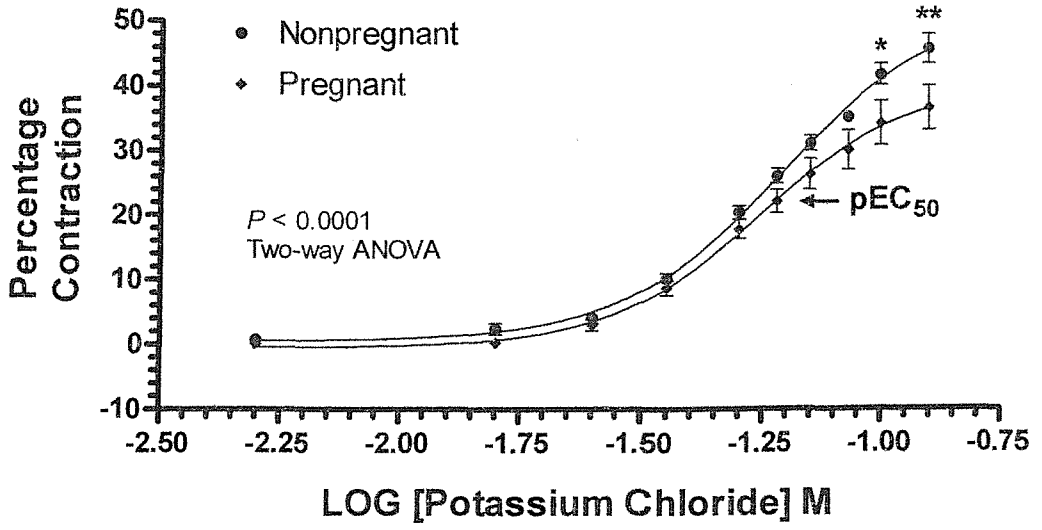


Figure 4.2A.: Concentration-effect curves for potassium chloride-induced contraction of mesenteric resistance arteries from nonpregnant (●, $n = 4$) and pregnant rats (◆, $n = 4$).

The myograph chamber was then washed four times with PSS and a 10 min recovery period allowed before the vessels were submaximally contracted with 3-5 μM phenylephrine and endothelium-dependent relaxation responses to acetylcholine subsequently determined by adding increasing concentrations of acetylcholine at two min intervals (final bath concentration $1x10^{-9}$ -$1x10^{-5}$ M). Sensitivity and maximum relaxation to acetylcholine was similar in nonpregnant and pregnant groups (nonpregnant rats, pEC_{50} 7.52 ± 0.09, $n = 10$, *vs* pregnant rats 7.66 ± 0.17, $n = 8$; not significant; nonpregnant rats, maximum relaxation 94.32 ± 0.90, $n = 10$, *vs* pregnant rats 97.03 ± 1.3, $n = 8$; not significant) (Figure 4.3A). Control contractions to phenylephrine without the addition of acetylcholine confirmed a time-dependent decrease in tension (<10%) which was not significantly different between arteries from nonpregnant and pregnant rats.

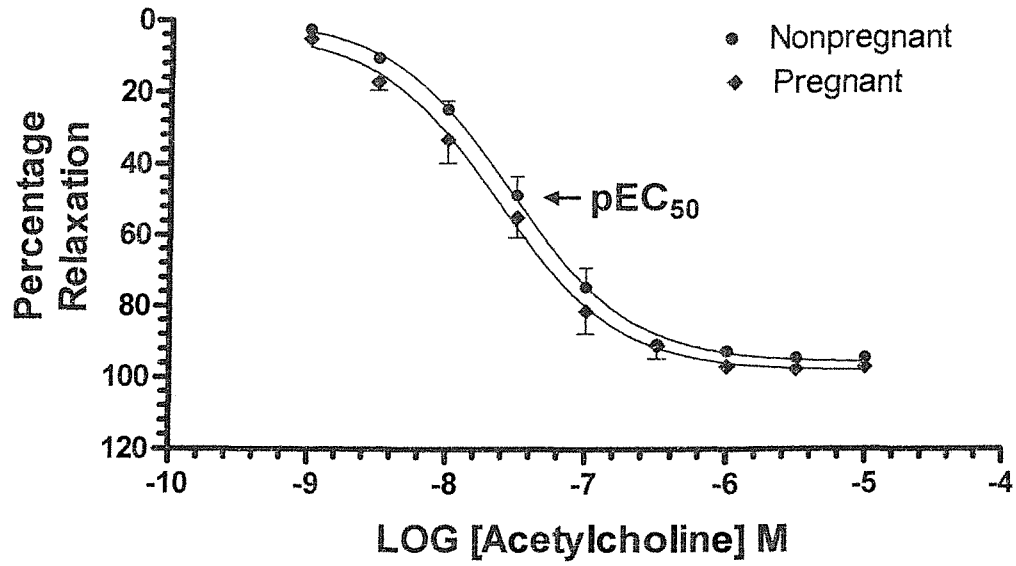


Figure 4.3A.: Acetylcholine-induced relaxation after precontraction with 3-5 μM phenylephrine in mesenteric arteries from nonpregnant (•, $n = 10$) and pregnant rats (♦, $n = 8$).

After the maximum response was reached (defined as no additional relaxation to a higher concentration of acetylcholine), the bath was washed four times with PSS and the arterial segments were equilibrated for 10 minutes.

After equilibration, arteries were again precontracted with 3-5 μM phenylephrine and exposed to cumulative concentrations of spermine NONOate at two min intervals ($1x10^{-9}$ -$1x10^{-5}$ M). There were no differences between nonpregnant and pregnant groups in terms of sensitivity (pEC_{50} values) or maximal response to spermine NONOate (nonpregnant rats, pEC_{50} 6.18 ± 0.31, $n = 8$, *vs* pregnant rats 6.55 ± 0.20, $n = 7$; not significant; nonpregnant rats, maximum relaxation 90.23 ± 2.11, $n = 8$, *vs* pregnant rats 93.99 ± 2.37, $n = 7$; not significant) (Figure 4.4A).

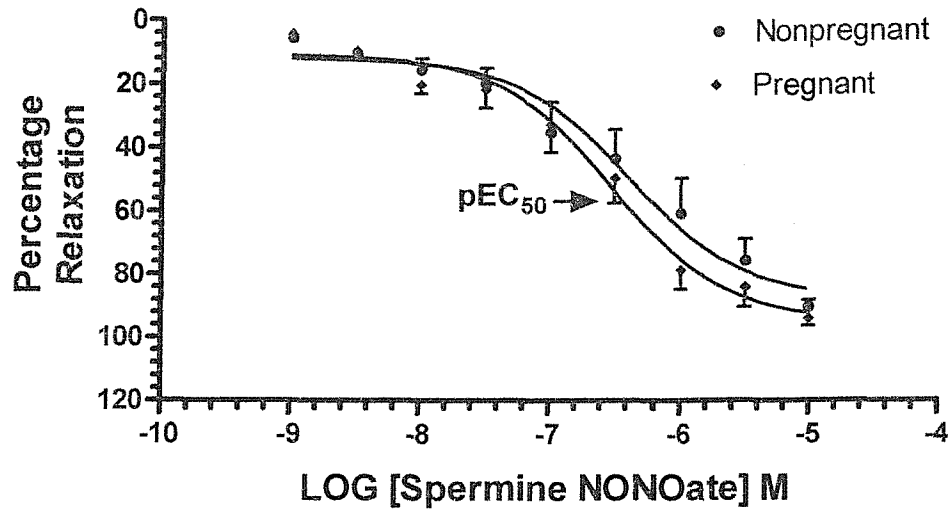


Figure 4.4A.: Spermine NONOate (SPN) concentration-effect curve for mesenteric arteries from nonpregnant (•, $n = 8$) and pregnant rats (♦, $n = 7$), following precontraction with 3-5 μM phenylephrine.

After a washout period (10 minutes), the cyclooxygenase inhibitor indomethacin (10 μM) was added to the bath for 20 minutes and the arteries were again preconstricted with PE in the continued presence of indomethacin. A second concentration-effect curve to ACh was then determined in the continued presence of indomethacin. Incubation of arteries from nonpregnant and pregnant rats with 10 μM indomethacin did not influence the level of precontraction elicited by PE compared with the previous response obtained in the absence of indomethacin (nonpregnant rats with indomethacin 2.73 ± 0.16 $mNmm^{-1}$, $n = 8$, *vs* 2.60 ± 0.18 $mNmm^{-1}$ $n = 10$, without indomethacin, $P = 0.6$, by unpaired t test; pregnant rats with indomethacin 3.02 ± 0.24 $mNmm^{-1}$, $n = 7$, *vs* 3.57 ± 0.26 $mNmm^{-1}$ $n = 8$, without indomethacin, $P = 0.2$, by unpaired t test). In arteries from nonpregnant rats there was no significant difference in sensitivity (pEC_{50}) and maximum response to ACh in the presence of indomethacin. The responses in these vessels did not differ before and after indomethacin (these were: nonpregnant rats in the absence of indomethacin: pEC_{50}: 7.52 ± 0.09, $n = 10$, *versus* 7.43 ± 0.05 in the presence of indomethacin, $n = 8$; not significant; maximum relaxation: nonpregnant rats in the absence of indomethacin: 94.32 ± 0.90 %, $n = 10$, *versus*

91.40 ± 1.70 % in the presence of indomethacin, $n = 8$; not significant) [(Figure 4.5A (i)].

Indomethacin decreased the effects of ACh in the arteries from pregnant rats by shifting the concentration-effect curve to the right evidenced by a decreased sensitivity (pEC_{50}) to ACh (pEC_{50}: with indomethacin: 6.60 ± 0.21, $n = 7$, *versus* 7.66 ± 0.17, $n = 8$, without indomethacin; $P = 0.005$) [(Fig. 4.5A (ii)]. Indomethacin had no significant effect on the maximal relaxation in response to ACh in arteries from pregnant rats (max. relax.: with indomethacin: 89.7 ± 3.3%, $n = 7$, *vs* 97.03 ± 1.3%, $n = 8$, in the absence of indomethacin; $P = 0.05$) [(Fig. 4.5A (ii)]. Time control PE-induced contractions in the absence of ACh showed no significant difference in time-dependent relaxation between nonpregnant and pregnant arteries.

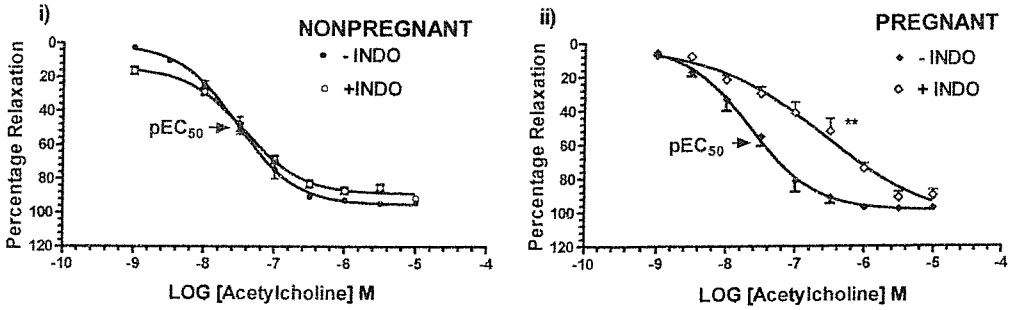


Figure 4.5A (i) and (ii).: Acetylcholine concentration-effect curves after precontraction with 3-5 μM phenylephrine in arteries from (i) nonpregnant rats in the absence (•, $n = 10$) and presence (o, $n = 8$) of 10 μM indomethacin and (ii) pregnant rats in the absence (◆, $n = 8$) and presence (◊, $n = 7$) of 10 μM indomethacin.

After washout for 10 min, indomethacin and the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) were added to the bath for 20 minutes and a third concentration-effect curve to ACh was carried out.

In preliminary experiments, the concentration of L-NAME required to achieve a reproducible rightward shift in the concentration-relaxation curve in

response to acetylcholine was verified in mesenteric arteries from five nonpregnant rats. In the presence of 50 μM L-NAME for 20 min, the pEC_{50} for ACh-evoked relaxation was 7.19 ± 0.11 and the maximum relaxation to ACh was 71.5 ± 4.7% (n = 5). Increasing the concentration of L-NAME to 100 μM shifted the pEC_{50} to 7.04 ± 0.24 (P = 0.6) and maximum relaxation to ACh to 57.0 ± 11.0 % (P = 0.3), which were not significantly different compared with the lower concentration of L-NAME (Figure 4.6A). Increasing the concentration of L-NAME to 1 mM shifted the pEC_{50} to 6.86 ± 0.26 and maximum relaxation to ACh to 60.2 ± 11.0 % (n = 5), which were not significantly different compared with the lower concentrations of L-NAME (Figure 4.6A).

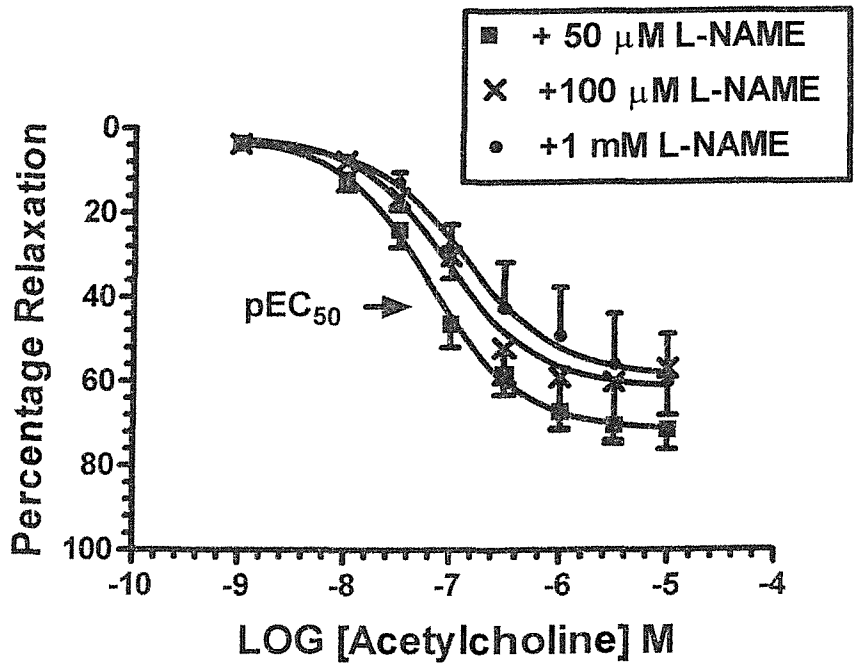


Figure 4.6A.: Endothelium-dependent vasodilator function in arteries from five nonpregnant rats: concentration-dependent relaxation of submaximally PE-preconstricted arteries in the presence of 50, 100 μM or 1 mM L-NAME. See text for pEC_{50} and maximal relaxation values.

Effect of indomethacin and L-NAME on ACh-induced vasodilatation in arteries of nonpregnant and pregnant rats.

The addition of 100 μM L-NAME (in the continued presence of 10 μM indomethacin) increased the level of pre-contraction to PE in both nonpregnant and pregnant animals when compared with that obtained in the presence of indomethacin alone (nonpregnant rats with indomethacin and L-NAME, tension = 3.48 ± 0.23 $mNmm^{-1}$, $n = 6$, *vs* 2.73 ± 0.16 $mNmm^{-1}$ $n = 8$, indomethacin alone, $P = 0.02$; pregnant rats with indomethacin and L-NAME, tension = 3.75 ± 0.21 $mNmm^{-1}$, $n = 6$, *vs* 3.02 ± 0.24 $mNmm^{-1}$ $n = 7$, indomethacin alone, $P = 0.045$). The maximum relaxation to ACh (in the continued presence of indomethacin and L-NAME) in both nonpregnant and pregnant groups was significantly attenuated when compared with the response to indomethacin alone (nonpregnant rats with indomethacin and L-NAME, max. relax.: 54.13 ± 13.5%, $n = 6$, *vs* 91.40 ± 1.7%, $n = 8$, indomethacin alone, $P < 0.0001$; pregnant rats with indomethacin and L-NAME, max. relax.: 61.30 ± 8.0%, $n = 6$, *vs* 89.73 ± 3.3%, $n = 7$, indomethacin alone, $P < 0.01$) [(Fig. 4.7A (i), (ii)].

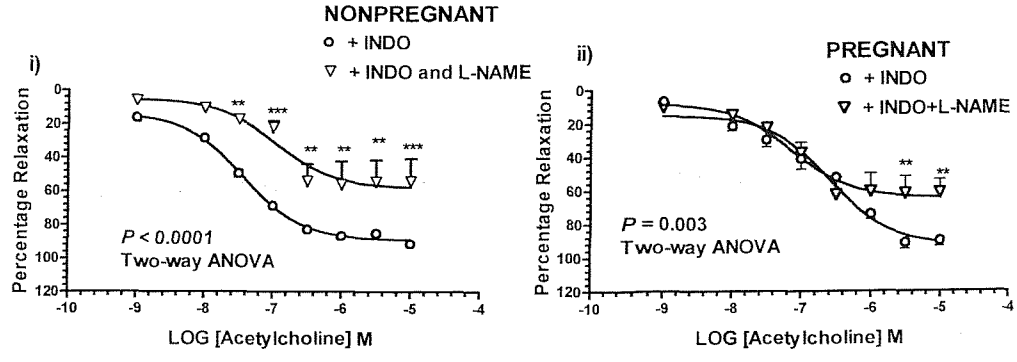


Figure 4.7A (i) and (ii).: A comparison of the acetylcholine concentration-effect curves after precontraction with 3-5 μM phenylephrine in arteries from (i) nonpregnant and (ii) pregnant rats. Responses were carried out in the presence of indomethacin alone (o) and after pre-incubation in 10 μM indomethacin and 100 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME, ∇).

Reproducibility of acetylcholine-induced relaxations without indomethacin and/or L-NAME.

Because each tissue was subjected to three acetylcholine concentration-response curves, control, plus indomethacin, and indomethacin plus L-NAME, and because the ACh concentration-response curves in control, plus indomethacin, and indomethacin plus L-NAME-treated arteries were performed at different times, separate time control experiments without the addition of indomethacin and/or L-NAME to the PSS were carried out in mesenteric arteries obtained from one group of female nonpregnant rats (n =3) [Figure 4.8A (i)] and one group of pregnant rats (n =3) [Figure 4.8A (ii)]. Concentration-response curves were obtained by cumulative addition of acetylcholine (final bath concentration $1x10^{-9}$ - $1x10^{-5}$ M) to arteries submaximally contracted to a stable plateau by adding 3-5 μM PE. Concentration-response curves in response to ACh were obtained by adding increasing concentrations of ACh at two min intervals. Three concentration-response curves to acetylcholine were carried out over the same period involved in the procedures described above. Therefore, the following protocol was used: (1) contraction in response to 5 μM noradrenaline in 125 mM potassium solution for 2 min followed by four washes and a 10 min equilibration period; (2) contraction in response to 5 μM noradrenaline in PSS for 2 min followed by four washes and a 10 min equilibration period; (3) contraction in response to a maximally depolarising K^+ physiological salt solution (PSS containing 125 mM KCl) for 2 min followed by four washes and a 10 min equilibration period; (4) contraction in response to 5 μM noradrenaline in 125 mM potassium solution for 2 min followed by four washes and a 10 min equilibration period; (5) contraction in response to phenylephrine, addition of acetylcholine, followed by four washes and a 10 min equilibration period; (6) concentration-response curve with phenylephrine, followed by four washes and a 10 min equilibration period; (7) concentration-response curve with KCl, followed by four washes and a 10 min equilibration period; (8) contraction in response to PE and concentration-response curve with acetylcholine, followed by four washes and a 10 min equilibration period; (9) contraction in response to PE and concentration-response curve with spermine NONOate, followed by four washes and a 30 min equilibration period; (10) contraction in response to PE and concentration-response

curve with acetylcholine, followed by four washes and a 30 min equilibration period; (11) contraction in response to PE and concentration-response curve with acetylcholine, followed by four washes. In both nonpregnant and pregnant groups, these three ACh concentration-effect curves were approximately indistinguishable [Figure 4.8A (i) and (ii)], minimising the probability that differences in the response to ACh were related to timing. Data from a particular tissue were rejected if the contractions produced by PE differed by more than 10%.

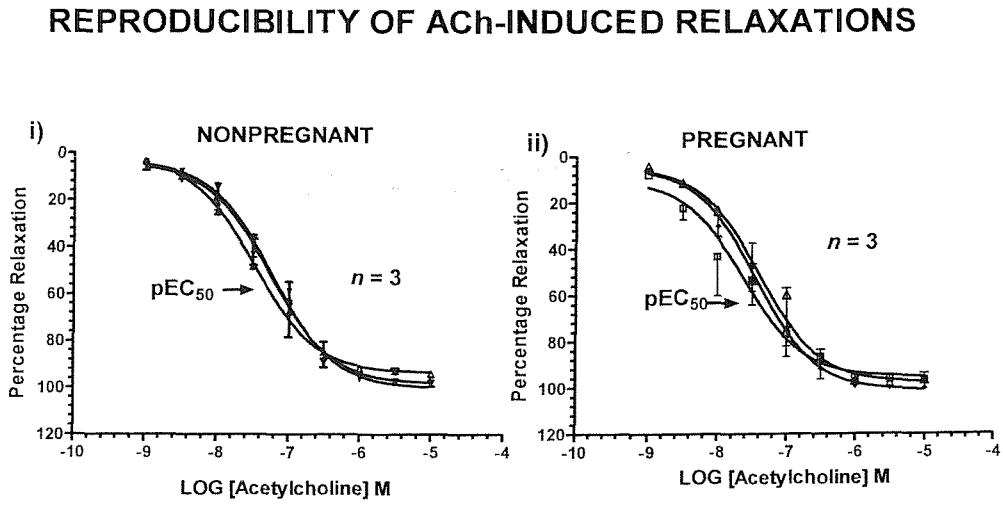


Figure 4.8A (i) and (ii).: The responsiveness to ACh was tested by three dose response curves to ACh in isolated mesenteric arteries from (i) nonpregnant and (ii) pregnant rats in the absence of indomethacin and/or L-NAME. Tone was induced with PE (3-5 μM) during the entire protocol.

4.3 RESULTS

Effects of feeding an 18% and a 9% casein diet on body weight and vascular function in the small mesenteric arteries from virgin Wistar rats.

4.3.1. Effect of feeding a low protein diet (90 g protein/kg diet) upon weight gain in virgin Wistar rats.

The rats in both the 18% and 9% casein groups gained weight significantly during the experimental period (18-19 days) (18% casein group: $P < 0.0001$; 9% casein group: $P < 0.0001$) (by paired Student's 't' test) (Table 4.1). However, there were no significant differences in the mean weight gain (39-40 g) between the two groups ($P = 0.9$). There were no significant differences in the average weights of the rats in either group either at the start (18% casein, 198.3 ± 6 g, *n* 9, *versus* 9% casein, 198.5 ± 5 g, *n* 10; $P = 0.9$) or at the end of the dietary regime, when vascular function was assessed (18% casein, 238.4 ± 5, *n* 9, *versus* 9% casein, 237.6 ± 6 g, *n* 10; $P = 0.9$). (Table 4.1). During the experimental duration, their weight increased, such that by day 18, virgin rats on 18% casein were on average approximately 20% above their mean weight at the start of the dietary regime. By day 18, virgin rats on 9% casein diet were 20% above their mean weight on experimental day 1.

TABLE 4.1

The effects of feeding an 18% and a 9% casein diet for 18-19 days upon weight gain in virgin Wistar rats.

VIRGIN RATS	18% Casein N = 9	9% Casein N = 10	P value
Initial Weight (g)	198.3 ± 6	198.5 ± 5	0.9
Weight Gain (g)	40.11 ± 6	39.10 ± 3	0.9
Final Weight (g)	238.4 ± 5 [a]	237.6 ± 6 [b]	0.9

Values are means ± SEM; *N* = number of animals used. 18% casein group: [a]$P <$ 0.0001 versus initial weight; 9% casein group: [b]$P < 0.0001$ versus initial weight.

4.3.2. Serum and plasma analyses

4.3.2.1. Serum 17β-oestradiol levels.

Table 4.2 depicts the data for the mean serum 17β-oestradiol concentration in virgin rats fed 18% and 9% casein for 18-19 days. The mean serum 17β-oestradiol concentration was not significantly different ($P = 0.17$, by unpaired Student's t test) between the two groups at the time of vascular function measurement.

4.3.2.2. Plasma cholesterol and triglyceride concentrations

Table 4.2 also depicts the data for the mean plasma cholesterol and TG concentrations. There were no significant differences in the mean plasma cholesterol concentrations in virgin 18% and 9% casein-fed groups ($P = 0.5$), nor were any differences in the mean plasma TG concentrations noted between the two groups ($P = 0.25$, by unpaired Student's t test), (Table 4.2).

TABLE 4.2

Concentrations of serum 17β-oestradiol (pmol/L) and of plasma cholesterol and triacylglycerols (mmol/L) in virgin rats fed 18% and 9% casein for 18-19 days.

	Dietary treatment		
Parameter	**18% CASEIN**	**9% CASEIN**	***P* value**
17β-oestradiol (pmol/L)	52.6 ± 5.3 (12)	42.5 ± 4.8 (12)	0.17
Cholesterol (mmol/L)	1.69 ± 0.08 (13)	1.79 ± 0.10 (14)	0.5
Triglycerides (mmol/L)	1.46 ± 0.15 (13)	1.68 ± 0.12 (14)	0.25

Data are expressed as mean ± S.E.M., with numbers of animals stated in parentheses.

4.3.3. Effects of feeding an 18% and a 9% casein diet on internal arterial diameters in the small mesenteric arteries from virgin rats.

The mean internal diameter of the mesenteric arteries from virgin rats fed either an 18% or a 9% casein diet were similar, (18% casein: 311.0 ± 12.0 μm, *n* 18 arteries *versus* 9% casein: 319.1 ± 10.35 μm, *n* 16 arteries; $P = 0.6$).

4.3.4. Vasoconstrictor responses to phenylephrine and KCl.

Arteries from control and low protein fed virgin rats constricted in a concentration-dependent manner in response to the α_1 adrenoreceptor agonist phenylephrine (PE). There were no significant differences in the concentration of PE required to achieve a constriction equal to 50% of the maximum (EC_{50}) between the two groups [pEC_{50} (-log EC_{50}): 18% casein: 5.57 ± 0.04, *n* 11, *versus* 9% casein: 5.53 ± 0.04, *n* 10; $P = 0.6$, by unpaired Student's *t* test]. (Table 4.3) (Figure 4.1). In respect of the constrictor responses to PE the arteries from the 9% casein fed rats demonstrated similar maximum constrictor tension when compared with those from 18% casein fed rats [maximum constrictor response (percentage of the contractile response to KPSS (125 mM KCl)-induced tension): 18% casein: 93.0 ± 4.65 %, *n* 11, *versus* 9% casein: 96.78 ± 4.37 % *n* 10; $P = 0.6$]. (Table 4.3) (Figure 4.1). The contractions induced by exposure of the vessels to 125 mM potassium containing physiological saline solution (KPSS) in the 'run up' procedure were not significantly different in arteries from 18% and 9% casein fed rats (3.87 ± 0.20 $mNmm^{-1}$, *n* 12, *versus* 3.85 ± 0.18 $mNmm^{-1}$ *n* 13, respectively ($P = 0.9$, by unpaired Student's *t* test). Arteries isolated from 18% and 9% casein fed virgin rats contracted to the receptor-independent contractile agent KCl in a dose-dependent manner (Figure 4.2) and there were no significant differences ($P = 0.6$) in the pEC_{50} values obtained for KCl (1.22 ± 0.009, *n* 10, *versus* 1.21 ± 0.008, *n* 10, respectively) (Table 4.3). There was no difference in the maximum contraction to KCl between the two groups (18% casein: 39.22 ± 1.93 %, *n* 10, *versus* 9% casein: 39.83 ± 1.53 % *n* 10; $P = 0.8$, by unpaired Student's *t* test). (Table 4.3) (Figure 4.2).

TABLE 4.3

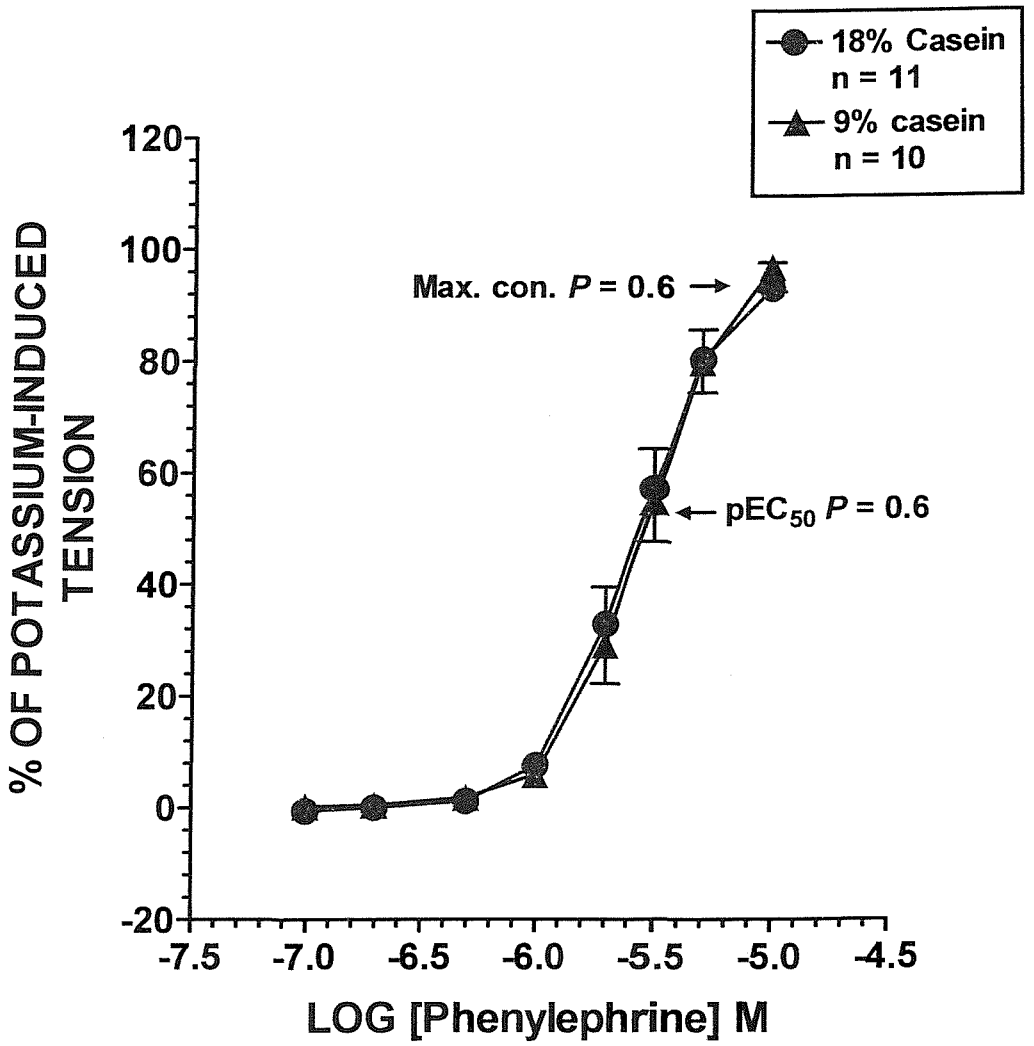
The effects of feeding an 18% and a 9% casein diet on responses to constrictor agonists in small mesenteric arteries from virgin Wistar rats.

		Phenylephrine (PE) (10^{-7} to 10^{-5} mol/l)		
		Max. Contraction		
Group *n*		**mN/mm**	**% KPSS**	**pEC_{50} (M)**
18% Casein	11	3.57 ± 0.17	93.0 ± 4.65	5.57 ± 0.04
9% Casein	10	4.17 ± 0.26[a]	96.78 ± 4.37	5.53 ± 0.04
		Potassium chloride (KCl) (5mmol/l to 125 mmol/l)		
		Max. Contraction		
Group *n*		**mN/mm**	**% KPSS**	**pEC_{50} (M)**
18% Casein	10	1.58 ± 0.06	39.22 ± 1.93	1.23 ± 0.01
9% Casein	10	1.69 ± 0.09	39.83 ± 1.53	1.20 ± 0.03

	KPSS (125 mM)	
		Maximum tension
Group	***n***	**mN/mm**
18% Casein	12	3.87 ± 0.20
9% Casein	13	3.85 ± 0.18

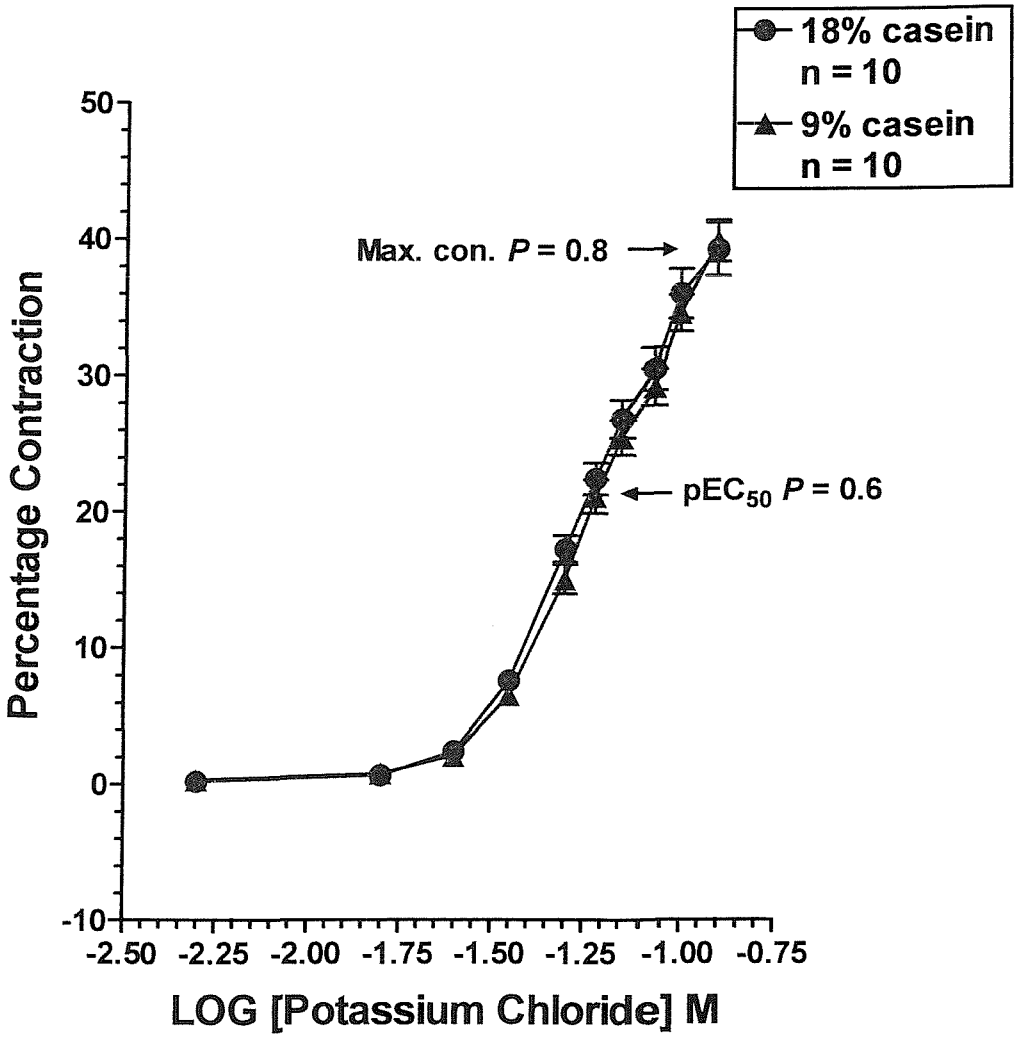
Data are expressed as mean ± SEM. *n* = number of animals examined. Maximal response is expressed either as absolute value (mN/mm) or as a percentage of the contractile response to KPSS (125 mM KCl)-induced tension. pEC_{50}, -log EC_{50} where EC_{50} is the concentration which produces 50% maximum response; [a] 9% casein *versus* 18% casein, (PE) $P = 0.06$, by the unpaired Student's *t* test.

Figure 4.1.: Concentration-response curves to phenylephrine in mesenteric small arteries from virgin rats fed an 18% (●; n = 11) or 9% casein diet (▲; n = 10).



Data are expressed as a percentage of the contractile response to a depolarising potassium buffer (125 mM KCl) and shown as mean and standard error (where not present, error bars lie within the symbols). Number of rats (n) stated. There was no significant difference in response to the adrenergic agonist phenylephrine. Max. con. = maximum constriction.

Figure 4.2.: Concentration-response curves to potassium chloride (KCl) in mesenteric arteries from virgin rats fed an 18% (●; n = 10) or 9% casein diet (▲; n = 10).



Data are means ± SEM (where not present, error bars lie within the symbols). Number of rats (n) stated. Responses are expressed as percentage of the response to KPSS. There was no significant difference in the contractile response to KCl between the two groups (by unpaired Student's *t* test). pEC_{50} = -log EC_{50}, where EC_{50} is the concentration which produces 50% maximum response.
Max. con. = maximum constriction.

4.3.5. Effect of the low protein diet on vasodilator responses to acetylcholine (ACh) and spermine NONOate (SPN) in virgin rats.

In respect of endothelium-dependent relaxation, before evaluation of ACh responses, preconstriction to PE was not different between arteries from virgin 18% casein and 9% casein fed rats (tension = 2.55 ± 0.17 $mNmm^{-1}$, *n* 11, *versus* 2.62 ± 0.20 $mNmm^{-1}$, *n* 10, respectively, $P = 0.6$, by unpaired Student's *t* test). As shown in Fig. 4.3, arteries from control and low protein fed virgin rats responded to the endothelium-dependent vasodilator acetylcholine (ACh) with dose-dependent relaxations that completely reversed the PE-induced preconstriction. The concentration-response curve to ACh was visibly shifted to the right in arteries from the 9% casein group ($P = 0.0006$, by Two-way ANOVA). On the 9% casein diet, the sensitivity to ACh was significantly reduced by 4% ($P = 0.03$) and the maximum relaxation to ACh by 3% ($P = 0.04$), (by Bonferroni post-tests) in comparison to the 18% casein diet (Table 4.4 and Figure 4.3). The low protein diet was associated with blunted endothelium-dependent relaxation in virgin rats.

Concentration-dependent relaxation to increasing concentrations of spermine NONOate was observed in arteries from both groups submaximally contracted with phenylephrine. The contractions induced by PE (5 μM) were not significantly different in arteries from virgin 18% casein and 9% casein fed rats (tension = 2.58 ± 0.25 $mNmm^{-1}$, *n* 9, *versus* 2.73 ± 0.23 $mNmm^{-1}$, *n* 9, respectively, $P = 0.9$, by unpaired Student's *t* test). On the 9% casein diet the sensitivity to spermine NONOate was decreased by 11 % ($P = 0.0003$) and maximum relaxation by 7 % ($P = 0.009$) (Figure 4.4 and Table 4.4).

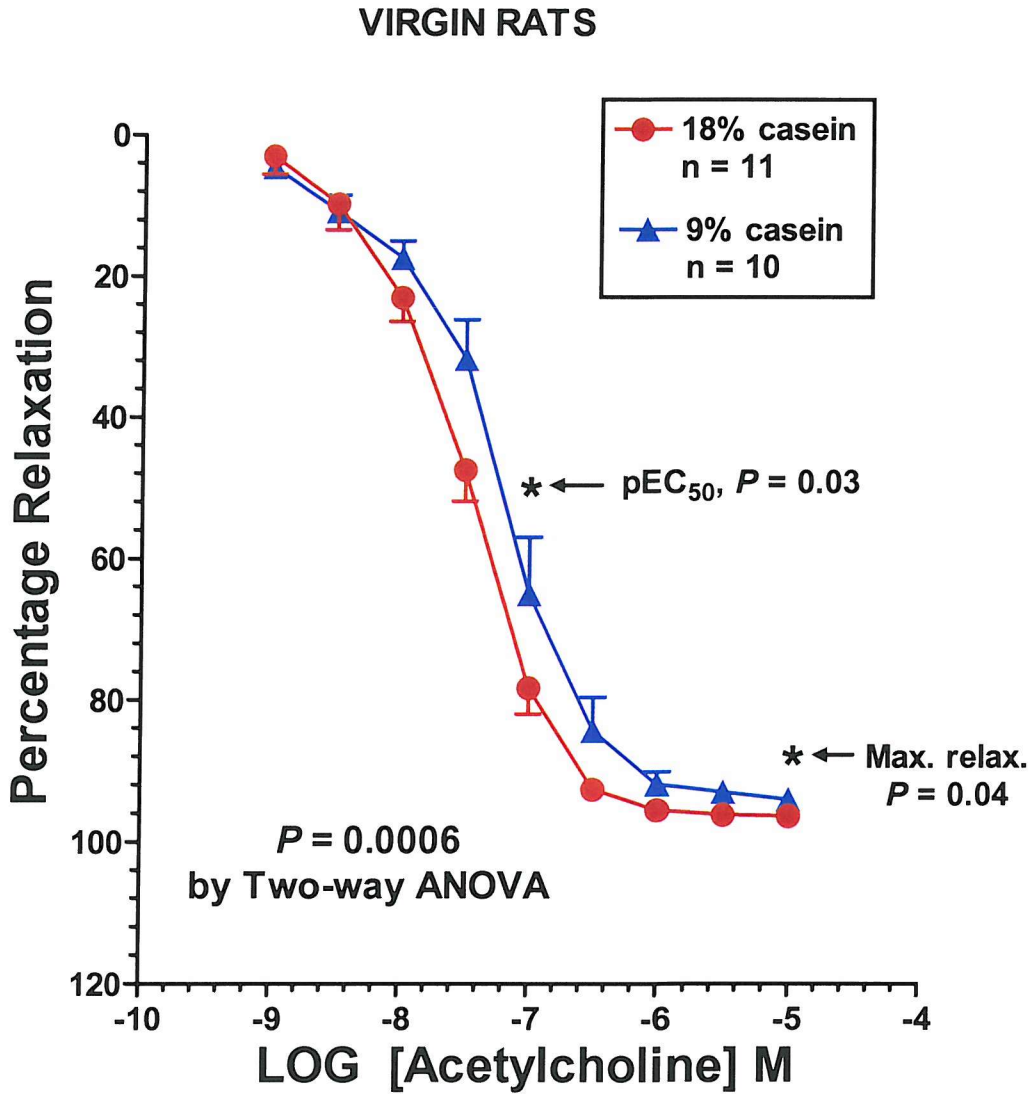
TABLE 4.4

The effects of feeding an 18% and a 9% casein diet on responses to dilatory agonists in small mesenteric arteries from virgin Wistar rats.

VIRGIN RATS		**18% Casein**	**9% Casein**	**P value**
Agonist		**Mean ± S.E.M.**	**Mean ± S.E.M.**	
ACh	**pEC_{50} (M)**	7.51 ± 0.06 (11)	7.20 ± 0.12 (10)	0.03
	Max. relax. (%)	96.36 ± 0.99 (11)	93.59 ± 0.77 (10)	0.04
SPN	**pEC_{50} (M)**	6.11 ± 0.09 (9)	5.46 ± 0.10 (9)	0.0003
	Max. relax. (%)	96.66 ± 1.8 (9)	89.22 ± 1.8 (9)	0.009

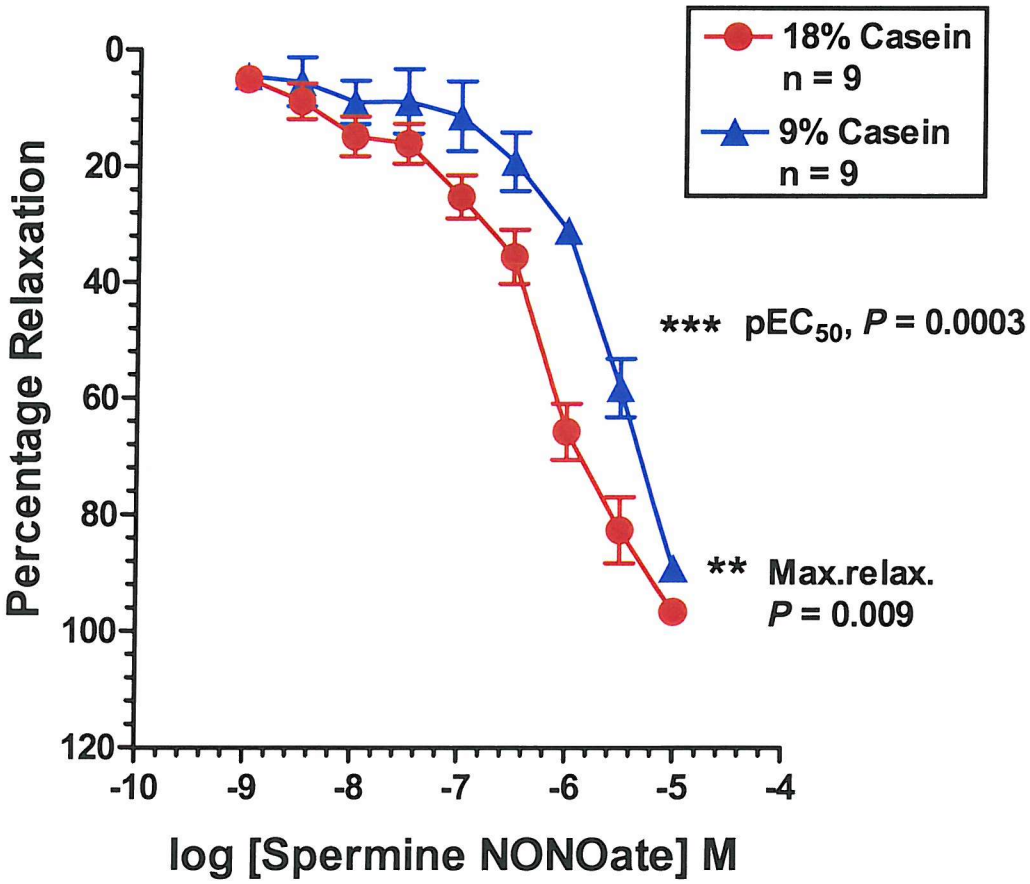
Table 4.4 summarizes data related to the dose-response curves presented in Figs. 4.3 and 4.4. Data are presented as the mean value ± S.E.M. Numbers in parentheses indicate the number of animals studied. ACh, 1 nmol to 10 μmol/l acetylcholine; SPN, 1 nmol/l to 10 μmol/l spermine NONOate; Data are expressed as pEC_{50} values, which are defined as the negative logarithm to base 10 of the EC_{50} values. Max. relax. = maximum relaxation expressed as the percentage of phenylephrine-induced precontraction.

Figure 4.3.: Concentration-response curves to acetylcholine (ACh) in mesenteric arteries from virgin rats fed an 18% (●; n = 11) or 9% casein diet (▲; n = 10).



Data are expressed as a percentage of PE-induced preconstriction and shown as mean and standard error. *n* equals the number of rats. There was a significant shift in sensitivity and maximum relaxation (Max. relax) to ACh-induced relaxation in the low protein-fed group. *Asterisks* indicate acetylcholine concentrations with different responses in 18% and 9% casein groups with Bonferroni post-tests.

Figure 4.4.: Endothelium-independent relaxation responses to spermine NONOate (SPN) in mesenteric arteries from virgin rats fed an 18% (●; n = 9) or 9% casein diet (▲; n = 9).

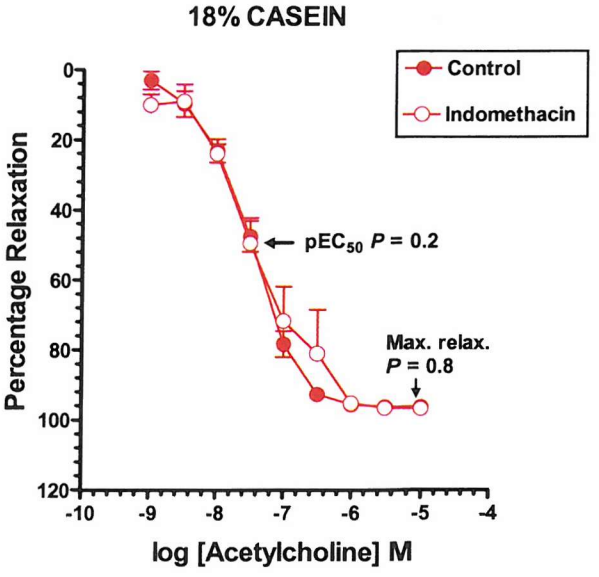


Data are expressed as a percentage of preconstricted tone and shown as mean and standard error (where not present, error bars lie within the symbols). *n* equals the number of rats. Max. relax. = maximum relaxation calculated as the percentage of phenylephrine-induced constriction $^{**}P < 0.01^{***}P < 0.001$.

4.3.6. Effect of inhibition of cyclo-oxygenase with indomethacin (INDO) on vasodilator responses to acetylcholine of phenylephrine precontracted mesenteric arteries isolated from virgin rats fed 18% casein.

Incubation of arteries from 18% casein fed virgin rats with 10 μM indomethacin did not influence the level of precontraction elicited by 5 μM PE compared with the previous response obtained in the absence of indomethacin (18% casein with indomethacin 2.42 ± 0.21 $mNmm^{-1}$, *n* 3, *versus* 2.55 ± 0.17 $mNmm^{-1}$ *n* 11, without indomethacin, $P = 0.2$, by Wilcoxon matched pairs test). Incubation of arteries from 18% casein fed rats with 10 μM indomethacin for 20 minutes effected no significant change in the subsequent vasodilator response to acetylcholine. The responses in these vessels did not differ before and after indomethacin (these were: 18% casein in the absence of indomethacin: pEC_{50}: 7.51 ± 0.06, *n* 11, *versus* 7.43 ± 0.11 in the presence of indomethacin, *n* 3; $P = 0.2$; maximum relaxation: 18% casein in the absence of indomethacin: 96.36 ± 0.99 %, *n* 11, *versus* 96.83 ± 0.27 % in the presence of indomethacin, *n* 3; $P = 0.8$) (Table 4.5) (Figure 4.5).

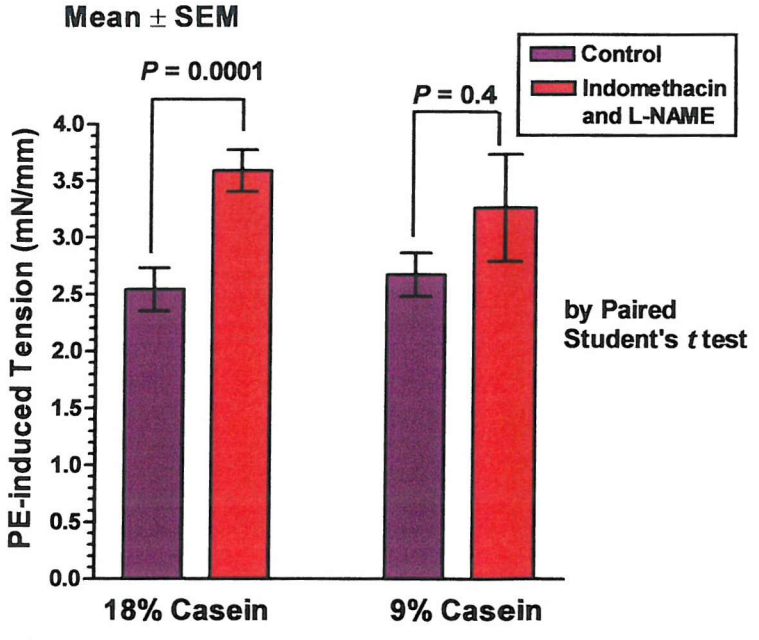
Figure 4.5.: Concentration-response curves to ACh in arteries from virgin rats fed an 18% casein diet before (●; n = 11) and after (○; n = 3) preincubation with 10 μM indomethacin.



4.3.7. Effect of indomethacin (INDO) and N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) on tension changes to phenylephrine in small arteries.

Mesenteric arteries from both groups were studied in control solution or in the presence of indomethacin and L-NAME. In the presence of indomethacin (10 μM) the subsequent addition of 100 μM L-NAME augmented the magnitude of pre-contraction to PE significantly by 41% compared to control conditions in arteries from rats on the 18% casein diet (18% casein with indomethacin and L-NAME, tension = 3.59 ± 0.18 mNmm^{-1}, *n* 6, *versus* 2.55 ± 0.17 mNmm^{-1} *n* 11, without the inhibitors, $P = 0.0001$, by paired Student's *t* test), (Fig. 4.6). In arteries from rats on the 9% casein diet, the combination of indomethacin with L-NAME caused an augmentation of the precontraction to PE by 25% compared to control (9% casein with indomethacin and L-NAME, tension = 3.26 ± 0.47 mNmm^{-1}, *n* 6, *versus* 2.62 ± 0.20 mNmm^{-1} *n* 10, without the inhibitors, $P = 0.4$, by paired Student's *t* test) (Fig. 4.6). In the presence of the inhibitors, preconstriction to PE was similar in arteries from 18% and 9% groups ($P = 0.5$).

Figure 4.6.: The effect of blockers on contraction evoked by phenylephrine.



4.3.8. Effect of indomethacin (INDO) and N $^{\omega}$-nitro-L-arginine methyl ester (L-NAME) on arterial vasodilator response to ACh.

Preincubation of arteries with indomethacin and L-NAME resulted in significantly blunted ACh relaxations in both dietary treatment groups leading to a significant shift in the pEC_{50} in the arteries from both groups and significant reduction in maximum relaxation in both groups [Figure 4.7 (a),(b)]. In arteries from rats on the 18% casein diet sensitivity to ACh was decreased by 12 % ($P < 0.0001$, by Two-way ANOVA) and maximum relaxation by 23 % ($P = 0.02$), (Table 4.5) [Figure 4.7(a)]. In arteries from rats on the 9% casein diet sensitivity to ACh was reduced by 12 % ($P < 0.0001$, by Two-way ANOVA) and maximum relaxation by 18 % ($P = 0.09$, by Two-way ANOVA) (Table 4.5) [Figure 4.7(b)]. However there were no significant differences in the sensitivity ($P = 0.3$) and maximum relaxation ($P = 0.9$) between the 18% and 9% groups in the presence of the inhibitors (Table 4.5).

TABLE 4.5

The effects of feeding an 18% and a 9% casein diet on responses to dilatory agonists in the presence of 10 μM indomethacin (INDO) and 100 μM L-NAME in small mesenteric arteries from virgin Wistar rats.

VIRGIN RATS		**18% Casein**	**9% Casein**	**P value**
Agonist		**Mean ± SE**	**Mean ± SE**	
ACh + INDO	**pEC_{50} (M)**	7.43 ± 0.11 (3)	Not measured	Not available
	Max. relax. (%)	96.83 ± 0.27 (3)	Not measured	Not available
ACh + INDO + L-NAME	**pEC_{50} (M)**	6.19 ± 0.21 (6)	6.36 ± 0.18 (6)	0.3
	Max. relax. (%)	74.03 ± 11.30 (6)	76.45 ± 12.41 (6)	0.9

Data are expressed as mean ± SEM. Number of rats (n) stated for each experiment. ACh, 1 nmol to 10 μmol/l acetylcholine; pEC_{50}, -log EC_{50} where EC_{50} is the concentration which produces 50% maximum response; Max. relax. = maximum relaxation calculated as the % of PE-induced constriction.

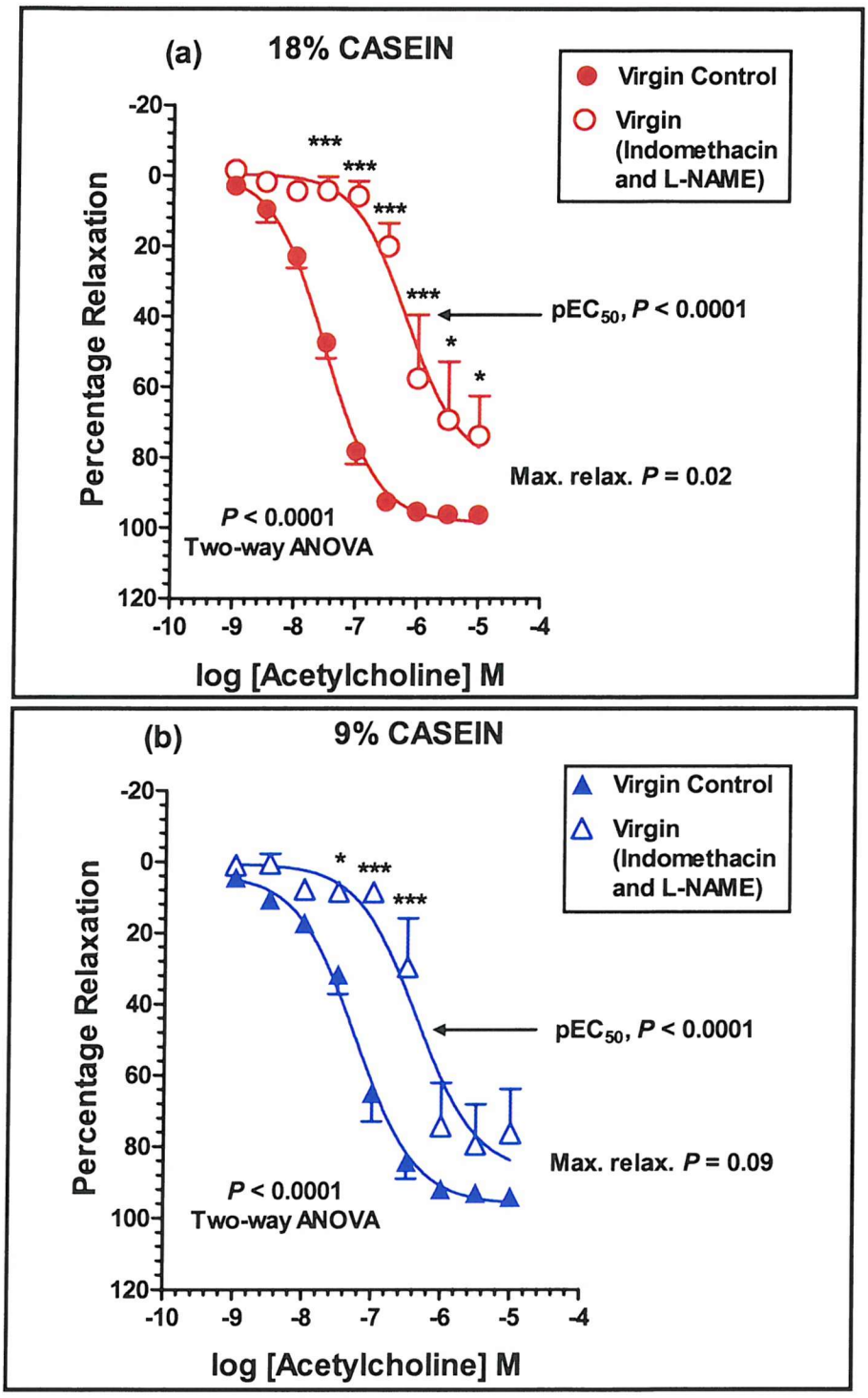


Figure 4.7 (a) and (b).: A comparison of the ACh concentration-effect curves after precontraction with 5 μM PE in arteries from (a) 18% casein and (b) 9% casein fed virgin rats. Responses were carried out in the absence of the inhibitors (control ●; *n*, 11, control ▲; *n*, 10) and after pre-incubation in 10 μM indomethacin and 100 μM L-NAME (○; *n*, 6, △; *n*, 6).

4.4 DISCUSSION

The present study investigated the influence of a low protein diet (9% casein) on *in vitro* vascular reactivity of isolated mesenteric arteries from virgin rats to PE, KCl, ACh and SPN. The present study has shown that vasorelaxation in response to the endothelium-dependent vasodilator acetylcholine was impaired in small mesenteric arteries from virgin rats fed 9% casein (Fig. 4.3). This observation is consistent with findings that have been reported previously in rats fed a low protein diet (6% casein) for two weeks (Tolins *et al*, 1995), in which decreased renal vasodilatation was observed compared with rats fed high protein diet (50%) for two weeks.

No significant differences in either 125 mM potassium-induced or dose-dependent PE-induced and KCl-induced contractions of the vessels were detected between the 18% and the 9% groups. Therefore, any differences between the groups do not appear to be related to an increase in vascular smooth muscle cell growth.

The responsiveness of mesenteric arteries to the NO donor SPN was suppressed (Fig. 4.4), suggesting that the ability of the vascular smooth muscle to respond to NO was altered by the consumption of a low protein diet. SPN is considered to be a pure NO donor, releasing NO on an almost equimolar basis (Ramamurthi and Lewis, 1997; Gerber *et al*, 1998).

ACh-induced vasodilatation has been known to be mediated by endogenous NO. ACh has also been shown to release vasodilator prostaglandins from several vascular beds (De Nucci *et al*, 1988). In a study of spinal resistance arteries in rabbits, Yashiro and Ohhashi, (1997) demonstrated that the ACh-induced vasodilatation was significantly reduced by 10 μM indomethacin, the concentration of which is known to inhibit significantly the cyclooxygenase activity in tissues (Koller and Kaley, 1990) and suggested that ACh produces an endogenous prostaglandin-mediated vasodilatation. In contrast, the current study demonstrated that the inhibition of cyclooxygenase-dependent pathways by 10 μM indomethacin had no significant effect on ACh-dependent relaxation in the 18% casein-fed animals (Fig. 4.5), and it is unlikely,

therefore that a dilator prostanoid plays a significant role in ACh-induced relaxation in this vascular bed. This is in agreement with a study of extrapulmonary, celiac strips and mesenteric arteries in the rabbit (Förstermann *et al*, 1986) which showed that the ACh-dependent formation of vasodilatory prostaglandins made only a limited contribution to the ACh-induced vasodilatation. The effect of inhibition of COX with indomethacin on the response to ACh of arteries from virgin rat fed 9% casein diet was not determined. The origin of impaired endothelium-dependent relaxation in the mesenteric arteries from low protein-fed virgin rats could lie in abnormalities of the production of different constrictor stimuli. For example, increased release of EDCF, which is a product of arachidonic acid cascade synthesized via the cyclooxygenase pathway, can also impair relaxation. In a study of mesenteric arteries in stroke-prone spontaneously hypertensive rats (SHRSP), Sunano *et al*, (1999) found that indomethacin improved the impaired dilator response to ACh and suggested that the defect was due in part to the production of a constrictor prostanoid. However, such factor may be unable to account for the dilator dysfunction reported here because the formation of such factor can only be evidenced following application of relatively high concentrations of ACh (1-10 μM) (Auch-Schwelk *et al*, 1990), whereas in the present study impaired endothelium-dependent relaxations in low protein-fed virgin rats were already apparent at 100-fold lower agonist concentrations.

In the presence of both indomethacin and L-NAME, the difference in ACh-induced relaxation between 18% and 9% casein-fed virgin rats was no longer evident. This could be interpreted as there being a reduced nitric oxide mediated component of relaxation in the virgin low protein-fed rats. Thus, the defect in endothelium-dependent relaxation to ACh in resistance arteries from low protein-fed virgin rats could result from a reduction in the amount of nitric oxide released from the endothelium or an acceleration in the breakdown of nitric oxide. Endothelium-independent mechanisms are also involved, as the vasodilator potency of the NO donor SPN was significantly attenuated in arteries of virgin 9% casein-fed rats (Fig. 4.4) and to a greater extent than the upstream endothelial-mediated mechanisms. This additional defect could reflect

reduced responsiveness of vascular smooth muscle and/or enhanced clearance of NO, and indicates that dietary protein restriction induces distinct abnormalities in the endothelium and vascular smooth muscle. Alternatively, it could be hypothesized that the blunted responsiveness to ACh in virgin low protein-fed rats probably reflects the combined effects of a decrease in both the NO producing ($_{ec}$NOS) and effector (soluble guanylyl cyclase) enzymes. It has recently been reported that, in the isolated perfused heart of the hypertensive rat, both the bradykinin- and sodium nitroprusside-induced vasodilator responses are attenuated compared to normotensive rats, and that, in the aortae from adult hypertensive rats, the endothelial NO synthase expression is not different compared with normotensive rats, whereas soluble guanylate cyclase expression is markedly decreased in the hypertensive rats (Bauersachs *et al*, 1998). These results support the notion that the release of NO by endothelial cells is unaltered in hypertensive rats. In the present experiments, endothelium-dependent vascular relaxation to ACh and the response to exogenous NO donor SPN was significantly attenuated in arteries from low protein-fed virgin rats. Alteration of muscarinic receptor-mediated $_{ec}$NOS stimulation would explain the reduced ACh response, but it does not explain the impaired response to the NO donor SPN. As ACh and SPN both induce vasodilatation by NO-mediated vascular smooth muscle relaxation, increased breakdown of NO would explain the results obtained from the present study. It is also possible that a defect in smooth muscle response per se that relates to a downregulation, inhibition, or saturation of one or more components of the vascular smooth muscle targets of NO, such as guanylate cyclase or the cGMP-dependent protein kinase, may be responsible for the observed findings. There is also evidence of unaltered $_{ec}$NOS protein expression in aortae of protein-deficient virgin rats, which is presented later (Chapter 6). However, there is no direct evidence that $_{ec}$NOS protein expression is unaltered in the mesenteric resistance vessels of the low protein-fed virgin rat. Therefore, the findings of the present study suggest an impairment of NO-dependent vasodilator function either at the level of or downstream from sGC.

The endothelium-dependent relaxation induced by acetylcholine in the mesenteric arteries was only partially blocked by a combined application of indomethacin and L-NAME (Fig. 4.7). Thus, elimination of the synthesis or action of NO and prostanoids only partially inhibited endothelium-dependent dilatation in the resistance vasculature. This indicates that a factor other than NO or a prostanoid is also involved in the relaxation. One of the factors that can induce relaxation of the mesenteric artery in response to muscarinic agonist ACh and other vasoactive substances is EDHF (Garland and McPherson, 1992). The EDHF component of the relaxation has been shown to be more important in smaller than larger arteries (Hwa *et al*, 1994). Based on studies in rat hepatic and mesenteric arteries, Edwards *et al* (1998), first showed the critical importance of endothelial cell hyperpolarization in the EDHF pathway. These workers concluded that EDHF was likely to be K^+ released as a consequence of endothelial cell K^+ channel opening and hyperpolarization. In male and female rats, relaxation to ACh in the mesenteric vascular bed is mediated by endothelium-derived NO and EDHF with the contribution of EDHF greater in female than male rats (McCulloch and Randall, 1998). Low protein feeding to virgin rats did not seem to affect the NO-independent component of the relaxation (EDHF) as the inhibition of the relaxation by L-NAME was not different between 18% and 9% casein-fed virgin groups.

There are a number of possible explanations for the adverse effects of the low protein diet on vascular reactivity in isolated mesenteric arteries from virgin female rats. A low protein diet may induce serum oestrogen deficiency in the rat (Ammann *et al*, 2000). This group investigated the effects of four isocaloric diets with varying levels of protein content (15, 7.5, 5, and 2.5% casein) on sex hormone status in adult female rats. Ammann and colleagues (2000), reported that after 16 weeks on a 2.5% casein diet, no oestrus sign in vaginal smear was observed. An earlier study in pregnant rats showed that plasma oestrogen concentrations were significantly reduced in rats whose dietary intake was globally restricted by 50% over the last 7 days of pregnancy (Gonzàlez *et al*, 1997). Oestrogen is likely to have an important influence on vascular function, with studies demonstrating that chronic oestrogen, for example due to long-term experimental exposure, enhanced

endothelium-dependent vasodilatory responses to both ACh (Vedernikov *et al*, 1997) and to flow (Cockell and Poston, 1997). These effects have been shown to be dependent on NO and appear to result from a genomic increase in the synthesis of NO synthase (Weiner *et al*, 1994). A reduction in oestrogen could therefore contribute to the reduced ACh responses in the low protein group. However, in the present study, although the mean serum oestrogen concentrations were numerically lower in rats fed 9% casein, there was no significant difference in the mean values between the two dietary groups (Table 4.2). Feeding a low protein diet (2% casein) to growing rats for 28 days has been shown to decrease serum concentrations of triacylglycerol and total cholesterol (Bouziane *et al*, 1992). However, in the present study, the mean plasma cholesterol and TG concentrations were numerically higher in rats fed 9% casein, but not significantly (Table 4.2). High LDL-cholesterol is a risk factor for atherosclerosis and cardiovascular events (Balligand, 2002). Dysfunction of the endothelium, e.g. the impairment of its capacity to produce NO, is an early step in atherogenesis. Balligand, (2002), identified a mechanism of endothelial toxicity of LDL-cholesterol that alters the activity of the endothelial isoform of NO synthase in the absence of changes in its expression. Data obtained in the present study, showing higher serum cholesterol and TG levels in the 9% casein group compared with the 18% casein group, are not significant and are therefore inconclusive as to whether increased cholesterol and TG levels could play a part in the diminished vasodilator response observed in the low protein group.

Another potential mechanism that could explain the impaired biological activity of the NO pathway observed in protein-deficient rats is L-arginine (Arg) deficiency. Arg is classified as a nutritionally essential amino acid for young mammals and for adults at times of stress and illness because endogenous Arg synthesis cannot meet arginine needs for optimal growth and health (Visek, 1986). As a precursor for the syntheses of protein and NO, Arg plays central roles in nutrition and metabolism (Wu and Morris, 1998). As a substrate for $_{ec}$NOS, Arg is essential for maintaining the enzyme in the active dimerization state (Harrison, 1997).In young mammals, dietary Arg deficiency results in a decrease in plasma Arg concentrations. Feeding 0.0 or 0.3% Arg diet to 30-day old male rats resulted

in marked growth retardation and decreased serum Arg concentrations compared with feeding 1% Arg diet. In addition, decreasing dietary Arg concentrations from 1% to 0% caused progressive decreases in constitutive and inducible NO synthesis in young rats (Wu *et al*, 1999). *In vitro* studies have demonstrated that increasing extracellular arginine concentrations increased NO synthesis by $_{ec}$NOS in endothelial cells (Arnal *et al*, 1995) and by iNOS in activated macrophages (Norris *et al*, 1995) in a concentration-dependent manner. Wu *et al*, (1999) found that although Arg serum levels were similar in rats fed the 5% casein diet and the 0.3% Arg diet, urinary excretion of nitrate by rats fed the 5% casein diet was only ~45% of that by rats fed the 0.3% Arg diet. It can be suggested that in protein-deficient rats factors other than decreased plasma Arg availability (e.g., decreased cNOS activity) contribute to the impaired constitutive NO synthesis.

Gross *et al* (1991), reported that plasma and liver glutamine levels increased in rats fed arginine-devoid diets for 13 days. An increase of glutamine concentration in the serum of 9% casein-fed rat dams has been shown by Rees *et al* (1999). Glutamine has been shown to decrease NO production in intact blood vessels (Swierkosz *et al*, 1990). It has also been shown that increasing extracellular L-glutamine concentrations from 0 to 2 mM dose-dependently decreases NO synthesis by bovine aortic endothelial cells under basal and stimulated conditions (Arnal *et al*, 1995). Recent evidence has shown that ecNOS expression and the intracellular concentrations of NOS substrates or cofactors, such as BH4, Ca^{2+}, and NADPH, are unaffected by glutamine or its metabolites (ie. ammonia, alanine, aspartate, glutamate) (Wu *et al*, 2001). In the same study it was shown that the metabolism of glutamine to glucosamine-6-phosphate in cultured bovine venular endothelial cells was required for inhibition of NO synthesis by glutamine (Wu *et al*, 2001).

Supplementation of the 18% and 9% casein diets with equal amounts of methionine (5 g/kg diet) resulted in proportionately higher levels of methionine in the 9% casein group (Appendix 2). The metabolic pathway converting methionine into cysteine involves the formation of homocysteine, which is condensed with serine to form cystathionine and finally cysteine (Selhub, 1999).

Homocysteine is converted to methionine by methionine synthase, which requires 5-methyltetrahydrofolate and methylcobalamin (vitamin B12) as cofactors. There is evidence to show that increasing extracellular concentrations of homocysteine decreases NO synthesis by endothelial cells (Zhang *et al*, 2000) and platelets (Mutus *et al*, 2001) thereby impairing endothelium-dependent relaxation. Although this could be relevant, Rees *et al* (1999, 2000), found no corresponding increase in serum methionine concentrations in nonpregnant rats fed low protein diet, despite the fact that this excess methionine cannot be used for protein synthesis, suggesting that it was being metabolized. Dietary methionine loading or hyperhomocysteinemia causes endothelial dysfunction. Impaired endothelium-dependent vasodilatation has been reported in humans after an oral methionine load (Bellamy *et al*, 1998), and in monkeys fed on a diet enriched in methionine and deficient in folate and choline (Lentz *et al*, 1996). Little is known about the mechanism for the inhibition of endothelial NO synthesis by homocysteine. Böger and coworkers (2001), recently reported that incubation of human EAhy.926 endothelial cells with 300 μM L-methionine increased N^G,N^G-dimethyl-L-arginine (asymmetrical dimethylarginine; ADMA) levels in the culture medium by 30% compared with the control. ADMA is an endogenous competitive inhibitor of NOS (Vallance *et al*, 1992). ADMA is synthesized by methylation of the side-chain guanidino nitrogen atoms of L-arginine residues within proteins, due to the action of methyltransferases that utilize S-adenosylmethionine as a methyl group donor (Gary and Clark, 1998). S-adenosylmethionine is an intermediate in the demethylation of methionine to homocysteine. Therefore, increased availability of S-adenosylmethionine during experimental hyperhomocysteinaemia induced by oral methionine loading, may stimulate the formation of ADMA, which will then inhibit endothelial NOS. There is evidence showing that inhibition of S-adenosylmethionine-dependent methyltransferase decreased the LDL-cholesterol-induced increase in ADMA release in cultured human endothelial cells (Böger *et al*, 2000).

Another possible explanation for the increase in ADMA levels after methionine loading may be decreased activity of dimethylarginine dimethylaminohydrolase, the enzyme that degrades ADMA. There is recent evidence

showing that homocysteine dose-dependently decreased the activity of recombinant human dimethylarginine dimethylaminohydrolase in a cell-free system (Stuhlinger *et al*, 2001). Although intracellular ADMA levels were not measured in the above study, these results suggest that methionine is converted to homocysteine in endothelial cells and that homocysteine decreases endothelial NO synthesis by increasing ADMA formation owing to an inhibition of its degradation.

Enhanced oxidative stress could play a critical role in the deleterious effect of the diet on the endothelium by means of nitric oxide breakdown due to reactive oxygen species (Stamler *et al*, 1993). During the autoxidation of the sulfhydryl group of biological thiols, hydrogen peroxide is formed (Loscalzo, 1996). Methionine does not have a free thiol group, suggesting a role for homocysteine in arterial endothelial impairment. Increased levels of oxygen free radicals can react with NO, thus decreasing its bioavailability by producing peroxynitrite and other NO radicals. Peroxynitrite ($ONOO^-$) is a potent cytotoxic reactive nitrogen species that subsequently reacts with proteins, lipids, and DNA to induce tissue damage (Halliwell, 1997). Homocysteine has been reported to decrease intracellular glutathione and glutathione peroxidase, which are responsible for the elimination of oxygen free radicals (Upchurch *et al*, 1997). There is also evidence suggesting that a high fat diet increases free radical production (Slim *et al*, 1996). It would be of interest to determine whether the low protein diet-induced impairment in the ACh and SPN response is reversible in the presence of superoxide dismutase or catalase, which are scavengers of reactive oxygen metabolites.

Although the exact mechanisms responsible for the impairment of NO-mediated arterial response in the protein deficient virgin rats still need to be further investigated, the present study provides the first evidence that the function of microvessels is importantly affected by a low protein diet.

In summary, the present findings suggest that a low protein diet is associated with reduced ACh- and SPN-induced dilations of the mesenteric arteries of the virgin rat. It is suggested that the reduced responsiveness to endothelium-derived NO seen in resistance arteries of the low protein-fed virgin rat is likely to result, at least in part, from a reduction in the cyclic-GMP-mediated response in vascular smooth muscle cells.

CHAPTER 5

THE EFFECTS OF DIETARY PROTEIN RESTRICTION ON VASCULAR REACTIVITY IN ISOLATED MESENTERIC RESISTANCE ARTERIES FROM PREGNANT WISTAR RATS.

5.1. INTRODUCTION

The adaptations to pregnancy in mammals include significant increases in maternal blood volume and cardiac output. These changes are likely to have important effects on fetal growth, since the increase in uterine blood flow in pregnancy and thus supply of nutrients to the fetus will in part depend on them. During pregnancy in rats, maternal plasma volume increases by 62% (Lederman and Rosso, 1989) and cardiac output by 43% (Ahokas *et al*, 1983). However, plasma volume fails to increase during pregnancy if rats are fed a low protein diet (6% casein) (Rosso and Streeter, 1979), and the increase in cardiac output is blunted when the maternal diet is globally restricted by 50% (Ahokas *et al*, 1983; Rosso and Kava, 1980). The same dietary restriction also leads to decreases in total uterine and placental blood flows by 60-65% (Ahokas *et al*, 1983) and a reduction in the birthweight of the offspring (Ahokas *et al*, 1983; Rosso and Kava, 1980). There has recently been renewed interest in these maternal responses to dietary restriction since the offspring of rats subjected to dietary protein restriction (9% casein) develop hypertension in adulthood (Langley and Jackson, 1994; Langley-Evans *et al*, 1996). Thus a deficient maternal cardiovascular response to pregnancy could compromise development of the fetus and therefore contribute to fetal programming of adult disease.

The cardiovascular adaptation to pregnancy is believed to be initiated by a

reduction in peripheral resistance. The decrease in peripheral resistance is substantial (approximately 25% in humans and monkeys) and it is completed in early pregnancy. As peripheral resistance falls, blood pressure is maintained by a rise in cardiac output. The initial vasodilatation is likely to involve much (or all) of the systemic circulation as it is unlikely to be achieved through dilatation and increased perfusion of the uterine circulation, which is minimal in early pregnancy. It was hypothesised that peripheral vasodilatation would be reduced in rats fed a low protein diet during pregnancy.

The low protein diet (9% casein) meets the recommended requirements of the non-pregnant rat (Clarke *et al*, 1978) and, when given in pregnancy, it has been shown to restrict fetal growth and to lead to hypertension in the offspring (Langley-Evans *et al*, 1996; Rees *et al*, 1999). The mesenteric circulation makes a major contribution to peripheral resistance in rats (Christensen and Mulvany, 1993), and therefore the ACh responses in small arteries from that vascular bed were investigated. This study has been presented to the Physiological Society (Koumentaki *et al*, 2001).

5.2 MATERIALS AND METHODS

5.2.1. Animals

Virgin female Wistar rats were purchased from Charles River UK and housed at the University of Southampton Biomedical Research Facility. The animals were fed a standard non-purified laboratory chow diet *ad libitum* (CRMX, Special Diet Services, Cambridge, UK) (Appendix 1) with free access to water. On attaining body weights of 220-250 g, virgin rats were caged overnight with a male breeder of the same strain. Mating was confirmed by detection of a vaginal plug and this day was denoted as day 1 (term = 22 days) and the experimental diets were started at this time point. The experimental diet composition is shown in Appendix 2. The methods of dietary administration were as described previously in Chapter 2 (sections 2.2.1-2.2.2).

A total of 16 pregnant animals were given free access to an 18% casein diet until 18-19 days of pregnancy. A further 16 pregnant animals were fed a 9% casein diet until 18-19 days of gestation. Two of the rats fed the 9% casein diet were found not to be pregnant and were therefore excluded from the study. Only those animals bearing 8-13 fetuses were included in the pregnant groups as finally constituted.

Experiments were performed at days 18-19 of pregnancy. The rats were killed by CO_2 inhalation and cervical dislocation and blood was collected by cardiac puncture. After weighing the rat, the abdominal wall was transected and the small intestine and intact mesentery were rapidly removed from the animal and placed in ice-cold physiological salt solution (PSS). The thoracic aorta was excised immediately, snap frozen in liquid nitrogen, and stored at -70°C for measurement of $_{ec}$NOS protein expression (as described in Chapter 6). The entire uterus was also removed and the fetuses and placentas were carefully detached from the uterine wall. The fetuses and placentas were separated from the amniotic membranes and fluid, were blotted dry, and weighed separately. The fetuses were killed by decapitation. As a representative sample, fetal and placental weights were measured from the first five fetuses delivered

from the right uterine horn in each female. All experimental procedures were approved and conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986.

The total weight gain of the rats during gestation was calculated by the difference between body weight at day 1 and day 18-19 of gestation. The net maternal weight gain during pregnancy was calculated as the difference between maternal weight at day 18-19 and body weight at day 1 of gestation.

5.2.2. Tissue and plasma sample collection and analysis

The blood obtained by cardiac puncture in pregnant dams at 18-19 days gestation (when vascular function was assessed) was dispensed into tubes containing either ethylenediamine-tetra-acetic acid (EDTA; 28 mmol/L) or no anticoagulant. The blood was then centrifuged, after standing at 4°C for 30 minutes, (2500g, 15 min, 4°C) and the plasma and serum samples obtained were frozen and stored at -20°C for later measurements of blood analytes [cholesterol, triacylglycerols (TGs) and 17β-oestradiol]. 17β-oestradiol concentration in serum was determined using a commercially available radioimmunoassay (RIA) (Codes 12264/12268, Estradiol Maia, Serono Diagnostics, UK) as described in section 4.2.3. Plasma cholesterol and triglyceride measurements were performed by the Chemical Pathology Department, Southampton General Hospital as described in section 4.2.4.

The thoracic aorta was rapidly dissected, cleaned with phosphate buffered saline (PBS), collected into sterile tubes, snap frozen in liquid nitrogen and subsequently stored at -70°C until analysis. Homogenates (30% wt/vol) were prepared in ice-cold RIPA buffer containing 1 x PBS, pH 7.4, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 10 mg/ml phenylmethylsulfonyl fluoride (PMSF), and 100 mM sodium orthovanadate at 0 to 4°C with the aid of a tissue grinder fitted with a motor-driven ground glass pestle. The homogenates were then transferred to microcentrifuge tubes, were centrifuged at 14,000xg for 20 minutes at 4°C, and the supernatant was removed and centrifuged again for 5 minutes at 14,000xg at 4°C and

the supernatant was used for Western blot analysis of endothelial constitutive ($_{ec}$NOS) protein (see *Chapter 6*).

5.2.3. Assessment of vascular function

Twenty-two pregnant Wistar rats were studied. (18% casein: n = 12; 9% casein: n = 10). The small intestine and intact mesentery were removed from the animal and placed in ice-cold physiological saline solution (PSS). In order to obtain arteries of approximately equal diameter in 18% and 9% casein-fed animals, third branch mesenteric arteries were routinely dissected. Following dissection, arteries were mounted as ring preparations on a small vessel myograph (see section 3.2, Chapter 3).

A total of seven arteries failed to produce a maximum active tension equivalent to an effective pressure of 100 mmHg (13.3 kPa) on the last NAK application and were therefore excluded from the study.

After the routine run-up procedure, cumulative concentration-response curves, at increments of 2 min duration, were constructed to PE (final bath concentrations $1x10^{-7}$-$1x10^{-5}$ M) (section 3.4: *Experimental protocol*). The PE responses were studied in arteries from 12 pregnant rats fed 18% casein and 10 rats fed 9% casein. Following repeated washing and recovery (10 minutes) a dose response curve to KCl (5mM - 125 mM) was constructed. The myograph chamber was then washed four times with PSS and a 10 min recovery period allowed before the arteries were precontracted with a submaximal concentration of PE (3-5 μM) to achieve approximately 80% of the maximum response and relaxation induced by ACh ($1x10^{-9}$ - $1x10^{-5}$ M) was then assessed. The ACh responses were studied in arteries from 10 rats in each group. A similar protocol was used to assess endothelium-dependent relaxation to BK ($1x10^{-9}$ - $1x10^{-5}$ M). The experimental protocol was very long therefore the BK response curve was performed in arteries from six rats fed 18% casein and four rats fed 9% casein. Following the BK concentration-effect curve, the chamber was washed four times with PSS and a further 10 min recovery period allowed before the arteries were submaximally precontracted for 2 min with the

period allowed before the arteries were submaximally precontracted for 2 min with the concentration of PE required to produce approximately 80% of the maximum response. Endothelium-independent relaxation to SPN was subsequently assessed by adding increasing concentrations of SPN at 2 min intervals ($1x10^{-9}$ - $1x10^{-5}$ M). A similar protocol was used to assess 8-br-cGMP ($3x10^{-6}$ - $1x10^{-3}$ M) responses.

After washout for 10 minutes indomethacin (10 μM) was added to the bath for 20 minutes and the arteries were again precontracted with PE in the continued presence of indomethacin. A second concentration-effect curve to ACh was then determined in the continued presence of indomethacin. The effect of indomethacin alone on ACh relaxation was tested in small mesenteric arteries from 9% casein-fed pregnant rats only. After washout for 10 minutes, indomethacin and N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) were added to the bath for 20 minutes and a third concentration-effect curve to ACh was carried out. Each artery was only subjected to one concentration of the inhibitor. Arteries were preconstricted with 2-4 μM PE, the concentration being adjusted in order to evoke similar preconstrictor tone to that observed without inhibitors.

5.2.4. Drugs

All general chemicals are described in the *Drugs and solutions*, (section 3.5, Chapter 3). All drugs were added to the vessel chamber and final concentrations are reported.

5.2.5. Data calculation and statistical analysis

The methods of data analysis and statistics were as described previously in Chapter 3 (section 3.6), and Chapter 4 (section 4.2.6). The maximum and half-maximum effective concentration (EC_{50}, expressed as -log mol/L = pEC_{50}) were calculated as the mean ± SEM of the individual values by nonlinear regression using the curve fitting programme 'Prism 3.0' (GraphPad Software Inc., San Diego, CA., USA) (see section 3.6). The effect of the diet on contractions of mesenteric arteries to PE and KCl and on relaxations in response to ACh and SPN, was tested by comparing the pEC_{50} values and maximum responses between the groups with use of the two-tailed Student's unpaired *t* test. Student's unpaired *t* test was chosen because data were consistent with a Gaussian population as indicated by testing for skewness. Skewness is a value that shows how skewed a curve is. The closer to zero the value for the skewness is, the more symmetrical is the distribution (GraphPad Software Inc., San Diego, CA., USA). A test was also used to detect how close a distribution is to normality. The Kolmogorov-Smirnov (KS) test was used to test for deviations from Gaussian distribution (GraphPad Software Inc., San Diego, CA., USA). Since the Gaussian distribution is also called the Normal distribution, the test is called a normality test (GraphPad Software Inc., San Diego, CA., USA). The Student's paired *t* test (parametric) was used for comparisons within each diet group. Dose-response analyses also employed two-way repeated-measures analysis of variance (ANOVA) (Prism 3.0, GraphPad Software Inc.). The effect of indomethacin and L-NAME on relaxation in response to ACh within each group was tested by two-way repeated-measures ANOVA followed by a Bonferroni *t* test. Relaxation responses to BK and 8-bromo-cGMP were not normally distributed and therefore responses to each BK and 8-bromo-cGMP concentration were compared between groups by the Mann-Whitney *U* test (two-tailed). Statistical analysis of serum 17β-oestradiol and plasma cholesterol and TG data was carried out using the unpaired Student's *t* test. Comparisons were considered significant when $P < 0.05$ and are reported as trends when $0.05 < P < 0.10$.

5.3 RESULTS

Part I: Effects of feeding an 18% and a 9% casein diet on body weight, fetal and placental weight and vascular function in the small mesenteric arteries from pregnant Wistar rats.

5.3.1. Effects of maternal protein restriction on body weight in pregnant animals.

Changes in total body weight and net maternal weight in 18% and 9% casein-fed rats over the course of gestation, days 1-18, are shown in Table 5.1. Net maternal weight is the total weight minus the weight of fetuses and placentas. As shown in Table 5.1, the average initial body weights of the two groups were the same at the start of the study period ($P = 0.9$, by unpaired Student's t test). Total body weight increased an average of almost 104 g in rats fed 18% casein *ad libitum* during gestation (days 1-18). Therefore, by day 18, 18% casein-fed pregnant rats weighed significantly 31% more than their mean weight on gestational day 1 ($P < 0.0001$), (by paired Student's t test) (Table 5.1). Like 18% casein-fed rats, 9% casein-fed pregnant rats gained an average of 111 g between days 1 and 18, which is 33% more than their mean weight on gestational day 1 ($P<0.0001$), (by paired Student's t test). The mean total weight gain between day 1 and days 18 or 19, was not statistically significantly different in 18% and 9% casein-fed rats ($P = 0.3$) (by unpaired Student's t test), (Table 5.1). Maternal net weight and net weight changes were not significantly different among the two groups after 18-19 days gestation (Table 5.1). Net maternal weight on days 18-19 in the dams fed the 18% casein diet remained 29% above the weight on gestational day 1 ($P < 0.0001$). Like 18% casein-fed rats, net maternal weight in the 9% casein-fed pregnant rats was 30% above their mean weight on gestational day 1 ($P < 0.0001$) (by paired Student's t test) (Table 5.1). There was no significant difference in maternal weights between 18% and 9% casein-fed pregnant rats at 18-19 days gestation ($P = 0.5$, by unpaired Student's t test), (Table 5.1).

5.3.2. Effects of maternal protein restriction on fetal and placental weight.

The effects of the two different dietary treatments on fetal and placental weight are shown in Table 5.1. By days 18-19 of gestation, the fetuses carried by the protein-deficient mothers were growth-retarded and weighed 13% less than those of mothers fed on 18% casein diet ($P = 0.005$) (Table 5.1), [Fig. 5.1 (b)]. Placental weight was also affected by protein restriction. By days 18-19 of gestation, placental weight in the protein-restricted group was significantly 14% less than that of 18% casein-fed group ($P < 0.0001$) (Table 5.1), [Fig. 5.1 (b)]. The numbers of fetuses carried by each mother were similar in animals fed on the 18% and 9% casein diets ($P = 0.7$) (by unpaired Student's *t* test), (Table 5.1). Mean weights of the conceptus mass for the two diet groups are shown in Table 5.1. Conceptus weight was not significantly altered by the dietary protein restriction ($P = 0.9$), (by unpaired Student's *t* test). There were no significant differences between groups in fetal death and resorption rate, which ranged from 0% to 3%.

TABLE 5.1

Maternal total body weight, weight gain, net maternal weight and net maternal weight changes, litter size and weight of the conceptus, fetal and placental weights on day 18 of pregnancy in rats fed on diets containing 18% or 9% casein.

	Dietary protein		
Parameter	**18% CASEIN**	**9% CASEIN**	***P* value**
Body weight (g)			
Initial	230.7 ± 8.2 (11)	231.2 ± 9.2 (10)	0.9
Final	335.8 ± 7.6 (12)	342.2 ± 7.0 (10)	0.5
Gain	104.1 ± 2.8 (11)	111.0 ± 6.1 (10)	0.3
Body weight change (%)	31.2 ± 1.0 (11)	32.6 ± 1.9 (10)	0.5
Net body weight (g)[1]	324.3 ± 8.0 (10)	328.7 ± 7.3 (10)	0.7
Net weight change (g)[2]	91.9 ± 3.4 (10)	97.5 ± 5.4 (10)	0.4
Net weight change (%)	28.6 ± 1.3 (10)	29.8 ± 1.7 (10)	0.7
Litter size	11.9 ± 1.1 (10)	11.4 ± 0.5 (10)	0.7
Conceptus weight (g)[3]	13.1 ± 1.8 (10)	13.5 ± 1.5 (10)	0.9
Fetal weight (g)	0.92 ± 0.03 (55)	0.80 ± 0.04 (50)	0.005
Placental weight (g)	0.36 ± 0.01 (55)	0.31 ± 0.01 (50)	<0.0001

Pregnant female rats were individually housed and given free access to either an 18% protein control diet or a 9% protein diet. Data are given as means ± SEM. [1] Total body weight on day 18 minus the weight of uterine contents. [2] Net maternal weight on day 18 minus initial body weight. [3] Includes fetal and placental weights combined. The numbers in parentheses indicate the number of animals studied. Comparisons between the two dietary treatment groups were made using the unpaired Student's *t* test and the *P* value denotes the statistical significance of the difference between the groups.

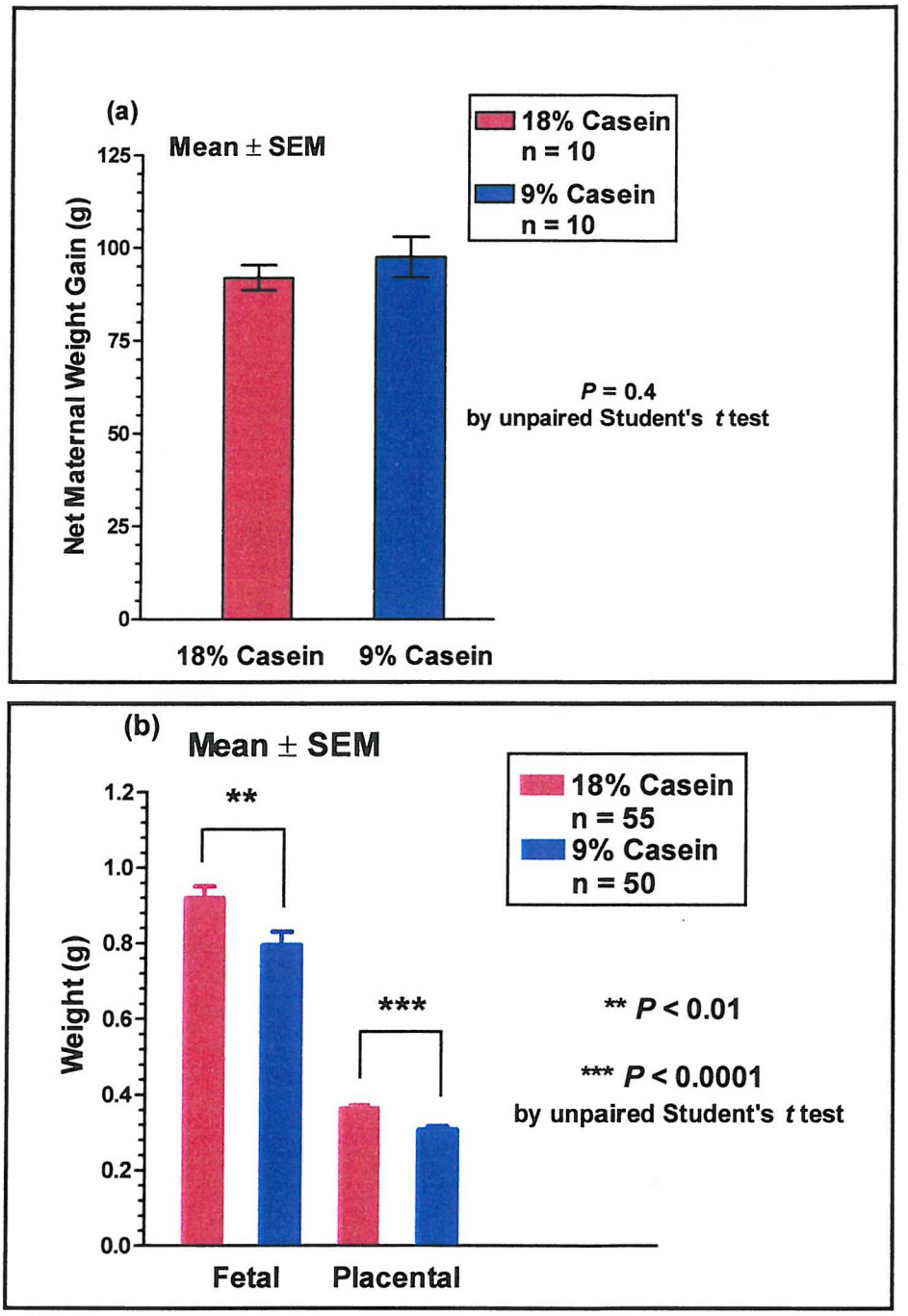


Figure 5.1. (a) and (b).: (a) Net maternal weight gain during days 1-18 of pregnancy and (b) fetal and placental weight at 18 days of gestation, in pregnant rats fed either 18% or 9% casein diets. Values are given as means ± SEM.

5.3.3. Serum and plasma analyses.

5.3.3.1. Serum 17β-oestradiol levels.

Table 5.2 depicts the data for the mean serum 17β-oestradiol concentration in 18% and 9% casein-fed pregnant rats on day 18 of gestation. The mean maternal serum 17β-oestradiol concentration was not significantly different ($P = 0.17$) between the two groups at the time of vascular function measurement.

5.3.3.2. Plasma cholesterol and triglyceride (TG) concentrations.

Table 5.2 also depicts the data for the mean maternal plasma cholesterol and TG concentrations. There were no significant differences in the mean plasma cholesterol concentrations in pregnant 18% and 9% casein-fed groups ($P = 0.6$), (Table 5.2). Plasma triglyceride concentrations were 10% higher in 18% casein-fed dams than in 9% casein-fed ($P = 0.07$, by Mann-Whitney test), (Table 5.2).

TABLE 5.2

Concentrations of serum 17β-oestradiol and of plasma triacylglycerols and cholesterol in 18% and 9% casein-fed maternal rats on day 18 of gestation.

	Maternal diet		
Parameter	**18% CASEIN**	**9% CASEIN**	***P* value**
17β-oestradiol (pmol/L)	75.3 ± 3.5 (12)	67.8 ± 6.4 (14)	0.17
Cholesterol (mmol/L)	1.76 ± 0.06 (16)	1.81 ± 0.10 (13)	0.6
Triglycerides (mmol/L)	4.06 ± 0.23 (16)	3.67 ± 0.54 (13)	0.07

Data are expressed as mean ± SEM., with numbers of animals stated in parentheses.

5.3.4. Effects of feeding an 18% and a 9% casein diet on internal arterial diameters in the small mesenteric arteries from pregnant Wistar rats.

The mean internal diameter of the mesenteric arteries from pregnant rats fed either an 18% or a 9% casein diet was similar, and not significantly different from the diameters measured in the nonpregnant groups (18% casein pregnant: 341.5 ± 12.7 μm, *n* 18 arteries *vs* 9% casein pregnant: 325.2 ± 15.5 μm, *n* 19 arteries; $P = 0.4$).

5.3.5. Reactivity of mesenteric resistance arteries from 18% and 9% casein-fed pregnant Wistar rats to PE and KCl.

The *in vitro* contractile responses of vessels obtained from pregnant 18% and 9% casein-fed rats to increasing concentrations of phenylephrine hydrochloride (PE) (10^{-7} to 10^{-5} mol/l), displayed the characteristic sigmoidal relationship, with no statistically significant differences between the two groups either by ANOVA ($P = 0.3$) or at any single concentration (Fig. 5.2). The effect of the two diets on the vasoconstrictor effect of PE is presented in Table 5.3. Maximal contractile responses to PE, expressed as a percentage of the maximal contractile response to KPSS (125 mM KCl), were similar in the vessels from the pregnant 18% *vs* 9% casein-fed animals ($P = 0.5$) (Table 5.3) (Fig.5.2). Similarly, the maximum amplitude of contraction induced by PE was not significantly different between arteries from pregnant 18% casein-fed rats when compared with those from 9% casein-fed, when the comparison was made in active wall tension values ($mNmm^{-1}$) ($P = 0.4$) (Table 5.3). Figure 5.2 shows that the dose-response curves of arteries from 18% and 9% casein-fed pregnant rats nearly overlap, with no difference in the sensitivity to PE (pEC_{50}), when expressed either as absolute tension or as a percentage of K^+-induced tension ($P = 0.3$) (Table 5.3).

Arterial segments were maximally constricted with KCl buffer. The maximum force induced by high K^+ (125 mM) was not significantly different whether the arteries

were obtained from 18% casein-fed or from 9% casein-fed pregnant rats ($P = 0.9$) (Table 5.3).

The response of arteries obtained from pregnant 18% and 9% casein-fed rats to cumulative concentrations of KCl is shown in Fig. 5.3. There were no significant differences observed in the pEC_{50} values ($P = 0.5$) for potassium depolarization constrictions. Maximal responses to KCl were similar in the vessels from the pregnant 18% *vs* 9% casein-fed animals ($P = 0.2$) (Table 5.3) (Fig. 5.3).

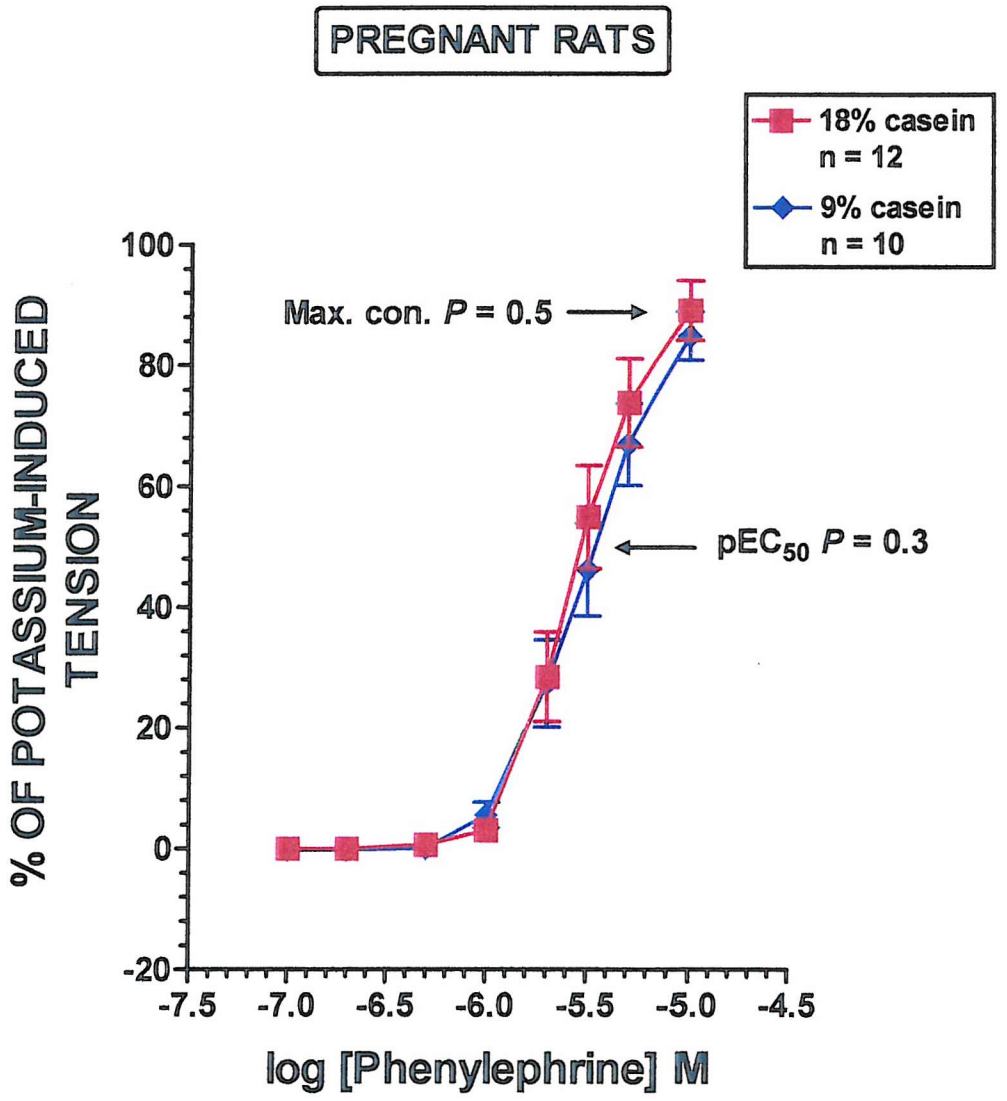
TABLE 5.3

The effects of feeding an 18% and a 9% casein diet on responses induced by PE, KCl and high K^+ (KPSS, 125 mM) in arteries from pregnant Wistar rats.

Phenylephrine (PE) (10^{-7} to 10^{-5} mol/l)				
		Max. Contraction		
Group	***n***	**mN/mm**	**% KPSS**	**pEC_{50} (M)**
18% Casein	12	4.25 ± 0.19	89.15 ± 4.93	5.56 ± 0.03
9% Casein	10	4.03 ± 0.26	84.88 ± 4.05	5.49 ± 0.04
Potassium chloride (KCl) (5mmol/l to 125 mmol/l)				
		Max. Contraction		
Group	***n***	**mN/mm**	**% KPSS**	**pEC_{50} (M)**
18% Casein	11	1.54 ± 0.11	35.21 ± 1.98	1.30 ± 0.02
9% Casein	9	1.86 ± 0.15	39.54 ± 2.07	1.31 ± 0.02
KPSS (125 mM)				
		Maximum tension		
Group		***n***	**mN/mm**	
18% Casein		12	4.45 ± 0.18	
9% Casein		9	4.49 ± 0.27	

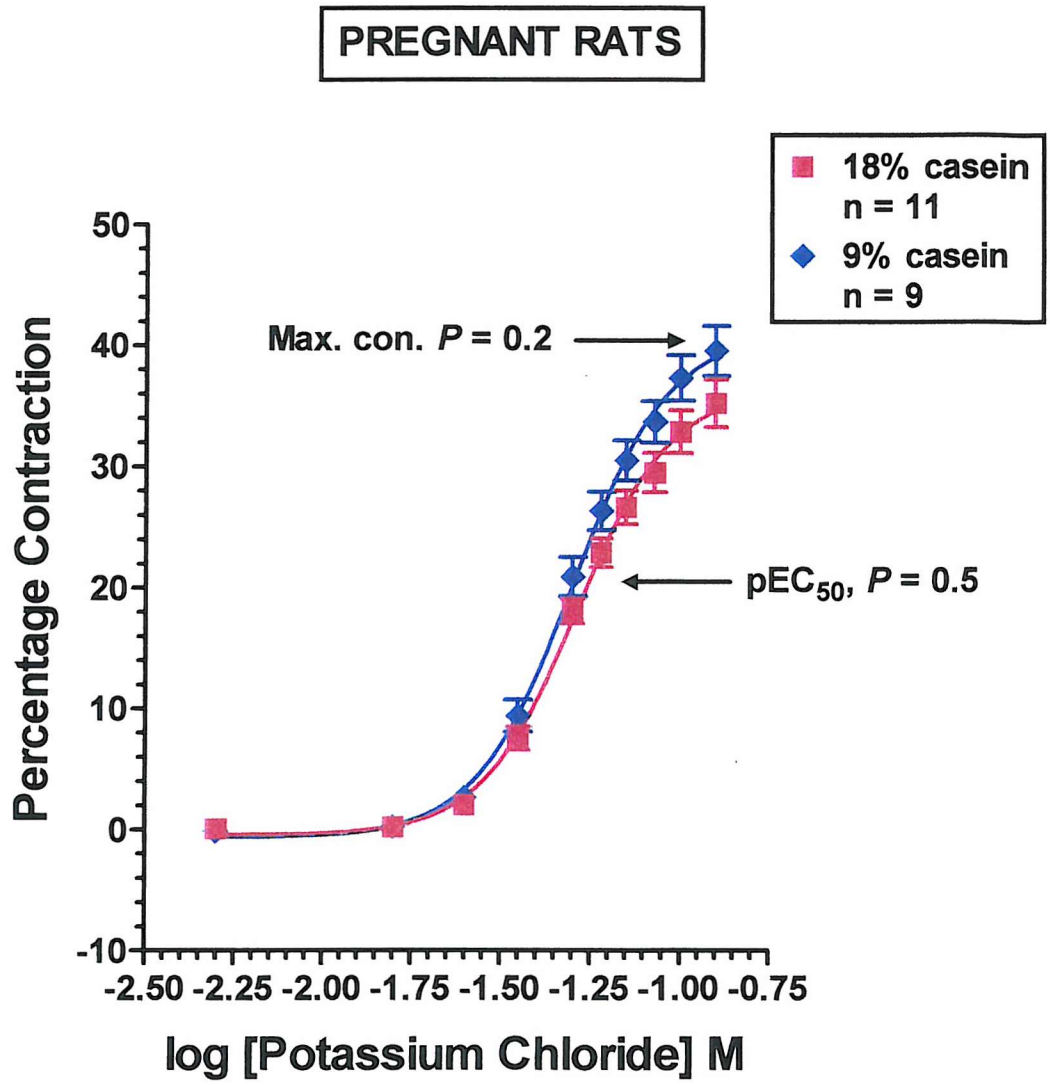
Table 5.3 summarizes data related to the dose-response curves presented in Figs. 5.2 and 5.3. Data are expressed as mean ± SEM. *n* = number of animals examined. Maximal response is expressed either as absolute value (mN/mm) or as a percentage of the contractile response to KPSS (125 mM KCl)-induced tension. pEC_{50}, -log EC_{50} where EC_{50} is the concentration which produces 50% maximum response.

Figure 5.2. : Arterial responsiveness to cumulative concentrations of phenylephrine (PE) for small mesenteric arteries from pregnant rats fed 18% (■; n = 12) or 9% casein (◆; n = 10) until 18-19 days of gestation.



Tension is expressed as a percentage of the maximal constriction induced by KPSS (K^+, 125 mmol/L). Data is means ± s.e.mean of the number of animals indicated by N. There was no statistically significant difference between the two groups for either sensitivity to PE ($P = 0.3$) or for maximum constrictor response ($P = 0.5$) (unpaired *t* tests). When absent, error bars lie within the symbols.

Figure 5.3.: Concentration-response curves to potassium chloride (KCl) in mesenteric arteries from pregnant rats fed an 18% (■; n = 11) or 9% casein diet (◆; n = 9).



Mesenteric arteries were contracted by KCl (5-125 mM). Vasoconstriction in response to KCl was expressed as a percentage of the maximal contraction obtained with 125 mM KPSS. Data represent means ± SEM.; *n* equals the number of rats. There was no statistically significant difference between the two groups for either sensitivity to KCl ($P = 0.5$) or for maximum constrictor response ($P = 0.2$) (unpaired Student's *t* test). When absent, error bars lie within the symbols.

5.3.6. Effect of the low protein diet on relaxation responses of isolated rat mesenteric arteries from pregnant Wistar rats to ACh and BK.

The application of incremental log concentrations of ACh from 1 nmol to 10 μmol/L to preparations precontracted with PE to 80% of maximal tension, induced dose-related endothelium-dependent relaxations of PE-induced tone in mesenteric resistance vessels from 18% casein fed pregnant rats achieving a maximum of 97.6 ± 1.1 % relaxation at an ACh concentration of 10 μmol/L (Table 5.4, Fig. 5.4). Arteries from pregnant rats on the 9% casein diet that were similarly exposed to ACh displayed an impairment of the relaxation responses, with a significant ($P = 0.0002$, Two-way ANOVA) rightward shift of the dose-response curve, as compared with vessels from pregnant rats on the 18% casein diet. As observed in the virgin rats, the sensitivity to ACh was significantly decreased by 4% in arteries from the pregnant rats on the 9% casein compared with 18% casein-fed ($P = 0.02$) but maximal relaxation to ACh was borderline ($P = 0.09$) (unpaired Student's *t* test) (Table 5.4 Fig. 5.4). Preconstrictor tension to PE prior to relaxation responses to ACh, was not different between arteries from pregnant 18% and 9% casein fed rats (tension = 3.82 ± 0.27 $mNmm^{-1}$, *n* 10, *versus* 3.49 ± 0.27 $mNmm^{-1}$, *n* 10, respectively, $P = 0.4$, by unpaired *t* test).

Similar results were obtained when bradykinin was used to induce endothelial-dependent relaxation. In the precontracted arteries from pregnant rats on the 18% or 9% casein diet BK (1 nmol to 10 μmol/l) elicited concentration-dependent arterial relaxation (Fig. 5.5). The pEC_{50} values for the relaxation to BK were not significantly different between the two dietary treatment groups ($P = 0.5$, by Mann-Whitney test, Table 5.4). At $3x10^{-7}$ mol/L bradykinin, relaxation was significantly attenuated by 42% ($P = 0.04$, Table 5.4) in arteries from pregnant rats on the 9% casein compared with pregnant rats on the 18% casein. At the highest bradykinin concentration of 10 μmol/L, the 9% casein group relaxed 28% (± 5%) compared to 44% (± 6%) in the 18% casein group ($P = 0.1$, Mann-Whitney test, Fig.5.5).

Before evaluation of BK responses, preconstriction to PE was no different between mesenteric resistance arteries from 18% and 9% casein-fed pregnant rats (tension = 4.11 ± 0.25 $mNmm^{-1}$, *n* 6, *versus* 4.44 ± 0.48 $mNmm^{-1}$, *n* 4, respectively, $P = 0.6$, by Mann-Whitney test).

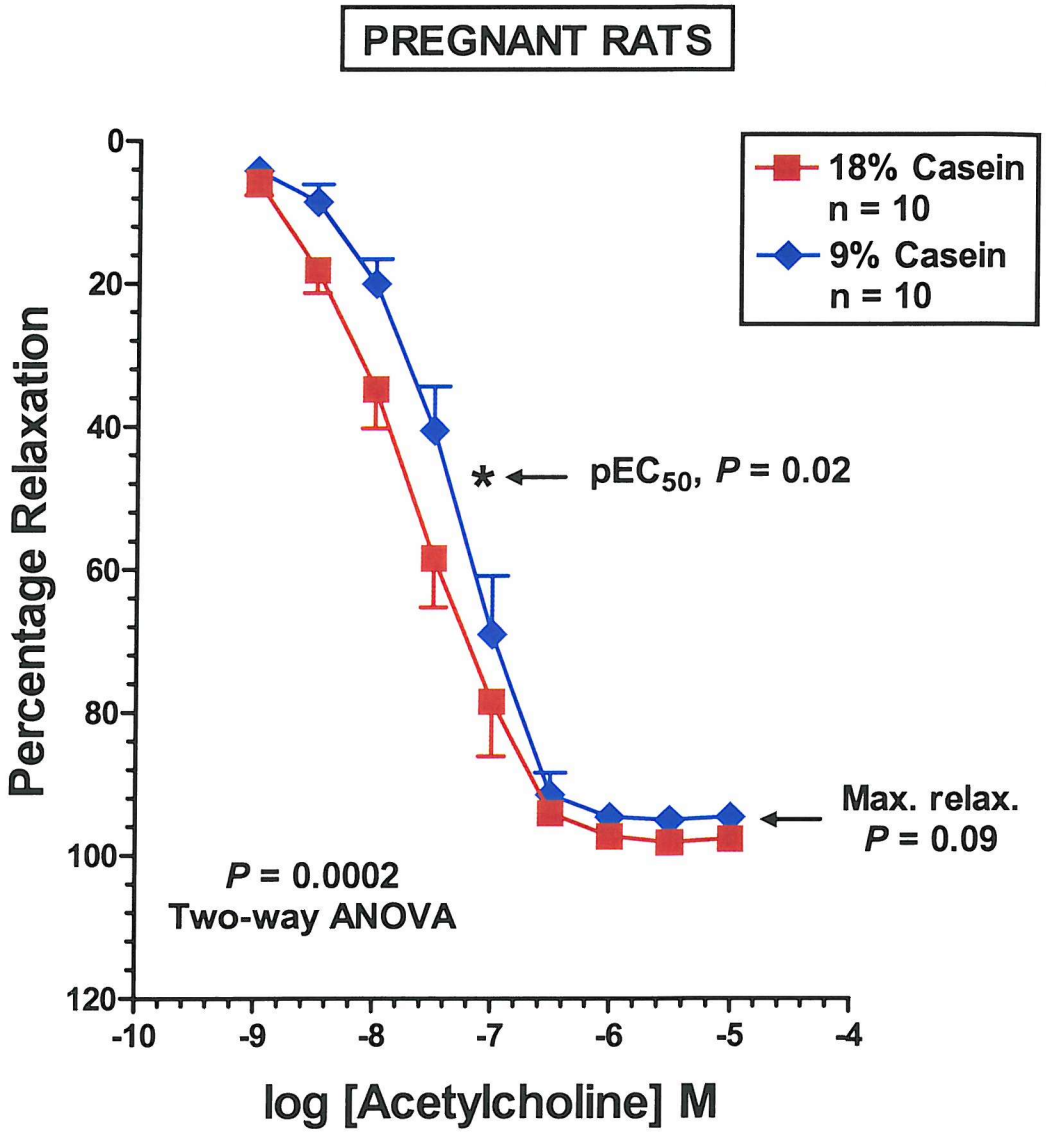
TABLE 5.4

The effect of dietary protein intake on vascular reactivity to ACh and BK of the isolated rat mesenteric arteries from pregnant Wistar rats.

PREGNANT RATS		**18% Casein**	**9% Casein**	**P value**
Agonist		**Mean ± SE**	**Mean ± SE**	
ACh	**pEC_{50} (M)**	7.64 ± 0.08 (10)	7.37 ± 0.07 (10)	0.02
	Max. relax. (%)	97.62 ± 1.14 (10)	94.59 ± 1.22 (10)	0.09
BK	**pEC_{50} (M)**	7.87 ± 0.28 (6)	8.05 ± 0.20 (4)	0.5
	Max. relax. (%)	55.84 ± 9.13 (6)	32.23 ± 4.31 (4)	0.04

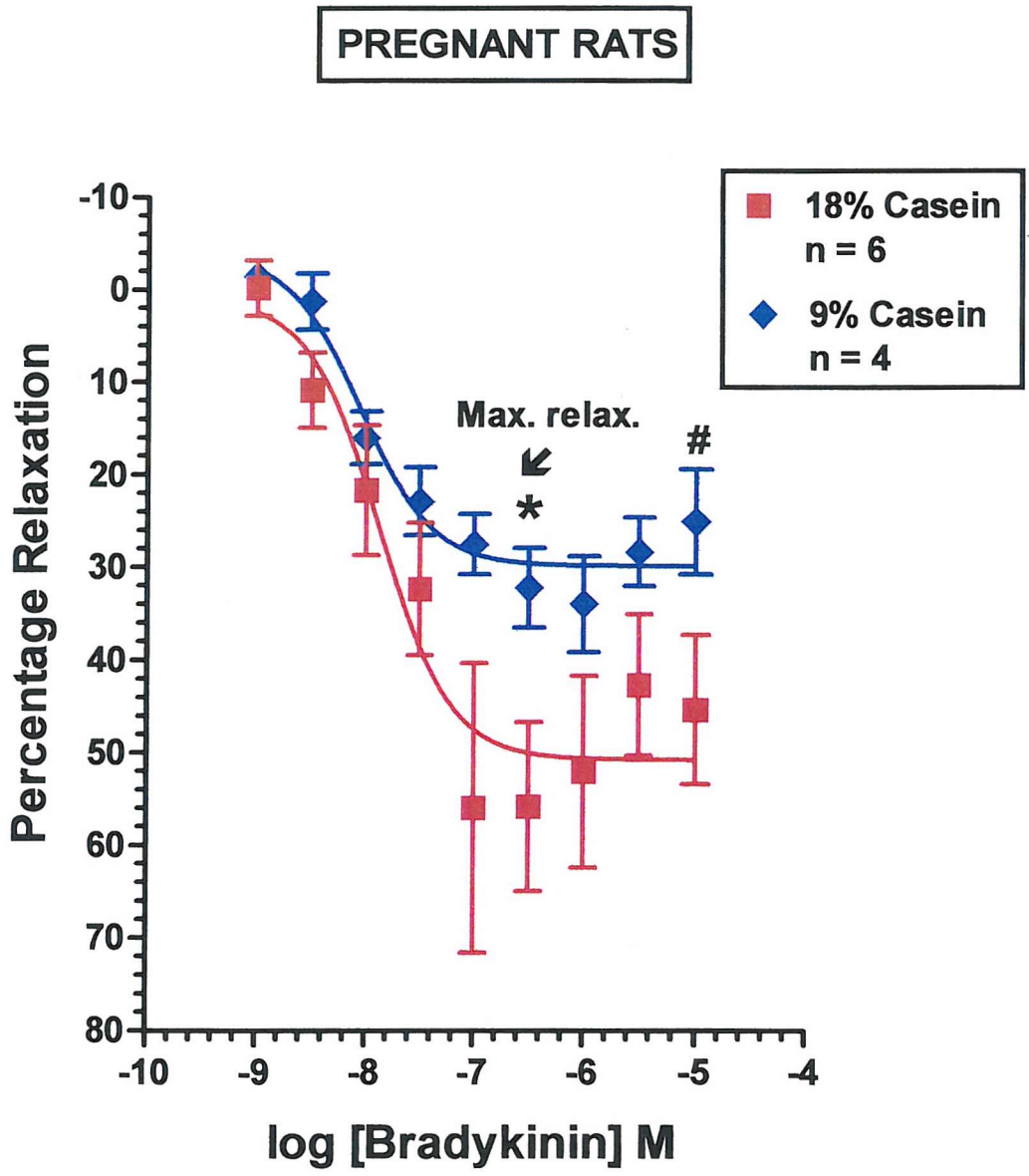
Table 5.4 summarizes data related to the dose-response curves presented in Figs. 5.4 and 5.5. Data are presented as the mean value ± S.E.M. Numbers in parentheses indicate the number of animals studied. ACh, 1 nmol to 10 μmol/l acetylcholine; BK, 1 nmol to 10 μmol/l bradykinin; Data are expressed as pEC_{50} values, which are defined as the negative logarithm to base 10 of the EC_{50} values. Max. relax. = maximum relaxation expressed as the percentage of phenylephrine-induced precontraction.

Figure 5.4.: **Concentration-response curves to acetylcholine (ACh) in mesenteric arteries precontracted with submaximal PE (5 μM) from pregnant rats fed an 18% (■; n = 10) or 9% casein diet (◆; n = 10).**



Values represent the mean ± S.E.M.; *n* represents the number of animals studied. Two-way ANOVA with repeated measures showed that low protein (9%) diet decreased the effects of acetylcholine in the isolated mesenteric artery of the pregnant rat by statistically shifting the concentration-relaxation curve to the right ($P = 0.0002$) which resulted in decreased pEC_{50} values ($P = 0.02$) in relation to the 18% casein control diet.

Figure 5.5.: Concentration (log *M*)-relaxation curves for bradykinin (BK) in mesenteric resistance arteries precontracted with PE from pregnant rats fed 18% (■; n = 6) or 9% casein (◆; n = 4) until 18-19 days of gestation.



Responses (means ± S.E.M.) were measured as a percentage of PE-induced precontraction. *n* equals the number of rats. pEC_{50} was similar in arteries from 18% and 9% casein fed pregnant rats ($P = 0.5$). Maximum relaxation to BK was significantly reduced in arteries from 9% casein fed pregnant rats in comparison to the 18% casein group ($P = 0.04$). $^{\#}$ $P = 0.1$ response *vs.* 18% casein.

5.3.7. Effect of the low protein diet on SPN and 8-br-cGMP-evoked vasodilatations of the isolated rat mesenteric arteries from pregnant Wistar rats.

Arterial segments were tested for endothelial-independent relaxation using spermine NONOate, an exogenous NO donor and a standard guanylate cyclase activator. The addition of SPN (cumulative doses: 1 nmol to 10 μmol/l) to the vessel bath produced concentration-dependent vascular relaxation of submaximal PE (5 μmol/l)-induced contraction (Fig. 5.6). The PE contraction was not significantly different between pregnant 18% and 9% casein-fed rats (tension = 3.91 ± 0.31 $mNmm^{-1}$, *n* 9, *versus* 4.15 ± 0.18 $mNmm^{-1}$, *n* 7, respectively, $P = 0.6$, by unpaired Student's *t* test). The concentration-response curve for the relaxation was almost identical in both diet groups; the degree of maximum relaxation attained with SPN ranged between 92 ± 4% (18% casein) and 95 ± 2% (9% casein) of the precontraction to PE (5 μmol/l); in contrast to virgin animals, arteries from pregnant, 9% casein-fed animals had similar maximum responses to SPN compared with pregnant, 18% casein-fed ($P = 0.8$, Table 5.5, Fig. 5.6). Sensitivity to SPN was not different between arteries from pregnant 18% and 9% casein-fed rats ($P = 0.4$, by unpaired Student's *t* test) (Table 5.5).

8-br-cGMP studied over the range 3 μmol to 1 mmol/l, elicited a concentration-dependent relaxation in PE precontracted mesenteric arteries taken from pregnant rats on the 18% or 9% casein (Fig. 5.7). PE-induced contraction before measurement of 8-br-cGMP responses, was found to be similar in arteries from both 18% and 9% casein groups (tension = 3.71 ± 0.29 $mNmm^{-1}$, *n* 4, *versus*, 3.83 ± 0.47 $mNmm^{-1}$, *n* 6, respectively, $P = 0.6$). The vasorelaxant effects of 8-br-cGMP were found to be similar in mesenteric arteries from both 18% and 9% casein-fed pregnant rats (Table 5.5, Fig. 5.7). There were no significant differences in the sensitivity to 8-br-cGMP ($P = 0.5$) and maximum 8-br-cGMP-induced relaxation ($P = 0.5$) between pregnant 18% and 9% casein-fed rats (Table 5.5, Fig. 5.7).

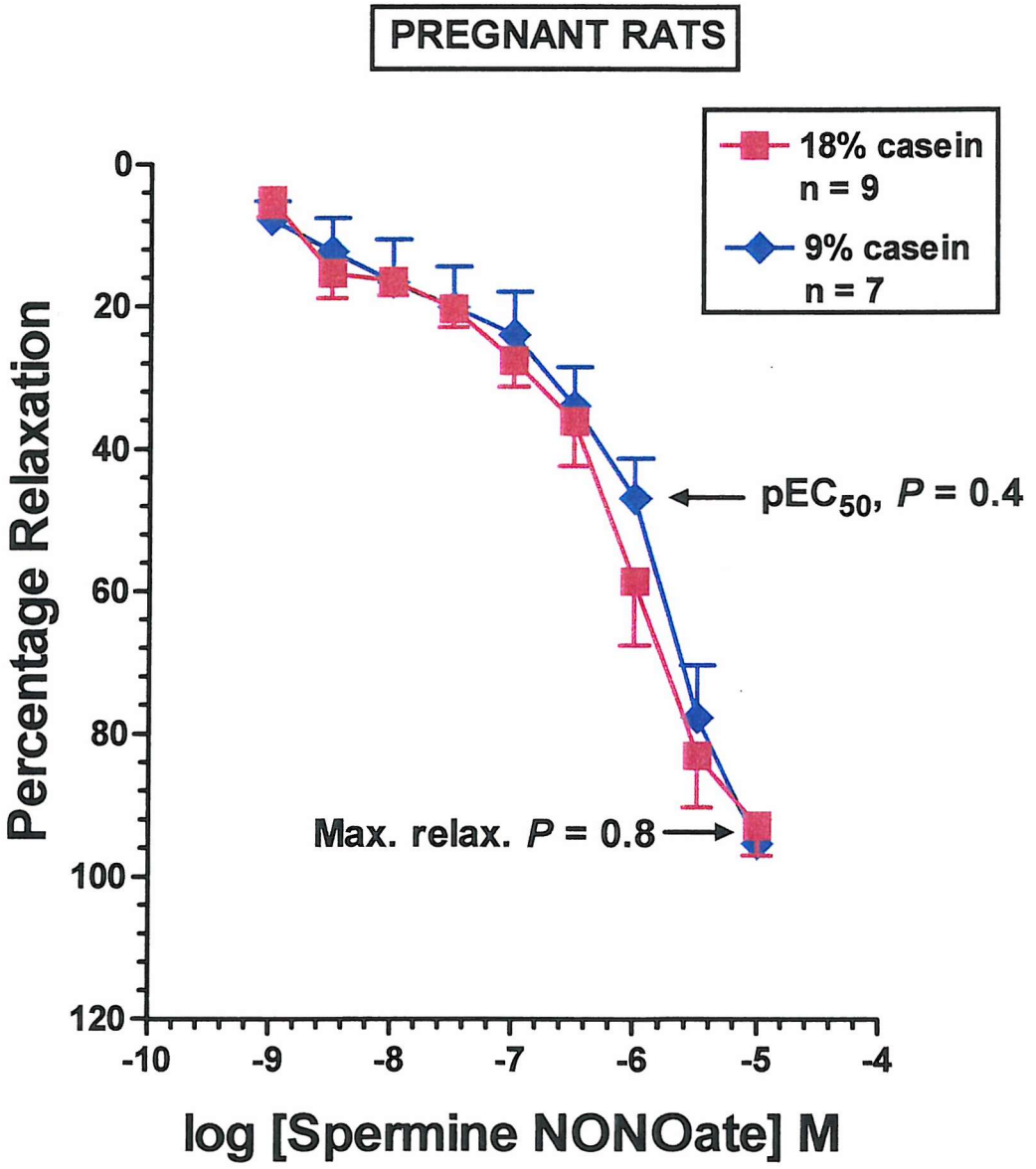
TABLE 5.5

The effect of dietary protein intake on vascular reactivity to 8-br-cGMP and SPN of the isolated rat mesenteric arteries from pregnant Wistar rats.

PREGNANT RATS		**18% Casein**	**9% Casein**	**P value**
Agonist		**Mean ± SE**	**Mean ± SE**	
SPN	**pEC_{50} (M)**	6.08 ± 0.11 (9)	5.81 ± 0.12 (7)	0.4
	Max. relax. (%)	92.89 ± 4.16 (9)	95.37 ± 2.26 (7)	0.8
8-br-cGMP	**pEC_{50} (M)**	3.92 ± 0.36 (4)	3.42 ± 0.16 (6)	0.5
	Max. relax. (%)	65.63 ± 10.74 (4)	78.05 ± 3.68 (6)	0.5

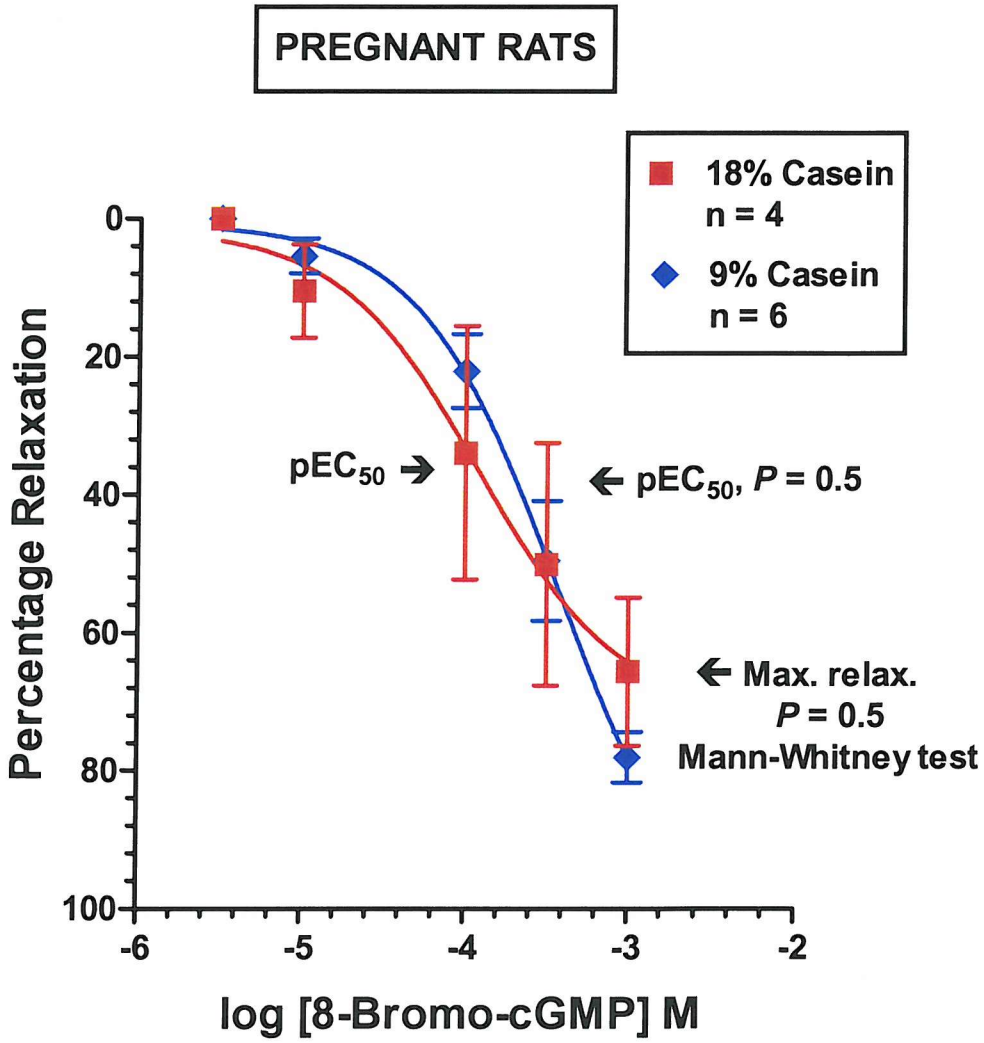
Table 5.5 summarizes data related to the dose-response curves presented in Figs. 5.6 and 5.7. SPN, 1 nmol to 10 μmol/l spermine NONOate; 8-br-cGMP, 3 μmol to 1 mmol/l 8-bromoguanosine-3':5'-cyclic monophosphate; Data are expressed as pEC_{50} values, which are defined as the negative logarithm to base 10 of the EC_{50} values. Max. relax. = maximum relaxation expressed as the percentage of phenylephrine-induced precontraction.

Figure 5.6.: Concentration-response curves to spermine NONOate (SPN) in PE-preconstricted mesenteric arteries from pregnant rats fed an 18% (■; n = 9) or 9% casein diet (◆; n = 7).



Data are expressed as a percentage of phenylephrine-induced precontraction and shown as mean ± s.e.mean, where *n* equals the number of animals studied. There were no statistically significant differences between pregnant low protein-fed animals and pregnant controls in terms of sensitivity ($P = 0.4$) (unpaired *t* test) or maximum response ($P = 0.8$) to SPN. When absent, error bars lie within the symbols.

Figure 5.7.: Effect of 8-bromoguanosine-3':5'-cyclic monophosphate-8-Br-cGMP on PE-preconstricted mesenteric arteries of pregnant rats fed an 18% (■; n = 4) or 9% casein diet (◆; n = 6).

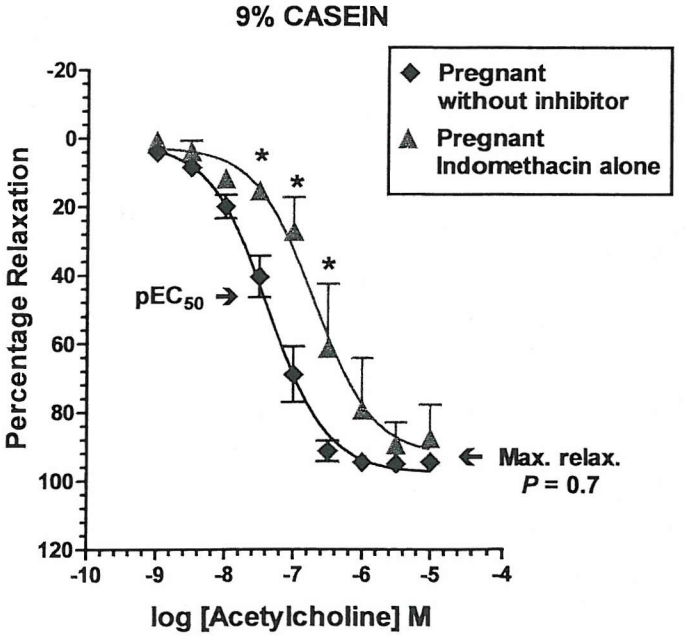


Phenylephrine (5 μmol/L) contraction was elicited, and then increasing concentrations of 8-bromo-cGMP were added and relaxation was measured as a percentage of the initial PE contraction. Data points represent the mean ± S.E.M. *n*, no of animals. There were no statistically significant differences between pregnant low protein-fed animals and pregnant controls in terms of sensitivity (pEC_{50} values) ($P = 0.5$) or maximum response ($P = 0.5$) to 8-bromo-cGMP (Mann-Whitney test). When absent, error bars lie within the symbols.

5.3.8. Effect of 10 μM indomethacin on relaxation responses to cumulative concentrations of ACh of isolated arteries from pregnant rats fed 9% casein.

The effect of 10 μM indomethacin on ACh-stimulated relaxation was determined in mesenteric arteries from 9% casein-fed animals only (Fig. 5.8). Indomethacin had no significant effect on the PE-induced tone compared with the untreated vessels (with indomethacin, tension = 3.40 ± 0.24 $mNmm^{-1}$, *n* 3, *vs* 3.49 ± 0.27 $mNmm^{-1}$, *n* 10, without indomethacin, $P = 0.5$, by Wilcoxon matched pairs test). Indomethacin decreased the effects of acetylcholine (ACh) in the isolated arteries from 9% casein-fed pregnant rats evidenced by a decreased sensitivity (pEC_{50}) to ACh (pEC_{50}: with indomethacin: 6.70 ± 0.16, *n* 3, *versus* 7.37 ± 0.07, *n* 10, without indomethacin) (Fig. 5.8). Indomethacin had no significant effect on the maximal relaxation in response to ACh in arteries from pregnant rats on the 9% casein (max. relax.: with indomethacin: 87.5 ± 9.5%, *n* 3, *versus* 94.6 ± 1.2%, *n* 10, in the absence of indomethacin; $P = 0.7$, by Mann-Whitney test), (Table 5.6) (Fig. 5.8).

Figure 5.8.: Effect of 10 μM indomethacin on ACh-induced relaxation of isolated arteries from pregnant rats fed 9% casein.



Relaxation of arteries from pregnant rats fed 9% casein before (◆) and after 10 μM indomethacin (▲). *$P < 0.05$ vs. without inhibitor for respective concentrations.

5.3.9. Effect of indomethacin and L-NAME on tension induced by phenylephrine (PE) in small arteries isolated from pregnant rats.

Incubation of arteries from 18% casein-fed pregnant animals in PSS containing indomethacin and L-NAME led to a reduction of the phenylephrine precontraction compared with the previous response obtained in the absence of indomethacin and L-NAME (18% casein with indomethacin and L-NAME, tension = 3.26 ± 0.56 $mNmm^{-1}$, *n* 5, *versus* 3.82 ± 0.26 $mNmm^{-1}$ *n* 10, without the inhibitors ($P = 0.3$) (Fig. 5.9).

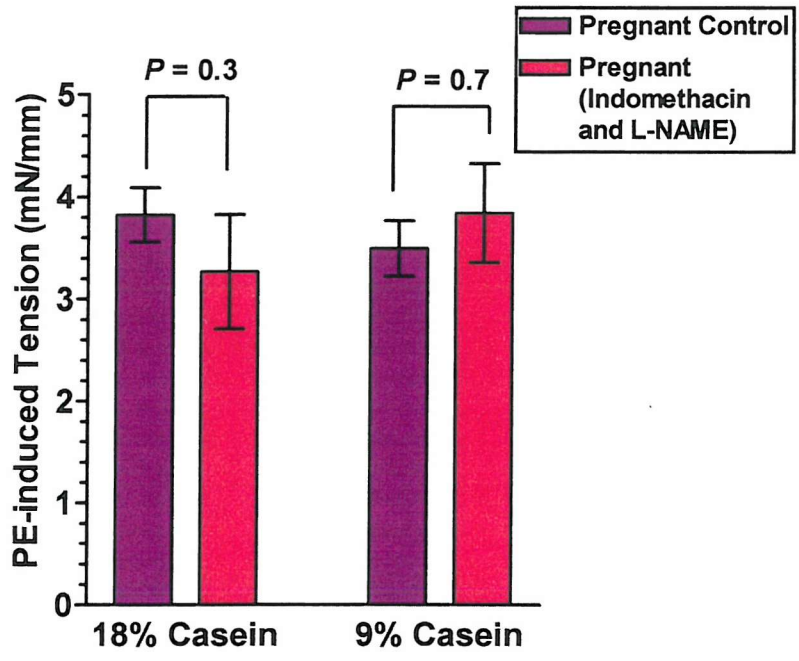


Figure 5.9.: Phenylephrine-evoked precontraction; effect of indomethacin and L-NAME. The effect of INDO (10 μM; 20 min incubation) and L-NAME (100 μM; 20 min incubation) on tension induced by PE in small arteries from 18% (*n* = 5) and 9% casein-fed (*n* = 5) pregnant rats, compared to arteries from 18% (*n* = 10) and 9% casein (*n* = 10) rats in PSS alone (control). Values represent mean ± S.E.M..

Incubation of arteries from 9% casein-fed pregnant animals in PSS containing indomethacin and L-NAME caused a slight augmentation of the precontraction to PE by 10% compared to control (9% casein with indomethacin and L-NAME, tension = 3.84 ± 0.49 $mNmm^{-1}$, *n* 5, *versus* 3.49 ± 0.27 $mNmm^{-1}$ *n* 10, without the inhibitors) ($P = 0.7$) (Fig. 5.9). In the presence of the inhibitors, preconstriction to PE was similar in arteries from 18% and 9% casein groups ($P = 0.4$).

5.3.10. Effect of nitric oxide synthase and cyclooxygenase inhibition on ACh-induced vasorelaxation in arteries from pregnant rats fed 18% or 9% casein.

Concentration-response curves to acetylcholine in the absence and in the combined presence of 10 μM indomethacin (an inhibitor of cyclooxygenase) and 100 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME, an inhibitor of NO synthase) are demonstrated in Fig. 5.10 (a) and (b). ACh-induced relaxation in normal PSS (5 mM KCl) was only partially inhibited in both groups by a combination of indomethacin and L-NAME. In the presence of INDO (10 μM) and L-NAME (100 μM) and contracted with phenylephrine, the concentration-response curves for ACh-induced relaxations were almost abolished, although relaxations were observed at the highest concentrations (1-10 μM) of ACh [Fig. 5.10 (a) and (b)]. In both groups, indomethacin and L-NAME decreased the effects of ACh in the isolated arteries of the pregnant rat by significantly shifting the concentration-response curves for ACh-induced relaxations to the right (18% casein: $P < 0.0001$; 9% casein: $P < 0.0001$ by Two-way ANOVA) [Fig. 5.10(a) and (b) respectively], which resulted in a significant reduction in the sensitivity to ACh by 21% in the 18% casein group ($P < 0.001$). In arteries from rats on the 9% casein diet the sensitivity to ACh was reduced by 12% ($P < 0.01$). Maximum residual relaxation to ACh (10 μM) in the presence of INDO (10 μM) and L-NAME (100 μM) was also significantly attenuated by 17% in arteries isolated from rats on the 18% casein ($P = 0.0027$, paired *t* test) [Fig. 5.10(a)] and by 48% in arteries from rats on the 9% casein ($P < 0.001$, paired *t* test) [Fig. 5.10 (b)] compared with the response in the absence of indomethacin and L-NAME.

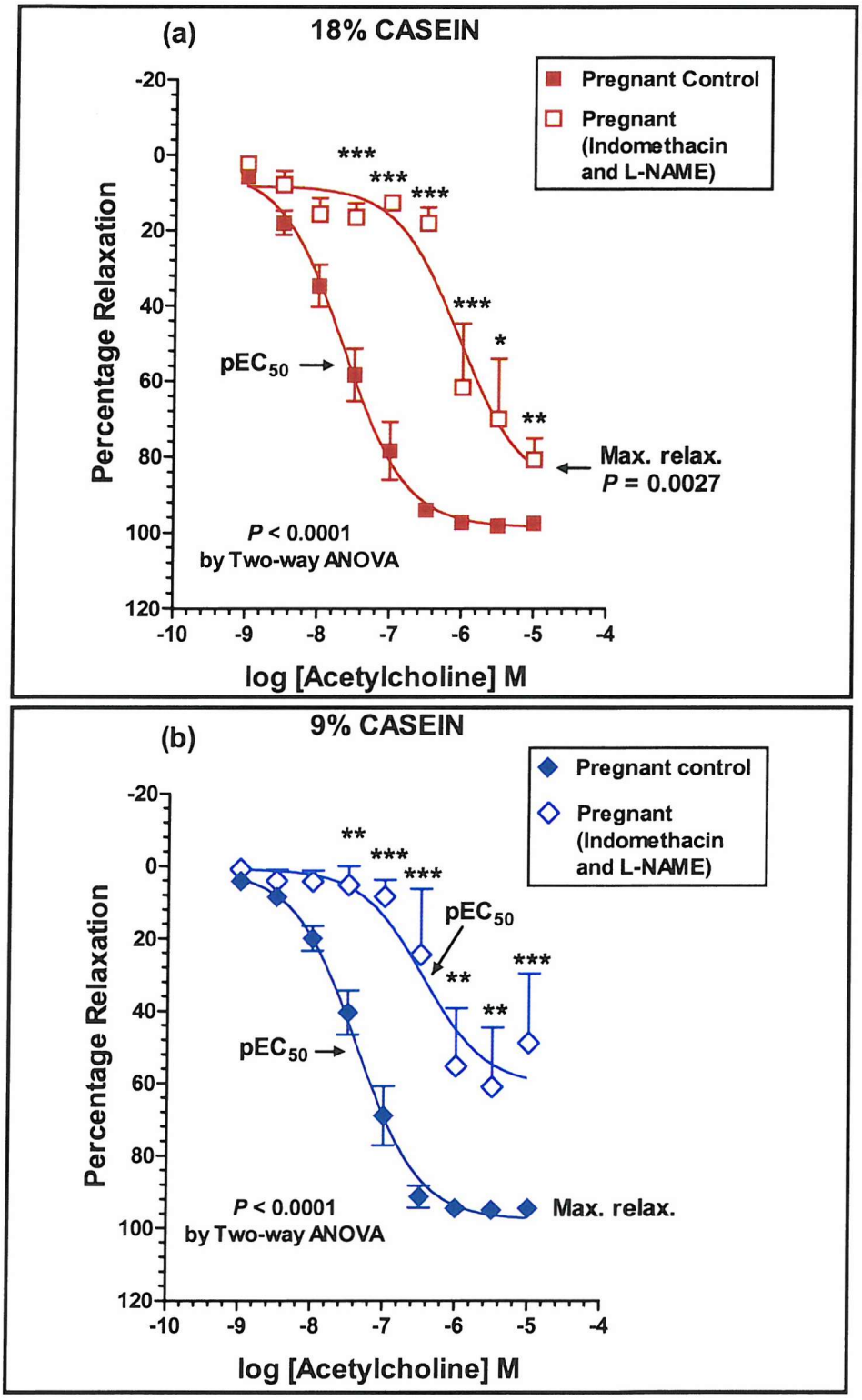


Figure 5.10 (a) and (b).: The response to ACh in mesenteric small arteries from (a) 18% casein fed pregnant rats in the absence (■; *n* 10) and presence (□; *n* 5) of both 10 μM INDO and 100 μM L-NAME and (b) 9% casein fed pregnant rats in the absence (◆; *n* 10) and presence (◇; *n* 5) of both 10 μM INDO and 100 μM L-NAME. ** $P < 0.01$ response *vs* control. *** $P < 0.001$ response *vs* control.

In the presence of both nitric oxide and cyclooxygenase blockade the values obtained for the sensitivity of the ACh response (pEC_{50}) and its maximum value in arteries from rats on the 18% casein diet were not significantly different from those obtained in arteries from rats on the 9% casein diet (pEC_{50}, $P = 0.5$ and max. relax., $P = 0.3$, by Mann-Whitney test) (Table 5.6 and Fig. 5.11).

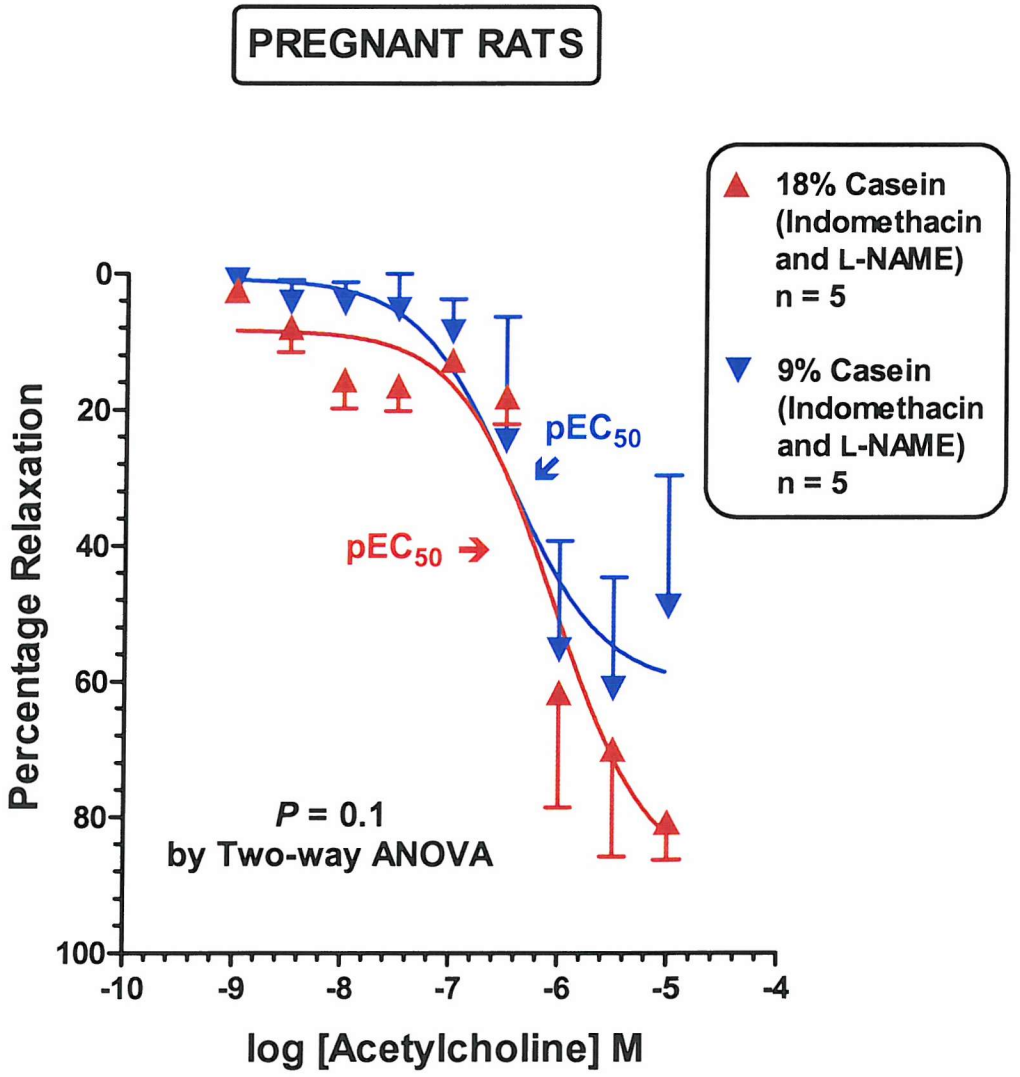
TABLE 5.6

The effects of 10 μM indomethacin (INDO) and 100 μM L-NAME on the measured relaxations induced by ACh (1 nmol to 10 μmol/l) in the isolated mesenteric arteries from pregnant Wistar rats fed 18% or 9% casein.

PREGNANT RATS		**18% Casein**	**9% Casein**	**P value**
Agonist		**Mean ± SE**	**Mean ± SE**	
ACh + INDO	**pEC_{50} (M)**	Not measured	6.70 ± 0.16 (3)	Not available
	Max. relax. (%)	Not measured	87.45 ± 9.5 (3)	Not available
ACh + INDO + L-NAME	**pEC_{50} (M)**	6.05 ± 0.20 (5)	6.46 ± 0.34 (5)	0.5
	Max. relax. (%)	80.78 ± 5.57 (5)	49.02 ± 19.2 (5)	0.3

Table 5.6 summarizes data related to the dose-response curves presented in Figs. 5.10 (a), (b) and 5.11. Data are expressed as pEC_{50} values, which are defined as the negative logarithm to base 10 of the EC_{50} values. Max. relax. = maximum relaxation expressed as the percentage of phenylephrine-induced precontraction. The significance of differences between the mean values was analysed non-parametrically using the Mann-Whitney test.

Figure 5.11.: Log dose-response curves for the vasorelaxation of the PE-induced tone in the 18% (▲; n = 5) and 9% (▼; n = 5) casein-fed pregnant rat isolated mesenteric arteries to ACh (1 nmol to 10 µmol/l) in the combined presence of 10 µM indomethacin (INDO) and 100 µM L-NAME.



Mesenteric arteries incubated in normal PSS solution were pretreated with 10 µM of the cyclooxygenase inhibitor INDO and 100 µM of the nitric oxide synthase inhibitor L-NAME in the bath medium for 20 min. A submaximal PE contraction was elicited, increasing concentrations of ACh were added, and the percent relaxation of PE-induced precontraction was measured. Data points are means ± S.E.M.; *n* represents the number of animals studied in each group.

Results: Part II. Comparison of nonpregnant *versus* pregnant (18% and 9% casein diet) groups.

5.3.11. Serum 17β-oestradiol and plasma cholesterol and triglyceride concentrations.

In the 18% casein-fed group, the mean maternal serum 17β-oestradiol concentration was significantly elevated in the pregnant rats by 30% ($P = 0.002$, by unpaired Student's t test) compared with virgin rats. In the 9% casein group, the mean serum 17β-oestradiol concentration was significantly raised in pregnant rats by 37% ($P = 0.005$, by unpaired Student's t test) compared with virgin rats.

In the 18% casein-fed group, the mean maternal plasma triglyceride concentrations were significantly elevated in the pregnant rats by 54% ($P < 0.0001$) compared with virgin rats. In the 9% casein group, plasma triglyceride levels were significantly raised in pregnant rats by 64% ($P = 0.001$, by unpaired Student's t test) compared with virgin rats. Pregnancy did not induce any significant changes or trends in cholesterol levels either in the 18% casein diet-fed ($P = 0.5$) or in 9% casein diet-fed animals ($P = 0.9$) (by unpaired Student's t test).

Vascular function

5.3.12. Effect of pregnancy on reactivity to α-adrenoreceptor agonist PE and KCl.

PE, an α_1-agonist, was applied at concentrations between $1x10^{-7}$ and $1x10^{-5}$ M. PE produced dose-dependent vasoconstriction in mesenteric arteries of pregnant as well as nonpregnant rats [Fig. 5.14(a) and (b)]. On the 18% casein diet, maximal absolute tension (mN/mm) elicited by PE (10^{-5} mol/L) was increased by 20% in pregnant animals ($P = 0.01$, unpaired Student's *t* test) (Table 5.7) [Fig. 5.13]. There were, however, no significant differences in contractility to PE in relation to percentage of KPSS (% KPSS, 125 mmol/L) with respect to maximal contraction ($P = 0.6$) and sensitivity (pEC_{50}: $P = 0.9$) (Table 5.7) [Fig. 5.14 (a)].

Pregnancy did not alter contractile sensitivity to PE in mesenteric arteries isolated from nonpregnant compared with pregnant rats that were fed 9% casein until 18-19 days of gestation or an equivalent period in the nonpregnant state (pEC_{50} values: $P = 0.4$) (Table 5.7) [Fig. 5.14 (b)]. Pregnancy did not affect maximal absolute tension (mN/mm) in response to PE in the 9% casein group ($P = 0.7$, by unpaired Student's *t* test) (Table 5.7) [Fig. 5.13]. Maximal contractile response expressed as percentage of KPSS was decreased by 13% but not significantly in the 9% casein pregnant group as compared with nonpregnant animals ($P = 0.06$, unpaired Student's *t* test) (Table 5.7) [Fig. 5.14 (b)].

At concentrations ranging from 5 to 125 mmol/L, KCl constricted arteries of nonpregnant as well as of pregnant rats in a concentration-dependent manner. There were no significant differences in contractility to KCl in relation to % of KPSS with respect to maximal contraction between 18% casein pregnant *vs* nonpregnant animals ($P = 0.16$) (Table 5.7). Maximal contraction to KCl was also not altered by pregnancy in the 9% casein group ($P = 0.9$) (Table 5.7). Sensitivity to KCl was significantly greater in the pregnant animals compared with virgins irrespective of the diet (pEC_{50} 18% casein pregnant *vs* nonpregnant; $P = 0.0004$) (pEC_{50} 9% casein pregnant *vs* nonpregnant; $P = 0.0001$) (Table 5.7).

TABLE 5.7

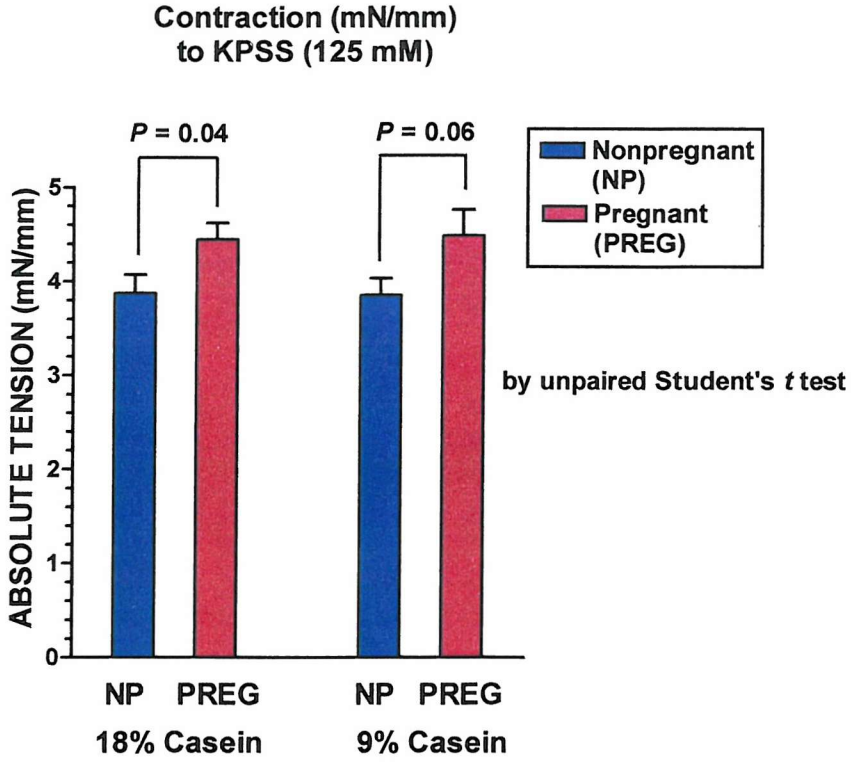
The effect of pregnancy on vascular reactivity of isolated mesenteric artery to α-adrenoreceptor agonist phenylephrine and KCl.

Phenylephrine (PE) (10^{-7} to 10^{-5} mol/l)				
		Max. Contraction		
Group	***n***	**mN/mm**	**% KPSS**	**pEC_{50} (M)**
18% Nonpregnant	11	3.57 ± 0.17	93.00 ± 4.65	5.57 ± 0.04
18% Pregnant	12	4.25 ± 0.19*	89.15 ± 4.93	5.56 ± 0.03
9% Nonpregnant	10	4.17 ± 0.26	96.78 ± 4.36	5.53 ± 0.04
9% Pregnant	10	4.03 ± 0.26	84.88 ± 4.05	5.49 ± 0.04
Potassium chloride (KCl) (5 mmol/l to 125 mmol/l)				
		Max. Contraction		
Group	***n***	**mN/mm**	**% KPSS**	**pEC_{50} (M)**
18% Nonpregnant	10	1.58 ± 0.06	39.22 ± 1.93	1.23 ± 0.01
18% Pregnant	11	1.54 ± 0.11	35.21 ± 1.98	1.30 ± 0.02
9% Nonpregnant	10	1.69 ± 0.09	39.83 ± 1.53	1.20 ± 0.03
9% Pregnant	9	1.86 ± 0.15	39.54 ± 2.07	1.31 ± 0.02

Values are expressed as means ± SEM.; Maximal contraction (mN/mm and % of contraction to KPSS, 125 mmol/L) and pEC_{50} values are given; *n* no. of animals. The asterisk indicates a statistically significant difference ($P = 0.01$) in the absolute maximal contractile response to PE between pregnant and nonpregnant animal values on the 18% casein diet.

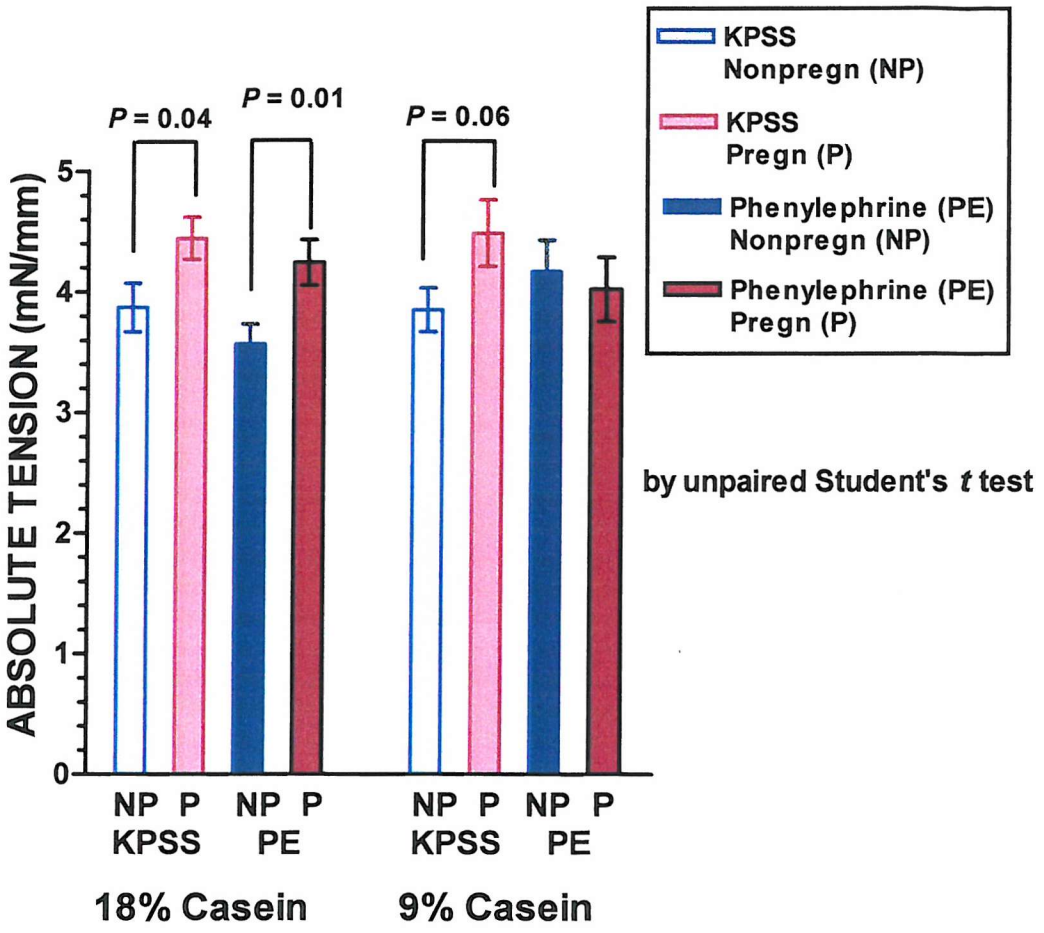
Pregnancy increased absolute tension (mN/mm) elicited in rat mesenteric arteries by depolarisation with 125 mM potassium (KPSS) in both diet groups; 18% casein group: KPSS [NP] *versus* KPSS [PREG]: 3.87 ± 0.20 $mNmm^{-1}$, *n* 12, *versus* 4.45 ± 0.17 $mNmm^{-1}$, *n* 12, respectively $P = 0.04$; 9% casein: KPSS [NP] *versus* KPSS [PREG]: 3.85 ± 0.18 $mNmm^{-1}$, *n* 9, *versus* 4.49 ± 0.28 $mNmm^{-1}$, *n* 13, respectively $P = 0.06$, (by unpaired Student's *t* test) (Figs. 5.12 and 5.13).

Figure 5.12.: A comparison of maximal contractile response to 125 mM KPSS between mesenteric resistance arteries isolated from nonpregnant (NP) and pregnant (PREG) rats that had been fed either an 18% or 9% casein diet during gestation or an equivalent period in the nonpregnant state.



Values are means ± SEM. Pregnancy increased absolute tension (mN/mm) of arteries to 125 mM KPSS irrespective of the diet.

Figure 5.13.: A comparison of maximal absolute tension (mN/mm) to KPSS (125 mM) and to phenylephrine (PE) (10^{-5} mol/L) between arteries isolated from nonpregnant (NP) and pregnant (P) rats that had been fed either an 18% or 9% casein diet during gestation or an equivalent period in the nonpregnant state.



Data are expressed as means ± SEM. In arteries from rats on the 18% casein diet pregnancy increased maximal absolute tension to KPSS ($P = 0.04$) and to receptor-operated agonist PE ($P = 0.01$). In arteries from rats on the 9% casein diet pregnancy increased the absolute tension to KPSS ($P = 0.06$) whereas tension in response to PE was not altered in pregnant animals
[9% casein: PE (NP) *versus* PE (P): $P = 0.7$]. (by unpaired *t* test).

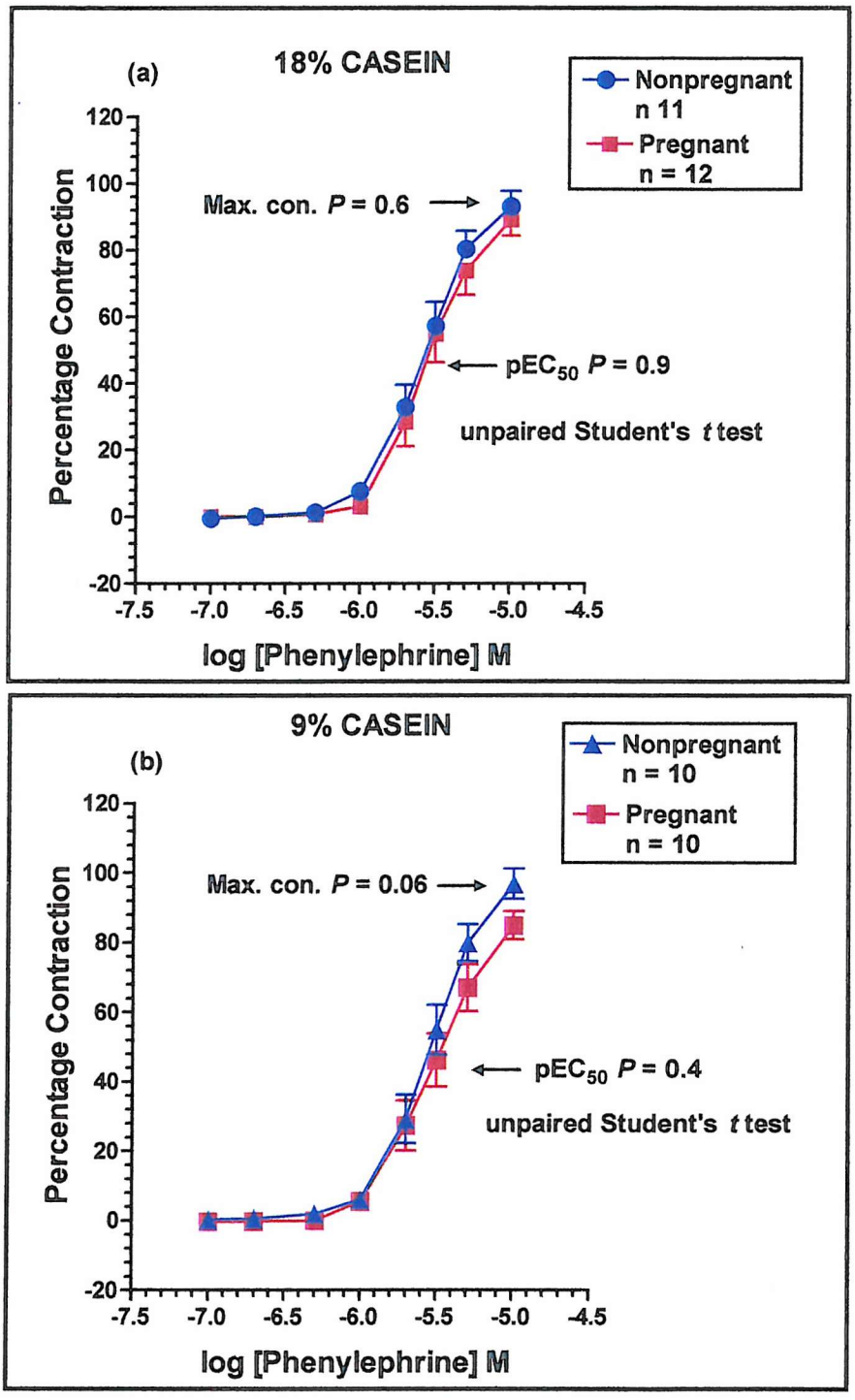


Figure 5.14 (a) and (b).: Concentration-response curves to cumulative doses of PE in nonpregnant and pregnant arteries isolated from (a) 18% and (b) 9% casein fed rats. *n*, no. of animals. Contractile sensitivity to PE was analysed as the entire dose-response curve using nonlinear regression analyses. Maximum contractile response to PE and EC_{50} (the PE dose yielding 50% of the maximum contraction, expressed as -log mol/L = pEC_{50}) were compared by Student's unpaired *t* test. Results of ANOVA and comparisons of pEC_{50} values were also noted in text.

5.3.13. Effect of pregnancy on reactivity to acetylcholine and spermine NONOate.

Concentration-response curves to acetylcholine and spermine NONOate in nonpregnant versus pregnant vessels were analysed by Two-way analysis of variance to determine the influence of gestation on arterial responses. The concentration-response curves of pregnant versus nonpregnant arteries to acetylcholine are shown in Fig. 5.15(a) and (b). On the 18% casein diet, the differences in the sensitivity ($P = 0.17$) and the maximum dilator responses ($P = 0.4$) to acetylcholine were not statistically significant (by Student's unpaired *t* test) [Figure 5.15 (a)]. Relaxant responses to acetylcholine were similar in arteries from pregnant and nonpregnant 9% casein fed rats ($P = 0.1$ by Two-way ANOVA) [Figure 5.15 (b)].

To determine whether pregnancy status altered or not endothelium-independent relaxation, responses to cumulative addition of spermine NONOate (SPN, 10^{-9} - 10^{-5} M), an exogenous nitric oxide donor, were measured in arteries preconstricted with submaximal PE (5×10^{-6} M). On the 18% casein diet the curves were not significantly different, nor were there any differences between pregnant and nonpregnant groups in terms of the sensitivity and maximum relaxation to spermine NONOate ($P = 0.8$ by Two-way ANOVA) [Fig. 5.16 (a)]. However, pregnancy increased SPN-stimulated relaxation of the arteries from 9% casein fed rats compared with the nonpregnant state ($P < 0.0001$ by Two-way ANOVA). Sensitivity and maximum relaxation to SPN were increased significantly in the pregnant low protein fed group: (pEC_{50}: 9% casein pregnant: 5.81 ± 0.12, *n* 7, *versus* nonpregnant: 5.46 ± 0.10, *n* 9; $P = 0.04$; maximum relaxation: 9% casein pregnant: 95.37 ± 2.26 *n* 7, *versus* nonpregnant: 89.22 ± 1.8, *n* 9; $P = 0.049$) (Table 5.8) [Figure 5.16 (b)].

TABLE 5.8

The effect of pregnancy on vascular reactivity of isolated mesenteric artery to acetylcholine and spermine NONOate.

	Acetylcholine (ACh) (10^{-9} to 10^{-5} mol/l)		
Group	***n***	**Maximal relaxation (%)**	**pEC_{50} (M)**
18% Nonpregnant	11	96.36 ± 0.99	7.51 ± 0.05
18% Pregnant	10	97.62 ± 1.14	7.64 ± 0.08
9% Nonpregnant	10	93.59 ± 0.77	7.20 ± 0.12
9% Pregnant	10	94.59 ± 1.22	7.37 ± 0.07
	Spermine NONOate (SPN) (10^{-9} to 10^{-5} mol/l)		
Group	***n***	**Maximal relaxation (%)**	**pEC_{50} (M)**
18% Nonpregnant	9	96.66 ± 1.80	6.17 ± 0.08
18% Pregnant	9	92.89 ± 4.16	6.08 ± 0.11
9% Nonpregnant	9	89.22 ± 1.78	5.46 ± 0.10
9% Pregnant	7	95.37 ± 2.26[a]	5.81 ± 0.12[b]

Table 5.8 summarizes data related to the dose-response curves presented in Figs. 5.15 (a), (b) and 5.16 (a), (b). Data are expressed as means ± SEM; Maximal relaxation (calculated as the percentage of precontraction to PE) and pEC_{50} values are given. *n* indicates the number of rats studied. Sensitivity ($^{b}P = 0.04$) and maximum relaxation ($^{a}P = 0.049$) to SPN were significantly greater in arteries from pregnant compared with nonpregnant rats on the 9% casein diet.

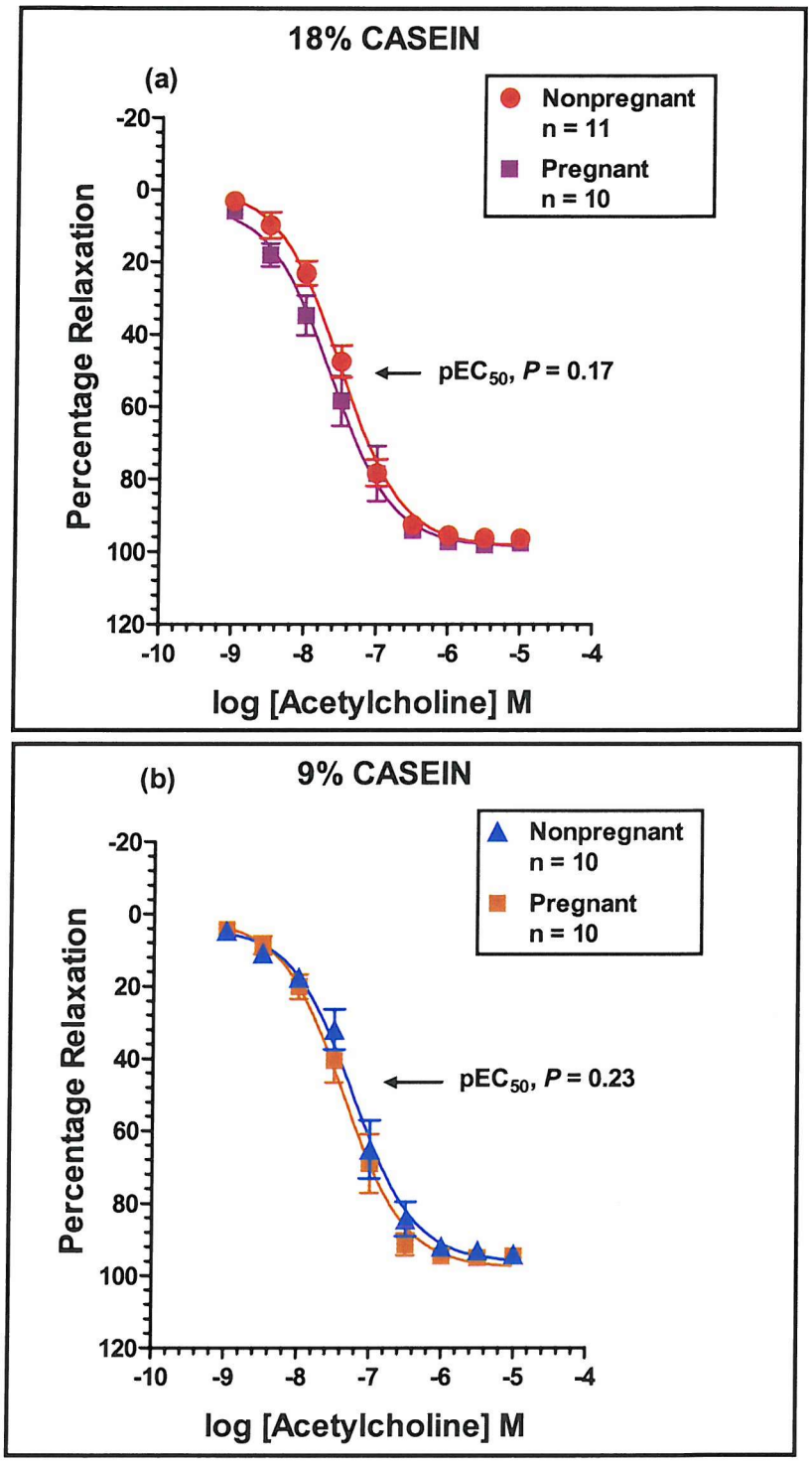


Figure 5.15 (a) and (b).: Concentration-response curves to cumulative addition of ACh in PE-preconstricted pregnant and nonpregnant arteries from (a) 18% and (b) 9% casein fed rats. Values are expressed as a percentage of preconstricted tone, and shown as means ± SEM (where not shown, error bars lie within the symbols).

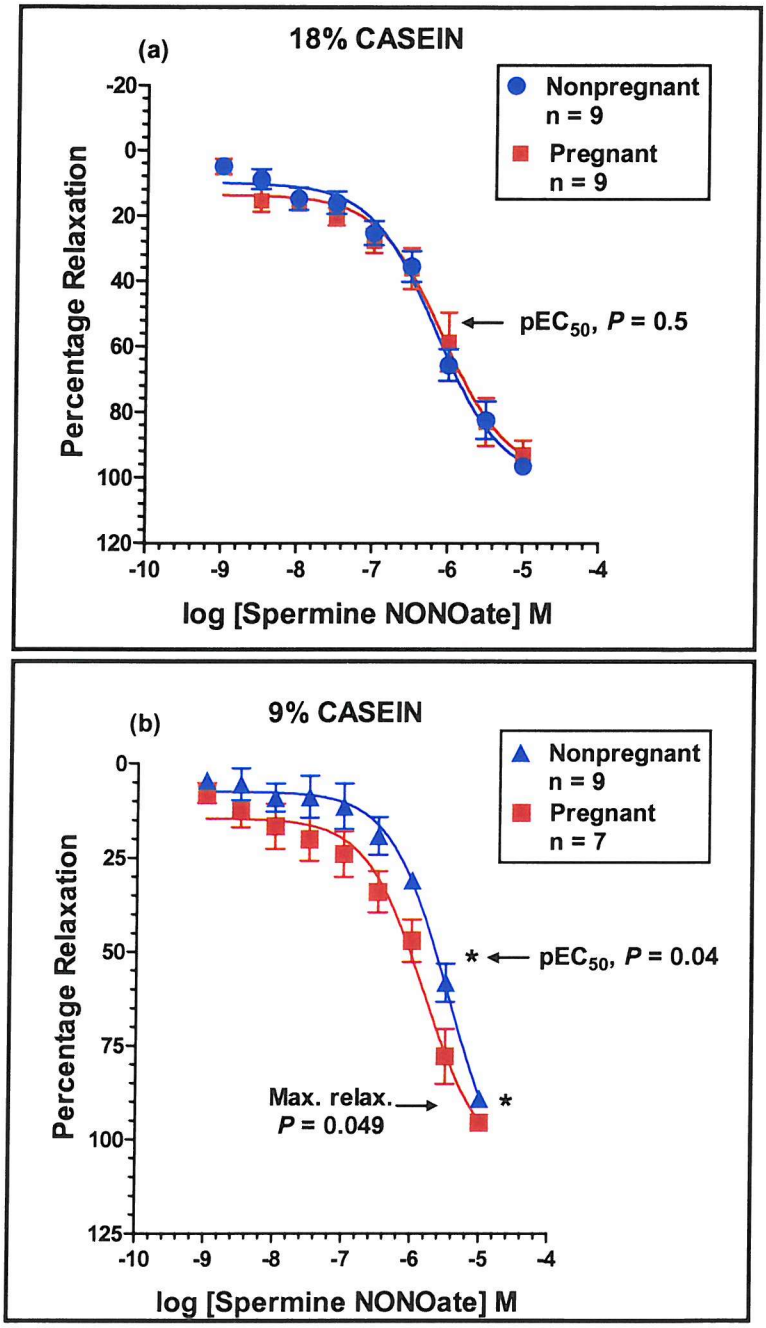


Figure 5.16 (a) and (b).: Dose-response curves to SPN in PE-preconstricted pregnant and nonpregnant arteries from (a) 18% and (b) 9% casein fed rats. Values are means ± SEM; *n*, no. of animals. In arteries from rats on the 9% casein, pregnancy shifted the concentration-response curve to the left ($P < 0.0001$ by Two-way ANOVA). *Asterisks* in **(b)** indicate SPN concentrations with different responses in pregnant and nonpregnant rats.

5.4 DISCUSSION

Part I: Effects of feeding an 18% and a 9% casein diet on body weight, fetal and placental weight and vascular function in the small mesenteric arteries from pregnant Wistar rats.

The present study has shown that a low protein diet was associated with impairment of vasorelaxation in response to two different endothelium-dependent vasodilators ACh and BK (Figs. 5.4 and 5.5). Both ACh and BK cause endothelial NO production by increasing intracellular Ca^{2+} and activating different G proteins (Liao and Homey, 1993). ACh binds both nicotinic receptors (receptor-operated cation channels in post synaptic membranes) and muscarinic receptors. The latter are G-protein-linked receptors and have been described by molecular cloning into a family of m 1 - 5 isoforms (Wess, 1996). Muscarinic receptors cause activation of phospholipase C and inhibition of adenylate cyclase. The ACh-induced NO formation via m2 muscarinic receptors in blood vessels is a $G_i\alpha$ protein-dependent pathway (Felder, 1995). Bradykinin is a potent vasodilator peptide which binds to B1 and B2 G-protein coupled receptors to mediate vasorelaxation of endothelial cells through generation of NO. The bradykinin receptor can be coupled to NO release via $G_q\alpha$ protein (Liao and Homey, 1993). It has been suggested that BK is a physiologically more relevant agonist than ACh *in vivo*, since it is synthesized in human vascular smooth muscle and endothelium (Graf *et al*, 1994). An altered response to both agonists (ACh and BK) each linked to different receptors (muscarinic and B), points to a defect in the NO synthase or in NO production, rather than to a specific muscarinic cholinergic receptor abnormality. In support of reduced NO synthesis in the mesenteric circulation of pregnant rats fed a 9% casein diet, ACh-induced NO levels have been reported to be less in mesenteric arteries from pregnant dams fed low protein (9% casein) than in 18% casein-fed pregnant rats (Brawley *et al*, 2002a). Blunted vasodilatation response to endothelium-dependent agonists appears to be part of an

overall change in the circulation, as decreases in vascular reactivity have also been found in the uterine circulation of pregnant rats fed a low protein diet (Itoh *et al*, 2002).

Pregnant rats showed a significant increase in body weight during the 18-day period of the experiment. This observation is similar to previous studies, which have examined the effects of a 9% casein diet on weight gain in pregnant rats (Langley-Evans *et al*, 1996; Rees *et al*, 1999). While the earlier studies showed an increase in fetal weight at day 18 in the protein restricted pregnant dams and a fall on the day of delivery, the fetuses in the present study were already of a lower weight at day 18 (Table 5.1).

The reduced ACh-induced relaxation in the low protein-fed pregnant rats could be due to a decrease in the synthesis or release of NO from endothelial cells or a change in the sensitivity of vascular smooth muscle to relaxation by NO. The sensitivity of vascular smooth muscle to relaxation by NO could be evaluated by its sensitivity to relaxation by exogenous NO donors such as SPN, which dissociates in a molar fashion to NO. In Chapter 4, it was demonstrated that compared with mesenteric arteries of 18% casein-fed virgin rats, arteries of 9% casein-fed virgin rats exhibited an attenuated vasodilator responsiveness to ACh and SPN (Figs. 4.3 and 4.4). This finding suggested an impairment of NO-dependent vasodilator function either at the level of or downstream from sGC. In contrast, in arteries from the pregnant rats, although ACh-induced relaxation was reduced (Fig. 5.4), responses to the NO donor were unaffected by the low protein diet (Fig. 5.6), suggesting that the endothelium-independent mechanisms of vascular relaxation and the sensitivity of the vascular smooth muscle to relaxation by NO were not impaired in low protein-fed pregnant rats. Furthermore, 8-bromo-cyclic GMP (a membrane permeable cyclic GMP analogue) was used to activate cGMP-dependent protein kinase. The endothelium-independent relaxation in response to 8-bromo-cyclic GMP was also not altered in arteries from pregnant rats on the 9% casein diet. There is no obvious explanation to account for the difference in the response to SPN compared with the virgin animals, but it is possible that the endocrine changes accompanying pregnancy exert

predominant and 'protective' effects on vascular smooth muscle. For example, elevated concentrations of progesterone and oestrogen may evoke vasorelaxation by reducing cell calcium entry (Shan *et al*, 1994; Barbagallo *et al*, 2001).

In the present study, the effect of inhibition of COX with indomethacin on the response to ACh was determined in arteries from pregnant rats fed the 9% casein diet only. The endothelium-dependent relaxant response to ACh of mesenteric arteries from pregnant rats fed 18% casein in the presence of indomethacin, which inhibits the production of prostanoids, was not measured, hence, it is not known whether altered dilator prostanoid activity played a role in the reduction of the endothelium-dependent relaxation observed in pregnant rats fed 9% casein. Indomethacin caused a significant decrease in sensitivity but not maximal relaxation to ACh in the pregnant 9% casein group tested (Fig. 5.8), suggesting that cyclooxygenase products play a role in ACh-induced vasodilatation in the pregnant low protein mesenteric vascular bed. Inhibition of COX has previously been shown to restore the vascular refractoriness to angiotensin II in pregnant women, suggesting that prostanoids are a pivotal mediator of vascular reactivity during pregnancy (Gant *et al*, 1987). Similarly, in a recent study using mesenteric resistance vessels from virgin and late pregnant rats fed either normal rat chow (21% protein) or a diet containing 20% saturated fat, it was shown that indomethacin effected a significant decrease in sensitivity to ACh in all groups, demonstrating that the COX pathway is involved in the vasodilatation induced by ACh (Gerber *et al*, 1999). However, others have reported that ACh-induced dilatation is not affected by COX inhibition in isolated mesenteric arterioles from ovariectomized or oestrogen treated rats (Meyer *et al*, 1997) and in the perfused mesenteric vascular bed from virgin and pregnant rats (Dalle Lucca *et al*, 2000).

To further investigate the possible role of NO synthesis and release in the proposed impaired endothelium-dependent relaxation pathway in the low protein-fed pregnant rats, it was found that pretreatment of the vessels with indomethacin and L-NAME significantly inhibited vascular relaxation by ACh in the pregnant 18% [Fig. 5.10 (a)] and 9% casein [Fig. 5.10 (b)] groups.

The prominence of indomethacin and L-NAME-resistant relaxation to ACh in the mesenteric arteries from pregnant rats irrespective of the diet, suggested that in addition to NO and prostacyclin, an endothelium-dependent vasodilator such as EDHF is important in these vessels (McCulloch *et al*, 1997). Similarly, it has been shown that relaxation to ACh is incompletely inhibited by NOS and COX inhibitors in arteries from pregnant rats (Pascoal *et al*, 1995; Gerber *et al*, 1998) and from pregnant women (McCarthy *et al*, 1994). Gerber *et al* (1998), showed that ACh-induced relaxation in 5 mM KCl was only partially inhibited by NOS, COX and soluble guanylate cyclase inhibitors in mesenteric arteries from nonpregnant and pregnant rats. The residual relaxation, which was greater in arteries from pregnant rats, was prevented by 25 mM KCl, which prevents relaxation dependent upon hyperpolarization, indicating pregnancy associated enhanced synthesis/reduced degradation of a hyperpolarizing factor. EDHF is believed to cause hyperpolarization of the cell membrane by the opening of a K^+ conductance ('selective' blockers have implicated the involvement of various different types of K^+ channels although results are sometimes varying). Hyperpolarization reduces the open probability of voltage-dependent Ca^{2+} channels attenuating Ca^{2+} influx, lowering intracellular free Ca^{2+} levels, resulting in relaxation. In the present study, when the EDHF-mediated component of vasorelaxation is unmasked in the absence of NO and prostacyclin, the maximum relaxant response to ACh was higher but not significantly in 18% casein-fed compared with 9% casein-fed rats and the response to the lower, and therefore more physiologically relevant, concentrations of ACh was reduced in arteries from pregnant rats on the 9% casein diet, implying that the defect in response to ACh was due partly to a reduction in the component attributable to EDHF. The EDHF-mediated hyperpolarization and relaxation are diminished in aged female rats when oestrogen levels are reduced by ovariectomy (Sakuma *et al*, 2002). In earlier studies it was shown that endothelium-dependent hyperpolarization to ACh was reduced in the mesenteric arteries of adult spontaneously hypertensive rats compared with Wistar Kyoto rats and the decreased hyperpolarization accounted, at least in part, for the

impaired relaxation to ACh in spontaneously hypertensive rats (Fujii *et al*, 1992). Further studies are necessary to elucidate the contribution of EDHF in the mesenteric artery from the low protein-fed rat.

Since the original observations linking poor maternal nutrition with an impairment in the maternal cardiovascular adaptation to pregnancy in rats (Ahokas *et al*, 1983b; Rosso and Streeter, 1979; Rosso and Kava, 1980), there have been major advances in our understanding of the initiation of the maternal cardiovascular responses to pregnancy. In the rat, NO is likely to play a major role (Conrad and Vernier, 1989; Conrad *et al*, 1993); for example the increased sensitivity of the mesenteric arteries to ACh in pregnancy is due to changes in the vascular responses to both NO (Davidge and McLaughlin, 1992; Pascoal *et al*, 1995; Gerber *et al*, 1998) and EDHF (Gerber *et al*, 1998). The importance of NO to the physiological mechanisms securing the cardiovascular adaptations to pregnancy in rats has been emphasized by a study showing that inhibition of NO caused a dose dependent reduction in the normal increase in plasma volume (Salas *et al*, 1995). The present study suggests, therefore, that a low protein diet may interfere with the normal enhancement of endothelium dependent relaxation in pregnancy; in turn, this may contribute to failure to expand the extracellular volume. Indeed, in a previous study it has been found that plasma volume was reduced in pregnant rats that were fed a 9% casein diet (Welham *et al*, 1998).

There are a number of possible explanations for the deleterious effects of the low protein diet on maternal vascular function. An earlier study showed that plasma oestrogen concentrations were significantly reduced in rats whose dietary intake was globally restricted by 50% over the last 7 days of pregnancy (Gonzàlez *et al*, 1997). A low protein diet may induce serum oestrogen deficiency in the rat (Ammann *et al*, 2000). Oestrogen is likely to have an important influence on vascular function, with a recent study demonstrating that blockade of the oestrogen receptor during rat pregnancy abolishes the normal enhanced response of the mesenteric vessels to ACh, and that this response is mediated through NO (Zhang *et al*, 2001). A reduction in

oestrogen could therefore contribute to the reduced ACh responses in the low protein group. However, in the present study, although the mean plasma oestrogen concentrations were numerically lower at day 18 of pregnancy in rats fed 9% casein there was no significant difference in the mean values between the two dietary groups (Table 5.2). There are also metabolic changes, linked with the low protein diet, which may have influenced vascular function. These include a reduction in the availability of L-arginine, which could adversely influence endothelial NO synthesis. In growing and pregnant rats, arginine becomes an essential dietary requirement, and plasma arginine concentration and whole body constitutive NO production are reduced in growing rats fed a low protein diet (5% casein) (Wu *et al*, 1999). It was reported in Chapter 4 that the virgin rats continued to gain weight significantly on both the 18% and 9% casein diets, thus the pregnant rats of the same age would require nutrition for their own growth as well as for the growth of the fetuses and placentae. In these circumstances the availability of dietary L-arginine may be critical. Another potential metabolic disturbance may have been caused by the relatively high methionine supplement (5g/kg), which was added in equal amounts to both diets and which was therefore proportionately higher in the 9% casein group (Appendix 2). The metabolic pathway converting methionine to cysteine involves the formation of homocysteine which is condensed with serine to form cystathionine and finally cysteine (Selhub, 1999). One possible consequence is an increase in the blood levels of homocysteine, which could impair endothelium-dependent vasodilatation (Tawakol *et al*, 1997). Although this could be relevant, others have found no differences in serum methionine (Rees *et al*, 1999) and homocysteine (Rees *et al*, 2000) concentrations in pregnant rats fed similar diets to those used in the present study.

The present study has examined the effects of a low protein diet on the maternal vasculature; although untested, it would be anticipated that the observed vascular disturbances would return to normal upon correction of the diet. The relevance of the present findings to programming of the offspring is indirect, but lies in the association between the low protein diet and reduced placental perfusion (Rosso

and Streeter, 1979; Rosso and Kava, 1980; Ahokas *et al*, 1983a; Lederman and Rosso, 1989), leading in turn to fetal growth retardation (Langley-Evans *et al*, 1996; Rees *et al*, 1999). The adaptations of the fetus to restricted nutrient availability are believed to lead to permanent changes in the circulation. Separate studies have shown that a low protein diet in rat dams is also associated with *permanent* alterations in the cardiovascular system of their offspring (Langley and Jackson, 1994; Langley-Evans *et al*, 1996). When are these changes evident? The vessels of the fetal rat are too small for myographic investigation but studies of the sheep fetus have shown that global restriction of the maternal diet is associated with a significant reduction in the vascular reactivity of small resistance branches of the fetal femoral artery (Ozaki *et al*, 2000). In rats, ACh induced relaxation is reduced in the mesenteric arteries of female rats who were exposed (in utero) to a globally restricted maternal diet (Holemans *et al*, 1999). Programming of the vasculature of the female fetus in this way may impair, in adult life, the ability to adapt to pregnancy, and in turn restrict the birthweight of the next generation.

In summary, the present study has shown that mesenteric vascular reactivity is reduced in pregnant rats consuming a low protein diet. The susceptibility of the vascular system to dietary change has important implications to an individual's current health and, in the case of females, potentially for the health of the next generation (Barker, 1994; Wheeler, 1999).

Part II. Comparison of nonpregnant *versus* pregnant (18% and 9% casein diet) groups.

Pregnancy is associated with a decrease in systemic vascular resistance which maintains or reduces maternal blood pressure despite the marked rise in blood volume and cardiac output. In rats in late pregnancy the decrease in mean arterial pressure is associated with blunted pressor responses and hyporeactivity to vasoconstrictors such as noradrenaline (NA) in rings of the superior mesenteric artery (Parent *et al*, 1990) and in perfused mesenteric vascular beds (Massicotte *et al*, 1987), although unchanged (Hart *et al*, 1986) or even increased responses to NA have also been reported (Gerber *et al*, 1999). Prostaglandins and NO are potent vasodilators which may contribute to attenuated vasoconstriction during pregnancy (Conrad and Colpoys, 1986; Davidge and McLaughlin, 1992). Enhanced endothelium-dependent vasodilatation has been described in rat isolated mesenteric arteries in pregnancy (Gerber *et al*, 1998), although similar ACh-mediated vasodilatation has also been reported in the mesenteric circulation in rats (Learmont *et al*, 1996). No differences in sensitivity to NO donors such as sodium nitroprusside has been reported between mesenteric arteries from late pregnant and nonpregnant rats (Pascoal *et al*, 1995; Learmont *et al*, 1996; Ballejo *et al*, 2002).

The present data show that serum levels of 17β-oestradiol were significantly higher in the late pregnant compared with the virgin rats irrespective of dietary intake, which is in agreement with the data in the literature (Garland *et al*, 1987; González *et al*, 1997). There was a significant rise in triglyceride levels in late pregnant rats compared with the virgin rats irrespective of the dietary regime which is also in agreement with a previous study in rat pregnancy (Gerber *et al*, 1999). The changes in circulating lipids during pregnancy in the rat, although not identical to the human, bear several critical similarities. During late gestation, the most pronounced change is the increase in maternal triglyceride-rich lipoproteins (primarily VLDL), which in both species largely results from both increased hepatic production and decreased clearance

from the circulation (Knopp *et al*, 1975). Human pregnancy is associated with pronounced hyperlipidaemia (Piechota and Staszewski, 1992; Belo *et al*, 2002). Belo *et al* (2002), found that as normal pregnancy progressed and the triglyceride levels rose there was a decrease in LDL size, corresponding to an increased proportion of atherogenic small dense LDL. Hormonal influence seems to be an attractive explanation for the lipid changes in pregnancy (Piechota and Staszewski, 1992; Belo *et al*, 2002). Despite important lipid alterations in pregnancy, this occurrence is well tolerated by the mother and may be associated with the need of providing metabolic precursors such as triglycerides to the growing fetus.

Effect of pregnancy on reactivity to α-adrenoreceptor agonist PE and KCl.

In the present study, pregnancy increased absolute contractile force of rat mesenteric arteries as assessed by depolarization in both diet groups (ie. KPSS, 125 mmol/L) (Figs. 5.12 and 5.13). This most likely reflects an increase in vascular smooth muscle mass induced during gestation. The present data contrast with the findings of others, who found similar maximal contractions to KPSS in mesenteric arteries of pregnant and virgin rats (Pascoal *et al*, 1995). Pregnancy also increased maximal absolute tension as measured by a receptor-operated agonist such as PE only in the mesenteric arteries from rats on the 18% casein (Fig. 5.13). To assess changes in the sensitivity of the mesenteric artery, the results obtained with PE were expressed as percentage of the response to the depolarizing KPSS solution (%KPSS, 125 mM) to correct for pregnancy-induced changes of vascular structure and the increased efficacy of the contractile apparatus. When the results were presented in this way [Fig. 5.14(a) and (b)], contractile sensitivity and maximum contraction in response to PE were similar in pregnant and virgin rats on the 18% casein and maximal contractile response to PE was decreased but not significantly in the 9% casein pregnant group as compared with virgin animals ($P = 0.1$). Pascoal *et al* (1995), reported similar dose-dependent PE-induced contractions in mesenteric vessels from virgin and pregnant rats. Although it has been well established that systemic arterial pressor responses

were decreased in animals as well as in women during late pregnancy, the mechanisms involved are still unclear. There are conflicting findings with *in vitro* and *in vivo* preparations with regard to the alterations in vascular reactivity in pregnancy. Some reports have shown decreased contractile responses to noradrenaline in the mesenteric artery (Parent *et al*, 1990), perfused mesenteric vascular bed (Chu and Beilin, 1993) or thoracic aorta (St-Louis and Sicotte, 1992) with pregnancy, but others have not found reduced contractile sensitivity to noradrenaline in the mesenteric artery (Hart *et al*, 1986). Another report showed enhanced constrictor sensitivity to noradrenaline in mesenteric arteries from pregnant rats fed a normal diet compared with virgin animals (Gerber *et al*, 1999). Vascular responses to PE have been reported to be decreased in isolated pressurized mesenteric arteries (D'Angelo and Osol, 1993), thoracic aorta (Khalil *et al*, 1998) and perfused mesenteric vascular bed (Ballejo *et al*, 2002) in late pregnant rats while in another study mentioned above reactivity has been unchanged (Pascoal *et al*, 1995). Such variation may be due to differences in generation or size of the vessel being studied, agonists employed, use of active *vs* passive tension, and conditions of flow *vs* no flow. Agonists such as noradrenaline, which bind to both α1- and α2-adrenergic receptors, may stimulate NO release through binding of α2-receptors on endothelial cells (Miller and Vanhoutte, 1985) and thus decrease contractile response. Such a mechanism may explain the pregnancy-associated decrease in contractile sensitivity to noradrenaline in isolated mesenteric arteries from pregnant rats (Learmont *et al*, 1996), whereas the response to PE, a pure α1-adrenergic agonist, was unchanged in the present study.

In contrast to previous studies (D'Angelo and Osol, 1993; Ralevic and Burnstock, 1996), arteries from pregnant rats on either diet were more sensitive to KCl than were those from virgin rats (Table 5.7). D'Angelo and Osol (1993) reported that in pressurized rat mesenteric arterial segments in pregnancy there was no change in constriction following depolarization with KCl, despite reduced sensitivity of the responses to noradrenaline. Hence, these workers concluded that changes in responsiveness in pregnancy are specific for receptor-mediated contraction and not for

a receptor-independent mechanism such as depolarization (D'Angelo and Osol, 1993). In the study by Ralevic and Burnstock (1996), responses to KCl were also unaffected by pregnancy. These observations and the present results are not consistent with the finding that rat mesenteric arteries are hyperpolarized in pregnancy and show reduced responsiveness to receptor-independent stimuli (K^+) (Meyer *et al*, 1993).

Effect of pregnancy on ACh-mediated vasodilatation.

The data presented here revealed that pregnancy did not influence endothelium-dependent relaxations to ACh in either group [Fig. 5.15(a) and (b)], suggesting no increase in ACh mediated release of either PGI_2 or nitric oxide. Similarly, it has been reported (Yamasaki *et al*, 1996) that ACh-induced relaxation is not increased in the late pregnant rat femoral resistance vessels under isometric conditions. Similar dose-dependent endothelium-dependent relaxations to ACh have been reported in the pregnant and nonpregnant state in the resistance vasculature of the subcutaneous tissue in women (McCarthy *et al*, 1994), in the mesenteric artery in Wistar rats (Learmont *et al*, 1996) and in the perfused rat mesenteric vascular bed (Ralevic and Burnstock, 1996; Dalle Lucca *et al*, 2000). However, previous work has suggested that increased receptor-mediated, endothelium-dependent vasodilatation may play a role in reducing pressor sensitivity and increasing vasodilatation in animal pregnancy (Weiner *et al*, 1991). In a study of isolated arteries from the pregnant guinea pig, increased synthesis of NO during pregnancy was suggested by the observation that ACh-induced vasodilatation in the carotid, uterine (Weiner *et al*, 1989b) and mesenteric (Kim *et al*, 1994; Pascoal *et al*, 1995) arteries was greater in pregnant animals than in nonpregnant animals. Results from a more recent study showed ACh-induced NO release to be also greater in mesenteric arteries from pregnant Sprague Dawley rats than in those from nonpregnant rats (Gerber *et al*, 1998). The present data failed to show an increase in the relaxation to ACh in the mesenteric circulation of the pregnant rat. This may be reflective of vascular bed specificity because evidence in favour of a role for NO in animal pregnancy shows receptor-mediated release

of NO to be greater in the uterine vascular bed compared with the systemic circulation (Weiner *et al*, 1991). Kim *et al* (1994), demonstrated greater ACh-stimulated relaxation in the mesenteric arteries, as compared to renal arteries in pregnant guinea pigs and concluded that pregnancy had a varied effect on endothelium-dependent mechanisms in different arteries. Some studies have suggested increased NO release in animal pregnancy, although this may be a reflection of basal release (Molnar and Hertelendy, 1992). Conrad and Vernier (1989), showed increased urinary cGMP levels in rat pregnancy and Conrad *et al* (1993), showed increased urinary nitrate excretion in rat pregnancy, arguing for increased nitric oxide-mediated vasodilatation in pregnancy. The results of these studies are consistent with enhanced NO-mediated vasodilatation in pregnancy, most likely as a result of increased synthesis in the uterine artery, but argue against this being a widespread effect. Another possible reason for the lack of effect of pregnancy on ACh-induced dilatation in the present study is that since the portion of ACh-induced dilatation in the mesenteric vascular bed attributable to NO is so small, it may not have been possible to detect any increase in the relaxation to ACh in the pregnant rats.

It would be of interest to determine whether pregnancy altered the BK response in mesenteric arteries but the BK response was not evaluated in arteries from virgin rats. However, the vasodilatory response to BK has been found to be significantly greater during pregnancy than in the nonpregnant state, both in the rat mesenteric circulation, (Learmont *et al*, 1996) and in the human cutaneous circulation (Knock and Poston, 1996), whereas the ACh response was unaffected by pregnancy in either vasculature. Both agonists promote NO formation by increasing intracellular Ca^{2+} and activating different G proteins (Liao and Homey, 1993). In view of the divergence in vasodilatory effect between ACh and BK it has been proposed that pregnancy may augment the sensitivity or activation of BK-dependent $G_q\alpha$ protein but not ACh-dependent $G_i\alpha$ protein (Knock and Poston, 1996).

Effect of pregnancy on endothelium-independent relaxation to SPN.

Direct endothelium-independent relaxations of the rat mesenteric arteries to SPN were augmented in pregnancy in arteries from rats on the 9% casein diet. Indeed, pEC_{50} values of the NO donor and maximal relaxation were increased [Fig. 5.16(b)]. SPN led to similar relaxations in mesenteric arteries from pregnant and virgin rats on the 18% casein diet [Fig. (5.16(a)]. Previous studies have shown that the sensitivity of the vascular smooth muscle to an exogenous nitrovasodilator (sodium nitroprusside) was identical in the mesenteric artery (McLaughlin and Conrad, 1995; Pascoal *et al*, 1995) and the perfused mesenteric vascular bed (Ballejo *et al*, 2002) from pregnant and nonpregnant rats. These findings suggested that the mesenteric vascular smooth muscle sensitivity is relatively unaffected by pregnancy, at least with regard to the cGMP transduction cascade. McLaughlin and Conrad (1995), showed that relaxation with sodium nitroprusside was not inhibited by endothelial removal or L-NAME, suggesting that the effect of sodium nitroprusside was not dependent on endothelial-derived NO. In the present study the SPN-induced relaxation was greater in arteries from pregnant rats on the 9% casein diet compared with virgin rats on the same diet and this could reflect an element of enhanced cGMP-mediated response in smooth muscle cells. In rat uterine arteries, pregnancy near term has been shown to result in unaltered (Ni *et al*, 1997) or reduced endothelium-independent vasorelaxation (Wight *et al*, 1998).

In summary, the present data failed to provide evidence of increased acetylcholine-mediated endothelium-dependent relaxation in the mesenteric resistance vasculature of rat pregnancy. It appears likely that the increased response to SPN of the mesenteric arteries from rats on the 9% casein diet during pregnancy suggests an increase in the responsiveness of the vascular smooth muscle to cGMP-mediated relaxation.

CHAPTER 6

EXPRESSION OF ENDOTHELIAL CONSTITUTIVE NITRIC OXIDE SYNTHASE ($_{ec}$NOS) AND SOLUBLE GUANYLATE CYCLASE (sGC) IN VASCULAR TISSUE (PILOT DATA).

6.1 INTRODUCTION

In Chapters 4 and 5 it was demonstrated that in isolated mesenteric arteries of virgin and pregnant rats fed 9% casein diet, the endothelium-dependent relaxation to acetylcholine is impaired. The results of those studies also suggested that the observed abnormalities may be explained, at least in part, by a deficit in the production of nitric oxide in the endothelial cells of the resistance vasculature.

NO is synthesized from L-arginine by NO synthase (NOS). NO synthases exist in three distinct isoforms: nNOS, iNOS, and ecNOS. nNOS (also known as type I, NOS-I) was first identified as constitutive in neuronal tissue. iNOS (also known as type II, NOS-II) was originally identified as being inducible by inflammatory cytokines and lipopolysaccharide (LPS) in macrophages and hepatocytes. ecNOS (also known as type III, NOS-III) was originally identified as constitutive in vascular EC. nNOS and ecNOS are collectively termed constitutive NOS (cNOS). However, it is now clear that all three NOS isoforms can be induced by different, appropriate stimuli through transcriptional and posttranscriptional mechanisms and can be constitutively expressed in some tissues or cells (Alderton *et al*, 2001). In addition all three isoforms can be found in the cytosol or particulate fractions of cells, or both.

The haemoprotein sGC is the predominant intracellular NO receptor in vascular smooth muscle cells (VSMCs) (Moncada *et al*, 1991), which mediates NO signalling via formation of cGMP. This enzyme is a heterodimer and consists in most

mammalian tissues of a larger α_1 (76- to 82-kDa) and a smaller β_1 (70- kDa) subunit (Nakane *et al*, 1990).

Dietary protein deficiency results in decreases in plasma Arg concentration and impairs both constitutive and inducible NO synthesis in young rats (Wu *et al*, 1999). Arg deficiency results in hypertension in animals and humans (Ignarro *et al*, 1999). Arg supplementation has been shown to increase NO synthesis and to prevent endothelial dysfunction in animal models of pulmonary or salt-induced hypertension and to improve NO-dependent endothelial relaxation in patients with major cardiovascular risk factors (hypercholesterolemia, smoking, diabetes, obesity, insulin resistance and aging) and with many common cardiovascular disorders (coronary and peripheral artery disease, heart failure, ischemia/reperfusion injury) (Maxwell and Cooke, 2001). Maternal dietary protein deficiency decreases arginine concentrations, constitutive and inducible NOS activities and NO production in placenta and endometrium of pigs during early gestation (Wu *et al*, 1998). In light of the foregoing, it was hypothesized that dietary protein deficiency may impair NO synthesis in virgin and pregnant rats by decreasing tissue $_{ec}$NOS expression.

In Chapter 4, it was demonstrated that compared with mesenteric arteries of 18% casein-fed virgin rats, arteries of 9% casein-fed virgin rats exhibited an attenuated vasodilator responsiveness to ACh and SPN. This finding suggested an impairment of NO-dependent vasodilator function either at the level of or downstream from sGC.

The purpose of the present study was therefore to clarify mechanisms underlying the reduced response to NO-mediated vasodilators in the 9% casein-fed virgin and pregnant rats. The specific goals of this study were to 1) develop a competitive PCR assay to measure changes in the expression of endothelial constitutive nitric oxide synthase ($_{ec}$NOS) and soluble guanylate cyclase (sGC) mRNA levels in mesenteric arteries from virgin and pregnant 18% and 9% casein-fed rats and 2) with the use of Western analysis, evaluate the expression of protein of the key enzymes of the NO/cGMP system: endothelial constitutive nitric oxide synthase ($_{ec}$NOS), and soluble guanylate cyclase (sGC) in mesenteric arteries and aortae from virgin and pregnant 18% and 9% casein-fed rats.

6.2 MATERIALS AND METHODS

6.2.1. Total RNA extraction and mRNA isolation (Fig. 6.1).

Mesenteric arteries were dissected free of connective tissue, fat and veins and collected into sterile tubes, snap frozen in liquid nitrogen, and stored at -70°C for RNA extraction. Two or three arteries were homogenised on dry ice in 200 µl of TRI REAGENT ™ (Product No. T9424, Sigma Chemicals, Poole, Dorset, UK), which contains guanidine thiocyanate and phenol in a monophasic solution. 40 µl of chloroform (Product No. C2432, Sigma Chemicals, Poole, Dorset, UK) were then added and after vortexing the mixture was cooled on ice for 10 minutes. After centrifugation in an eppendorf microcentrifuge tube (12,000xg, 20 minutes), 80 µl of the upper aqueous phase was transferred to a sterile tube, and an equal volume of isopropanol (Sigma Chemicals, Product No. I9516) was added and the RNA was allowed to precipitate by standing at -20 °C for 15 minutes before recovery by centrifugation (12,000xg, 15 minutes) and washing of the pellet in 75% ethanol (Sigma Chemicals, UK). The resultant RNA pellet was dried in a fumehood before being solubilized in 20 µl of nuclease free water (Promega, Southampton, UK). This RNA solution was stored at -20 °C until required for reverse transcription. Concentration and A_{260}/A_{280} ratio was checked and integrity assessed by examination of the ribosomal bands after gel electrophoresis. All materials used for RNA work were autoclaved before use or certified RNAase-free by the manufacturer.

6.2.2. Reverse transcription-polymerase chain reaction (RT-PCR) assay for ecNOS mRNA and sGC mRNA.

A first-strand cDNA synthesis kit (Advanced Biotechnologies Ltd., Epsom, Surrey, UK) was used to synthesize cDNA by reverse transcription from RNA extracted from the vessels. RT-PCR was performed essentially as described by the

manufacturer (Advanced Biotechnologies Ltd.). 2 µl of RNA solution was added to 1 µl of anchored oligo-dT (0.5 µg/µl) and 9 µl of sterile water. The mixture was heated briefly at 70°C for 5 minutes to remove any secondary structure, and then placed on ice to allow annealing of the random primers to the template RNA. After approximately 10 minutes, the following reverse transcription reagents (Advanced Biotechnologies Ltd.) were added: 4 µl 5X 1st strand synthesis buffer, 2 µl dNTP mix (5mM each), 1 µl M-MLV reverse transcriptase (25 units per µl) and 1 µl of RNAase Block Ribonuclease inhibitor (5 units per µl). The RT reaction was performed by incubation of the mixture at 42°C for sixty minutes. Then, to inactivate the RTase the mixture was incubated at 75°C for ten minutes. The cDNA could then be stored at -20°C until required for the polymerase chain reaction.

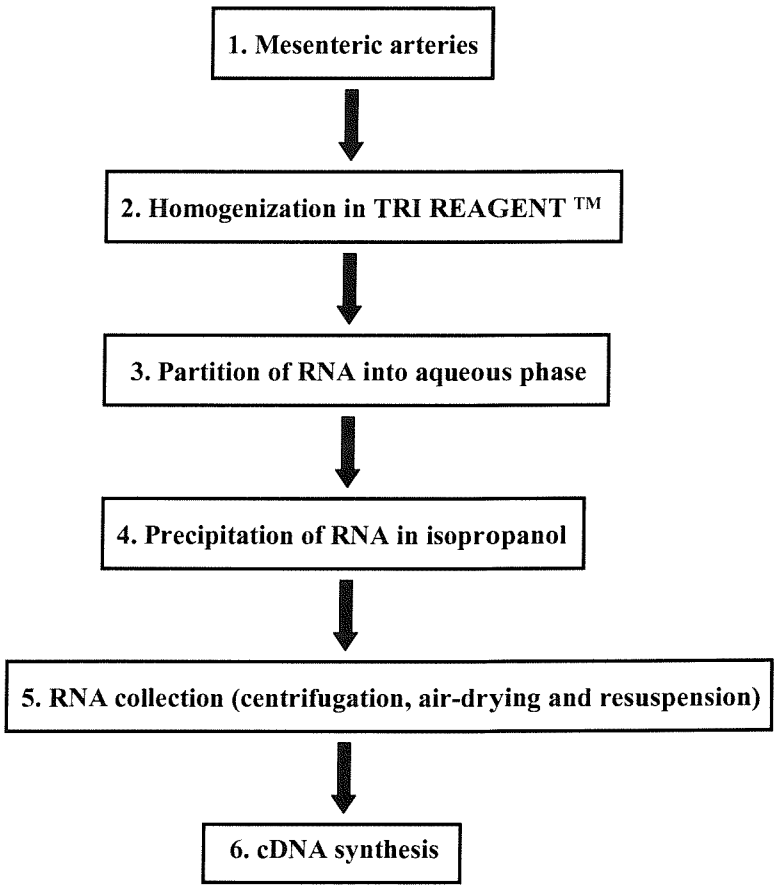


Figure 6.1.: RNA extraction and complementary DNA synthesis procedure.

6.2.3. PCR

Amplification of reverse transcription (RT)-generated cDNA by PCR was conducted essentially as described by the manufacturer (Advanced Biotechnologies 1st strand synthesis kit, Advanced Biotechnologies Ltd., Epsom, Surrey, UK). Oligonucleotide primers were synthesized in the Department of Molecular Microbiology, University of Southampton. 2 µl of each cDNA sample was added to 48 µl of PCR reaction mixture comprising 5 µl 10X reaction mixture buffer with 1.5 mM $MgCl_2$ (Advanced Biotechnologies Ltd.), 2 µl dNTP mix (5mM each), 1 µl sense primer (10 µM), 1 µl antisense primer (10 µM), 0.3 µl Taq DNA Polymerase (5 units per µl) (Red Hot Taq, Advanced Biotechnologies Ltd.), and 39 µl distilled water. The reaction mixture was overlaid with 30 µl of mineral oil and was then subjected to the appropriate program for the product of interest. For ecNOS the following oligonucleotide primers were used: sense 5' - CGT GCG CCA GGC TCT CAC TTA C - 3' and antisense 5' - GGC TGC AGC CCT TTG CTC TCA - 3'. The conditions of amplification were: denaturation at 91°C for 30 sec; annealing at 52°C for 60 sec; extension at 72°C for 60 sec, for a total of 40 cycles of amplification.

At the end of the assay the products generated by PCR were observed and an estimate of their size made following electrophoresis through agarose gels. A 10 µl aliquot of the PCR mixture was added to 4 µl of loading buffer (0.25% Bromophenol blue, Sigma Chemicals, UK; 40% w/v sucrose, Sigma Chemicals; in H_2O) and size fractionated through a 2% agarose gel (3:1 wide range: standard agarose, Sigma Chemicals, Poole Dorset, UK; designed for efficient separation of PCR products) in 1X Tris-borate EDTA (TBE) buffer, using a constant 150 V field. PCR product size was determined by comparison with a 100 bp DNA ladder (1 µl, Gibco BRL). To permit visualisation of the DNA bands, gels were stained for fifteen minutes in ethidium bromide (0.5 µg/ml, Sigma Chemicals), destained for ten minutes in water and examined on a 312nm UVP ultraviolet transilluminator (Genetic Research Instrumentation Ltd., Dunmow, UK). Gels were photographed using a D.S.34

Polaroid camera and hood and Polaroid 667 film (Genetic Research Instrumentation Ltd.).

The primers were expected to produce amplification band of 550 base pairs (bp) in size for ecNOS. For guanylate cyclase the following primers were used: sense 5' - GGT TTG CCA GAA CCT TGT ATC CAC C - 3' and antisense 5' - GAG TTT TCT GGG GAC ATG AGA CAC C - 3'. The conditions of amplification were: denaturation at 91°C for 30 sec; annealing at 62°C for 30 sec; extension at 72°C for 60 sec, for a total of 35 cycles of amplification to yield a product of 283 base pairs.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme responsible for catalysing the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate (Ercolani *et al*, 1988). Structurally, GAPDH is a tetramer of four identical 37,000 molecular weight subunits, with regulation of the enzyme being achieved through its interaction with the cell membrane and the action of glycolytic metabolites. Due to its essential role in the process of glycolysis, GAPDH is recognised as a housekeeping gene. The small amounts of mesenteric artery sample obtained in the present study and the desire to maximise the quantity of material available for $_{ec}$NOS and sGC analysis resulted in PCR used for studying this gene's expression. For the GAPDH, the following oligonucleotide primers were used: sense - 5' - TAT GAC AAC TCC CTC AAG AT - 3' and antisense 5' - AGA TCC ACA ACG GAT ACA TT - 3' but using an annealing temperature of 52°C and for only 30 cycles. This yielded a 320 bp product.

6.2.4. Western blot analysis of $_{ec}$NOS protein expression

These measurements were carried out to determine the $_{ec}$NOS protein in the aorta tissue preparations of 18% and 9% casein-fed pregnant rats. $_{ec}$NOS protein in mesenteric arteries was not investigated. Frozen rat aorta from each animal were homogenized in three volumes (wt/vol) ice-cold RIPA buffer containing 1 x PBS, pH 7.4, 0.1% SDS, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 10 mg/ml phenylmethylsulfonyl fluoride (PMSF), and 100 mM sodium orthovanadate at 0 to 4°C with the aid of a tissue grinder fitted with a motor-driven ground glass pestle as described in section 5.2.2. Insoluble debris was removed by centrifugation at 14,000xg for 20 minutes at 4°C, and the supernatant was removed and centrifuged again for 5 minutes at 14,000xg at 4°C. Solubilized protein was quantified using the Bio-Rad *DC* protein concentration assay kit (Catalog No. 500-0116, Bio-Rad Laboratories Inc., Hercules, CA) (Appendix 7) with bovine serum albumin as a standard.

Proteins (35 μg) were then analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Appendix 8) according to Laemmli (1970). Proteins were separated by size on 7.5% acrylamide gels (100 V, 2.5 h; Mini Protean II, Bio-Rad Laboratories Inc.) alongside positive controls (in aorta tissue preparations measured, 2.5 μg /lane human endothelial cell lysate was used) and Kaleidoscope prestained molecular mass marker proteins (Bio-Rad Laboratories Inc.). After electrophoresis, proteins were transferred from gel to PVDF (polyvinylidene difluoride) membrane electrophoretically (100 V, 1 h) in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol and 0.05% SDS) using a Bio-Rad Trans-Blot system. Molecular mass standards (Kaleidoscope prestained standards, Bio-Rad Laboratories Inc.) were identified directly on the PVDF membrane.

Once the proteins had been transferred from the polyacrylamide gel to the PVDF membrane, detection of $_{ec}$NOS protein proceeded by the use of antibodies. Prior to the addition of antibodies, the PVDF membrane was blocked for 12 h at 4°C in 25 ml of Tris-buffered saline (10 mM Tris, pH 7.5, 100 mM NaCl)

containing 0.1% (v/v) Tween 20 and 5% non-fat dry milk. Blocking the membrane was important so that antibodies did not bind nonspecifically to the membrane. After preincubation with blocking buffer, the membrane was then incubated with agitation for 2 h in Tris-buffered saline (10 mM Tris, pH 7.5, 100 mM NaCl) containing 0.1% (v/v) Tween 20, 1% non-fat dry milk and 10 µl of anti-$_{ec}$NOS antibody (250 µg/ml). Antiserum incubations were all performed at room temperature diluted as follows: $_{ec}$NOS mouse monoclonal Ab (N30020, Transduction Laboratories, Inc., Lexington, KY, USA), diluted 1:1000, 2 h, followed by anti-mouse IgG-Horseradish Peroxidase (HRP), diluted 1:3,000, 1 h. The membrane was washed for 30 minutes in a shaking bath, changing the wash buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20) every five minutes prior to one hour of incubation in blocking buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% (v/v) Tween 20, 1% non-fat dry milk) plus diluted anti-mouse horseradish peroxidase-conjugated secondary antibody. The washes were repeated before proteins were visualised using an enhanced horse radish peroxidase/luminol chemiluminescence reagent detection system as described by Amersham Pharmacia Biotech (ECL Plus, Amersham Pharmacia Biotech Ltd., Little Chalfont, Buckinghamshire, UK). The excess wash buffer was drained from the washed membranes. The membrane was then placed protein side up on a sheet of Saran Wrap (Dow Chemical Company). 3 ml of detection solution A (ECL Plus substrate solution containing tris buffer) and 75 µl of solution B (stock acridan solution in dioxane and ethanol, ECL Plus, Amersham Pharmacia Biotech Ltd.) were mixed and pipetted on to the membrane. The membrane was incubated with the chemiluminescent solution for 5 minutes at room temperature. Following the incubation, excess detection reagent was drained off from the membrane and the blots were placed protein side down on to a fresh piece of Saran Wrap, were wrapped up and placed protein side up, in an x-ray film cassette and subjected to autoradiography (Hyperfilm ECL, Amersham Pharmacia Biotech Ltd.) for 3-5 minutes.

6.2.5. Chemicals

The following chemicals were purchased from Sigma Chemicals (Poole, Dorset, UK): phenylmethylsulfonyl fluoride (PMSF) (Catalog No. P-7626), sodium dodecyl sulphate (SDS), polyoxyethylene [9] *p-t*-octyl phenol (NP-40), sodium deoxycholate, sodium orthovanadate, PVDF membrane (Catalog No. P-2938). Anti-$_{ec}$NOS mouse monoclonal antibody (Catalog No. N30020), anti-mouse IgG-Horseradish Peroxidase (HRP), human endothelial lysate positive control (Catalog No. 611450) were supplied by Transduction Laboratories (Lexington, KY, USA). Kaleidoscope molecular weight markers (Catalog No. 161-0324) was purchased from Bio-Rad (Bio-Rad Laboratories Inc., Hercules, CA). ECL Plus Western blotting kit (Catalog Nos. RPN 2132, RPN 2133) and Hyperfilm (Catalog No. RPN 2103) were purchased from Amersham Pharmacia Biotech UK Ltd.

6.3 RESULTS

6.3.1. RT-PCR identification of $_{ec}$NOS in mesenteric arteries

A representative ethidium bromide-stained gel with RT-PCR products for $_{ec}$NOS from RNA obtained from mesenteric arteries is illustrated in Figure 6.2. Following reverse transcription of mRNA isolated by mesenteric arteries, PCR carried out with specific primers for $_{ec}$NOS resulted in the amplification of $_{ec}$NOS mRNA (Figure 6.2, lanes 2-3). A band of the predicted size (550 bp) was detected in mesenteric arteries (Figure 6.2, lanes 2-3). As shown in Figure 6.2, lanes 6-7, $_{ec}$NOS-specific amplification of cDNA from endothelial cells yielded the expected product. RT-PCR reaction run in the absence of cDNA was used as negative control (Figure 6.2, lane 4).

6.3.2. RT-PCR identification of sGC in mesenteric arteries

Figure 6.3 (A) depicts an ethidium bromide-stained gel containing RT-PCR products for sGC from RNA obtained from mesenteric arteries (lanes 3-4). RT-PCR amplification of cDNA from arteries with the use of specific primers for sGC detected a PCR product of 283 bp in mesenteric arteries [Figure 6.3 (A), lanes 3-4]. The same amplified product was also observed in rat vascular smooth muscle cells (VSMCs) [Figure 6.3 (A), lanes 1-2]. RT-PCR amplification of cDNA from mesenteric arteries with the use of primers to distinguish the VEGF splice variants, detected products of three different sizes (calculated bands of 415, 550 and 650 bp in lanes 7-8) [Figure 6.3 (A)]. No signal was observed in the control PCR reactions when reverse transcriptase was omitted in RT reactions [Figure 6.3 (A), lane 5], demonstrating that the RT-PCR products were not derived from genomic DNA. RT-PCR reaction run in the absence of cDNA was also used as negative control [Figure 6.3 (A), lane 6]. As a positive control for RNA extraction and the RT reaction, the GAPDH RT-PCR product was detected in isolated arteries with the predicted size of 320 bp [Figure 6.3 (B), lanes 1-3].

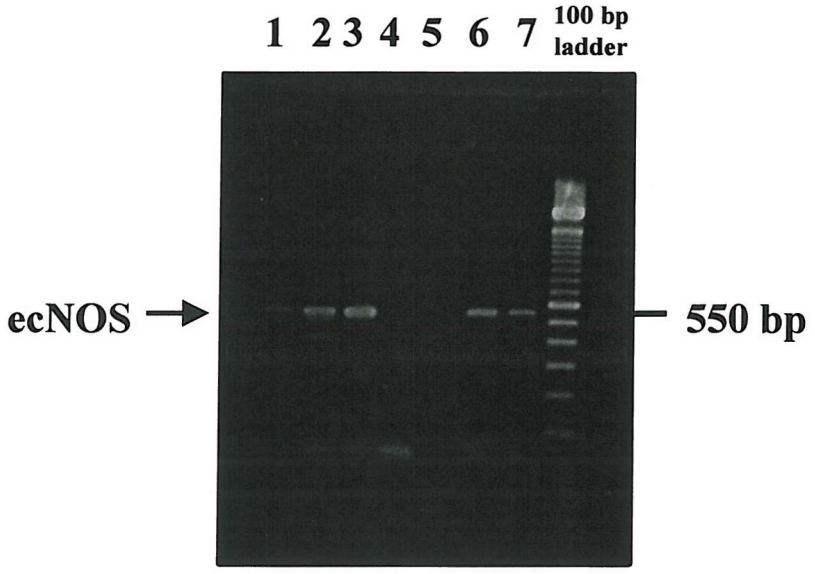


Figure 6.2.: Ethidium-bromide-stained agarose gel demonstrating RT-PCR products for ecNOS (550 bp) in RNA extracted from mesenteric arteries. *Lane 1*, endothelial cell culture; *Lanes 2 -3*, mesenteric arteries; *Lane 4*, (-ve) PCR; *Lane 5*, blank; *Lanes 6-7*, endothelial cell culture. The lower size marker is 100 bp.

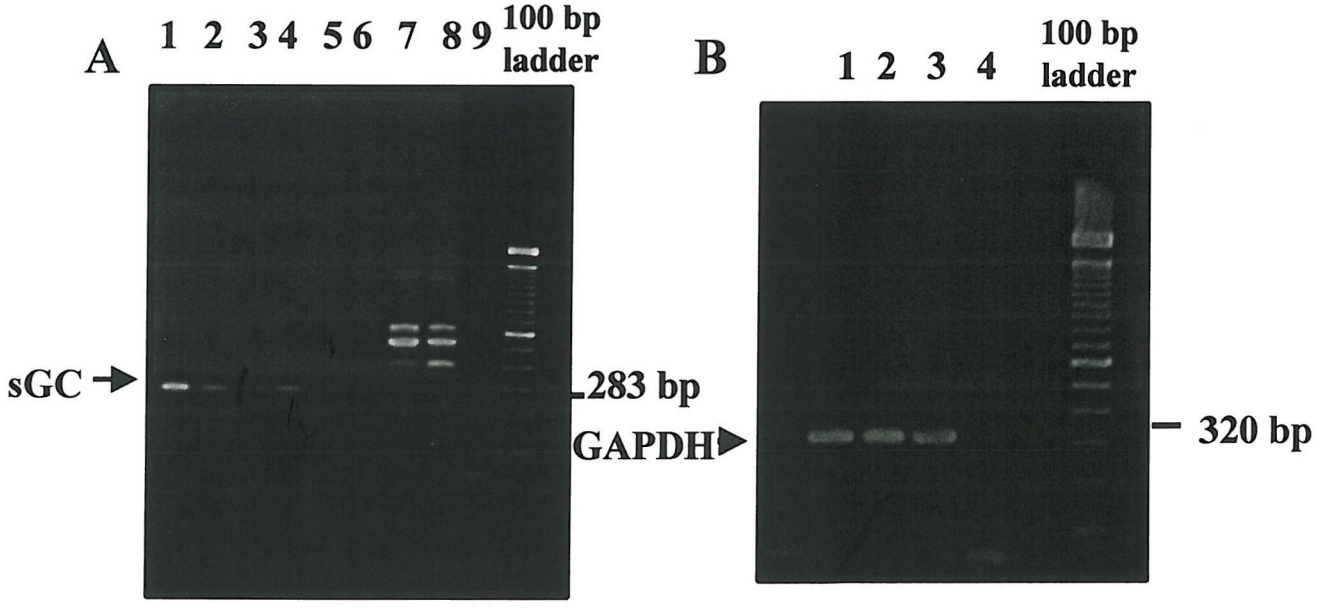


Figure 6.3.: (A) Expression of sGC mRNA as detected by RT-PCR in rat small mesenteric arteries. *Lanes 1-2,* VSMCs; *Lanes 3-4,* mesenteric arteries; *Lane 5,* -ve RT; *Lane 6,* -ve PCR; *Lane 7,* VEGF alternatively-spliced products-mesenteric; *Lane 8,* VEGF mesenteric; *Lane 9*, blank. (B) The expression level of GAPDH served as an internal control. *Lanes 1-3,* mesenteric arteries.

6.3.3. Quantification of the effects of a low protein diet on expression of $_{ec}$NOS protein in the rat thoracic aorta by Western immunoblot analysis.

Western immunoblot studies from 18% and 9% casein-fed pregnant and nonpregnant rats are shown in Figures 6.4 to 6.7. Figure 6.4 (A) illustrates the presence of an immunoreactive band consistent with $_{ec}$NOS in Western blot of rat aorta. Figure 6.5 depicts representative Western immunoblots of $_{ec}$NOS protein expression in aortae from 18% and 9% casein-fed pregnant rats. A single band at 140 kDa was detectable and was similar in size to the band with human endothelial cell protein positive control (C=control). The expression of $_{ec}$NOS protein was greater in the 18% P than in the 9% P aortae.

Figure 6.6 (A) and (B) illustrates typical Western immunoblots of $_{ec}$NOS protein expression in aortae from nonpregnant rats fed 18% casein or 9% casein for 18-19 days. Figure 6.6 (A) shows $_{ec}$NOS protein expression in aortae from 4 nonpregnant rats fed 18% casein and 3 nonpregnant rats fed 9% casein. Figure 6.6 (B) shows $_{ec}$NOS protein expression in aortae from 8 nonpregnant rats fed 18% casein and 4 nonpregnant rats fed 9% casein. As shown in Figure 6.6 (A) and (B), there was no significant difference in aorta $_{ec}$NOS protein expression between the 18% NP and 9% NP groups studied.

Figure 6.7 (A) shows expression of $_{ec}$NOS in aortae from 5 pregnant and 3 nonpregnant 18% casein-fed rats. There was no apparent difference in the relative optical densities of $_{ec}$NOS expression in the 18% P and NP aortae. Expression of $_{ec}$NOS protein was found to be increased in aortae from 9% NP when compared with 9% P [Figure 6.7. (B)].

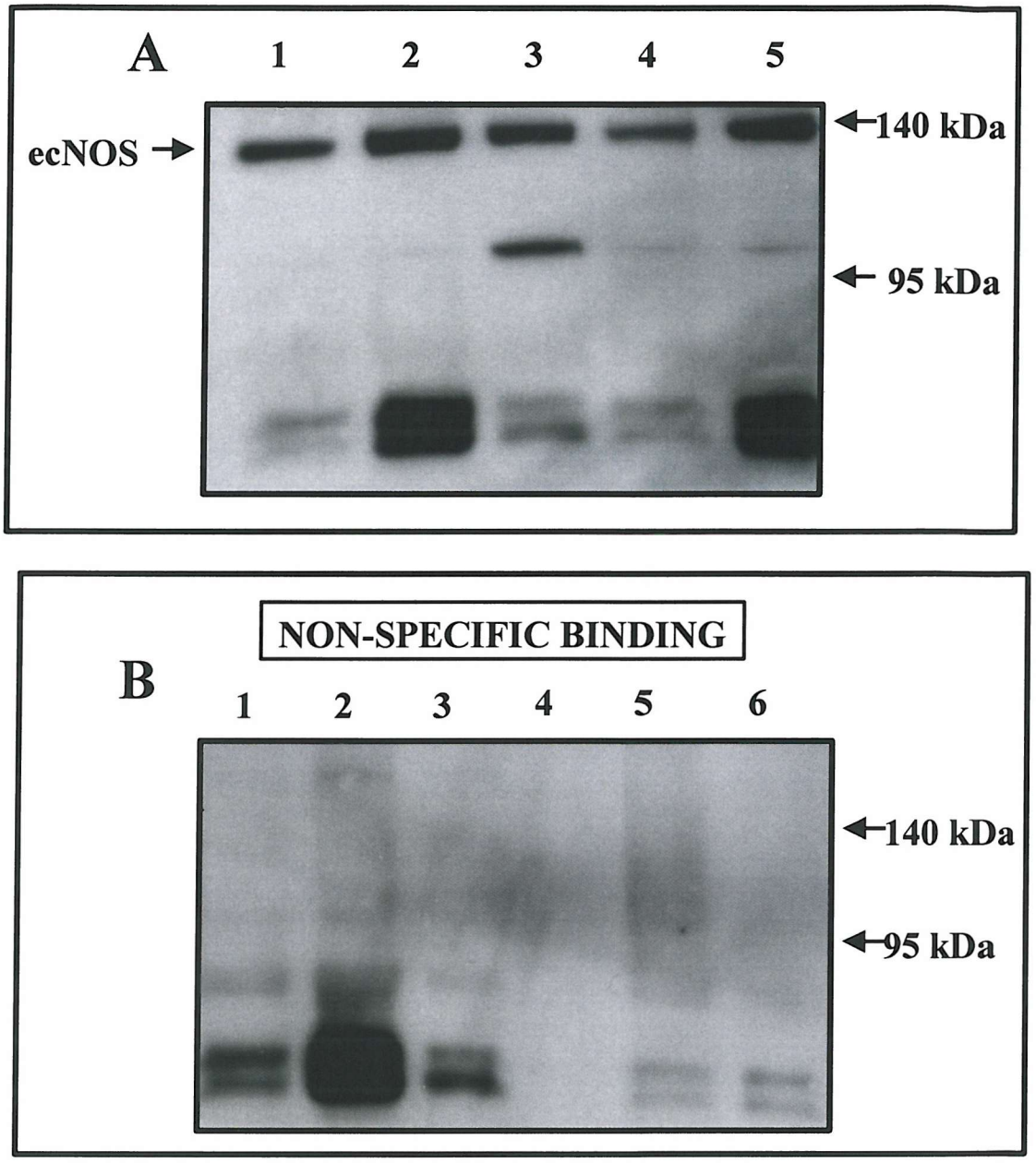


Figure 6.4.: Detection of ecNOS protein in rat aorta. **(*A*)** Western blots were probed with a monoclonal mouse anti-$_{ec}$NOS antibody followed by anti-mouse IgG-HRP linked secondary antibody. **(*B*)** Nonspecific: Aorta homogenates were incubated with secondary antibody (no primary antibody present). Proteins were extracted from aortas from pregnant rats placed on either 18% (*Lanes 1-2* in both blots) or 9% (*Lanes 4-5* in *A*, and *Lanes 5-6* in *B*) diet until 18-19 days of gestation or from virgin 18% casein-fed rats (*Lane 3* in both blots). *Lane 4* in (B) is blank. Note a 140 kDa ecNOS immunoreactive band only in (*A*).

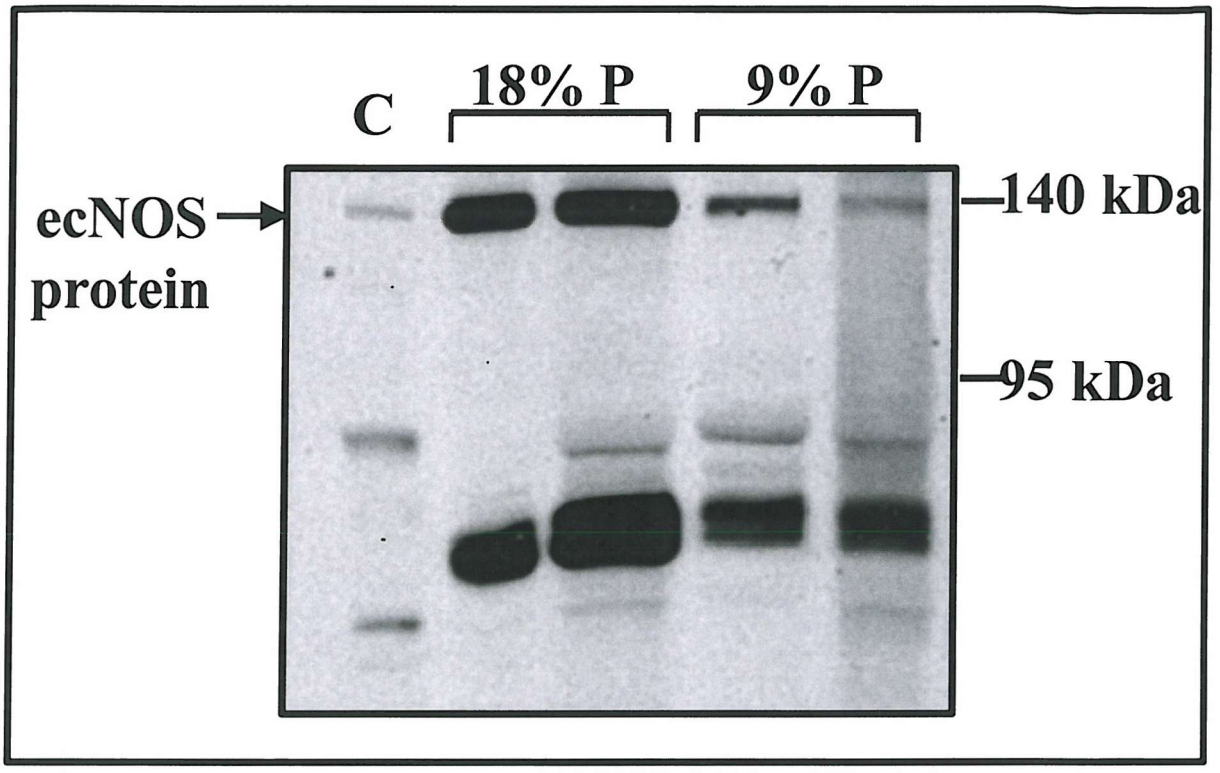


Figure 6.5.: Comparison of $_{ec}$NOS protein expression in the aorta from pregnant rats placed on either a 18% or 9% casein diet for 18-19 days. A representative Western blot is shown in which aorta homogenates were probed with a monoclonal mouse anti-$_{ec}$NOS Ab. The first lane shows human endothelial lysate positive control.

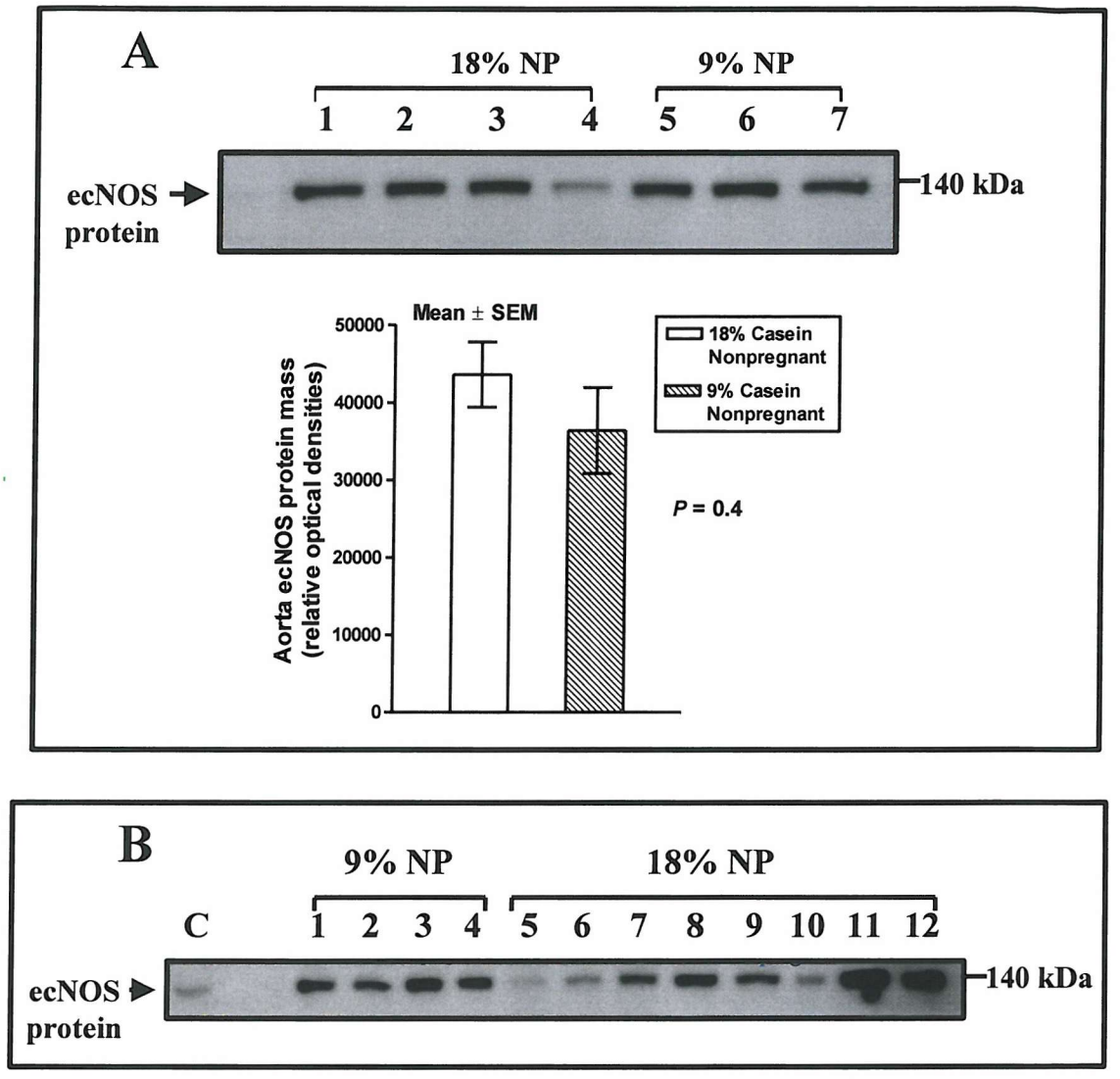


Figure 6.6. (A) and (B): Examples of Western immunoblots of $_{ec}$NOS protein expression in aortae from nonpregnant (NP) rats placed on either a 18% or 9% casein diet for 18-19 days. Proteins were separated by SDS-PAGE and subjected to immunoblot analysis using a monoclonal mouse anti-$_{ec}$NOS antibody as described in Methods. Each lane number represents a different rat. In *(A),* the blot and the results of densitometric analyses shown are representative of 2 independent experiments. Each data point represents the mean ± SEM derived from two separate experiments. In *(B)*, the immunoblot shown is also representative of two independent experiments. The first lane shows human endothelial lysate positive control.

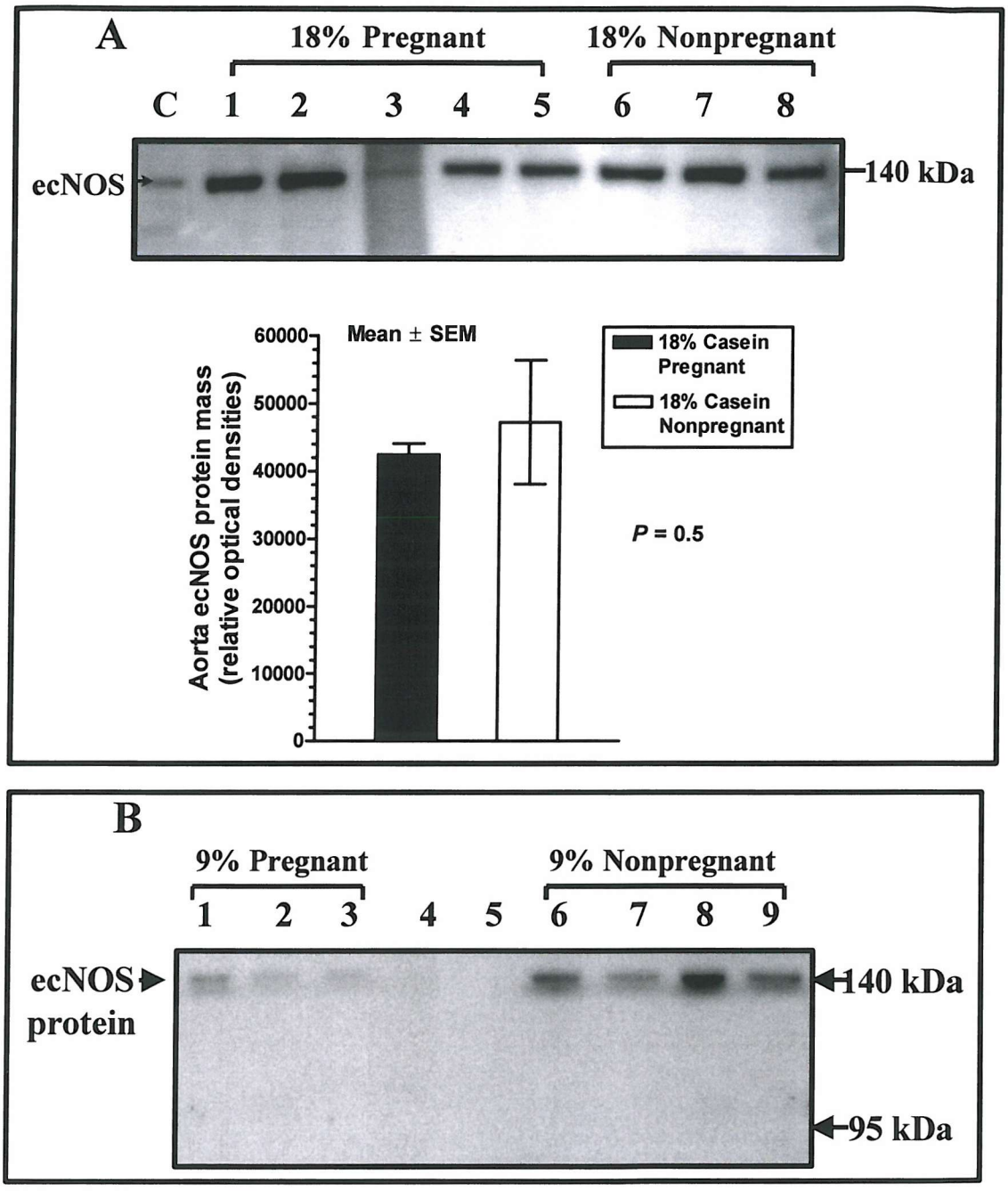


Figure 6.7. (A) and (B): $_{ec}$NOS protein expression in aortae from pregnant (P) and nonpregnant (NP) rats placed on either a 18% or 9% casein diet for 18-19 days. Each lane number represents a different rat. In *(A)*, representative Western blots and densitometric analyses from 2 separate experiments are shown. The positive control (C) was human endothelial lysate. In *(B)*, the immunoblot shown is representative of two independent experiments.

6.4 DISCUSSION

As discussed in Chapters 4 and 5, small mesenteric arteries of virgin and pregnant rats fed a low protein diet demonstrate impaired endothelium-dependent, NO-mediated vasorelaxation. The decreased release of NO may be explained by downregulation of $_{ec}$NOS, the key enzyme of endothelial, receptor-dependent NO liberation. $_{ec}$NOS is an important regulatory enzyme in the cardiovascular system catalysing the production of NO from arginine. The vasodilator potency of the NO donor spermine NONOate was significantly reduced in mesenteric arteries of virgin 9% casein-fed rats compared with 18% casein-fed. To elucidate 1) whether the attenuation of endothelium-dependent relaxation is the result of an alteration in the expression of the key enzymes of NO-mediated dilation, and 2) to investigate the role of the NO receptor sGC in the reduction in NO-donor-dependent (SPN-dependent) relaxation associated with low protein feeding in virgin rats only, determination of expression of mRNA and protein of $_{ec}$NOS and sGC in mesenteric arteries of virgin and pregnant 18% and 9% casein-fed Wistar rats by RT-PCR and Western blot, was one of the main objectives of the present study.

The data presented here demonstrate a technique for assessing $_{ec}$NOS and sGC mRNA levels in rat small mesenteric arteries. Because only small amounts of mRNA can be extracted from blood vessels, the quantification of gene expression in individual rats is difficult. PCR amplification products that had the expected size for $_{ec}$NOS (550 bp) and sGC (283 bp) gene products were obtained through use of mRNA isolated from rat mesenteric arteries. The size of $_{ec}$NOS gene product obtained (550 bp) was identical to that of the PCR $_{ec}$NOS gene product obtained from the positive control (endothelial cells) (Figure 6.2, lanes 1, 6-7). The size of sGC gene product obtained (283 bp) was identical to that of the PCR sGC gene product obtained from the positive control (VSMCs) [Figure 6.3 (A), lanes 1-2]. Chu *et al* (2002) have developed a real-time PCR method to quantify $_{ec}$NOS mRNA in blood vessels from C57BL/6 mice. Their findings provide the first quantitative measurements $_{ec}$NOS mRNA by using real-time PCR in the vessels of mice and suggested age- and

sex-related differences in the basal levels of $_{ec}$NOS mRNA in mice (Chu *et al* 2002). In addition, the $_{ec}$NOS region that was used for real-time PCR was amplified and sequenced for monkeys and other species. With modifications, this region may be used to design real-time PCR for $_{ec}$NOS in other species. With regard to low protein feeding, the present study failed to provide data on the potential alterations of the expression of mRNA of two key enzymes of vasorelaxation: $_{ec}$NOS regulating the synthesis of the most important vasodilator NO, and its target enzyme in smooth muscle cells, sGC.

Low protein-fed pregnant animals employed in the longitudinal study 1, in Chapter 2, exhibited a significant decrease in twenty-four-hour urinary excretion rate of NO_x at gestational day 18 (Fig. 2.7). Those findings point to a decrease in NO production in the low protein-fed animals. The observed decrease in NO production could be accompanied by and due to the downregulation of constitutive NOS or inducible NOS in low protein-fed rats. There are few published studies on the effect of dietary protein on constitutive and lipopolysaccharide (LPS)-induced NO production. Wu *et al*, (1999) reported that dietary protein deficiency reduced urinary nitrate excretion before and after lipopolysaccharide (LPS) treatment in young growing 30 days old male rats. The rats employed in the present study were female and were significantly older than those used in the study by Wu *et al*, (1999) (100 *vs*. 30 days of age). Inducible NO synthesis in young rats treated with lipopolysaccharide was also lower in rats fed 5% casein compared with rats fed 20% casein (Wu *et al*, 1999). Feeding a 5% casein diet to young 30-day old male rats resulted in 52% decrease in whole body constitutive NO production compared with rats fed a 20% casein diet (Wu *et al*, 1999). Serum concentrations of arginine were 20% lower in rats fed the 5% casein diet compared with rats fed the 20% casein diet (Wu *et al*, 1999). Their results suggested that dietary protein deficiency impaired constitutive NO synthesis and could not maintain maximal inducible NO synthesis by young rats in response to LPS treatment. The reduced $_{ec}$NOS protein in the thoracic aortae of the low protein-fed pregnant rats found in this study agrees with previous investigations which report that

dietary protein deficiency decreased cNOS activity in brain, heart, jejunum, lung, skeletal muscle and spleen of the rat (Wu *et al*, 1999). Wu *et al*, (1998) reported that maternal dietary protein deficiency decreased constitutive nitric oxide synthase activity in placenta and endometrium of pigs during early gestation. It is likely that the lack of all essential amino acids impaired the expression of enzymes involved in the synthesis of NOS protein in rats fed the low protein diet.

Another potential mechanism that could explain the impaired biological activity of the NO pathway observed in protein-deficient rats is L-arginine (Arg) deficiency. As a substrate for $_{ec}$NOS, Arg is essential for maintaining the enzyme in the active dimerization state (Harrison, 1997). As a precursor for the syntheses of NO and protein, Arg plays vital roles in nutrition and metabolism (Wu and Morris, 1998). On the basis of growth or nitrogen balance, Arg is classified as an essential amino acid for young mammals because endogenous Arg synthesis cannot meet arginine needs for optimal growth and health (Visek, 1986). In young mammals, dietary arginine deficiency results in a decrease in plasma arginine concentrations. Feeding 0.0 or 0.3% Arg diet to 30-day old male rats resulted in marked growth retardation and decreased serum Arg concentrations compared with feeding 1% Arg diet. In addition, decreasing dietary Arg concentrations from 1% to 0% caused progressive decreases in constitutive and inducible NO synthesis in young rats (Wu *et al*, 1999). *In vitro* studies have demonstrated that increasing extracellular arginine concentrations increased NO synthesis by $_{ec}$NOS in endothelial cells (Arnal *et al*, 1995) and by iNOS in activated macrophages (Norris *et al*, 1995) in a concentration-dependent manner. Wu *et al*, (1999) found that although Arg serum levels were similar in rats fed the 5% casein diet and the 0.3% Arg diet, urinary excretion of nitrate by rats fed the 5% casein diet was only ~45% of that by rats fed the 0.3% Arg diet. It can be suggested that in protein-deficient rats factors other than decreased plasma Arg availability (e.g., decreased cNOS activity) contribute to the impaired constitutive NO synthesis.

In adult nonpregnant rats Arg is regarded as a non-essential amino acid. Rees *et al* (1999) found that feeding 9% or 6% casein diet to pregnant rats resulted in no

differences in serum concentrations of arginine on day 19 of gestation compared with feeding the 18% casein diet. Analysis of the free amino acids in the maternal serum showed that on day 19 the diets containing 9% and 6% casein led to threonine concentrations that were reduced to 46 and 20% of those found in animals fed on the control (18% casein) diet. The other essential amino acids were unchanged, except for a small decrease in the branched-chain amino acids in animals fed on the 6% casein diet (Rees *et al*, 1999). Thoracic aorta was chosen for measurement of $_{ec}$NOS protein mass in the present study primarily because its large size would provide sufficient endothelial tissue to accommodate the requirements for this assay. However, as a conduit artery, the aorta tone is not highly influenced by the vasoactive agents. Nonetheless, the downregulation of $_{ec}$NOS in the aortae from pregnant low protein-fed animals may reflect similar changes in the smaller resistance vessels. $_{ec}$NOS and sGC protein expression in mesenteric arteries was not investigated.

In the virgin rats the most striking effect of the low protein diet was the reduction in the vasodilator responsiveness to both ACh and SPN in mesenteric arteries suggesting that the blunted responsiveness to ACh probably reflects the combined effects of a decrease in both the NO producing ($_{ec}$NOS) and effector (sGC) enzymes. The decrease in ACh-induced relaxation observed in small arteries from low protein-fed virgin rats did not seem to be due to an alteration of $_{ec}$NOS expression because no significant differences in the levels of $_{ec}$NOS in aortae of virgin rats maintained on either 9% or 18% casein for 18-19 days were found (Fig. 6.6). The unaltered $_{ec}$NOS protein expression in aortae of protein-deficient virgin rats found in this study conflicts with previous investigations which report that impaired NO synthesis in protein-deficient subjects is due to decreases in tissue cNOS expression (Wu *et al*, 1999). Mechanisms which may possibly contribute to the dilator dysfunction in virgin low protein-fed rats include an increased inactivation of NO as a result of an enhanced O_2^- production, or an alteration in the NO-mediated activation of sGC and subsequent cGMP formation in vascular smooth muscle cells.

Impaired maximal acetylcholine-induced, NO-dependent relaxation has previously been reported in aortic rings from rats with chronic heart failure (Bauersachs *et al*, 2002). The mechanism underlying the reduction of agonist-stimulated dilator responses in heart failure has been proposed to be a defective production of endothelium-derived NO. Reduced vascular $_{ec}$NOS expression has been reported in pacing-induced chronic heart failure in dogs and in monocrotaline-induced pulmonary hypertension and chronic heart failure in rats (Comini *et al*, 1996, Smith *et al*, 1996). In contrast, other studies have reported an increase in vascular $_{ec}$NOS in aortic rings of rats with chronic heart failure (Bauersachs *et al*, 1999). In the latter study, dilator responses, the expression of protein and mRNA of the $_{ec}$NOS, iNOS, sGC, and superoxide anion (O_2^-) and peroxynitrite production were determined in aortic rings from Wistar rats 8 weeks after myocardial infarction and compared with those in sham-operated animals. In rats with heart failure, the concentration-response curve of the endothelium-dependent vasodilator acetylcholine (after preconstriction with phenylephrine) was significantly shifted to the right, and the maximum relaxation was attenuated. Determination of expression levels of the two key enzymes for NO-mediated dilations, $_{ec}$NOS and sGC, revealed a marked upregulation of both enzymes in aortas from rats with heart failure, whereas iNOS expression was not changed. Pretreatment with exogenous superoxide dismutase partially restored the acetylcholine-induced relaxation in aortas from rats with heart failure. Aortic basal and NADH-stimulated O_2^- production assessed by use of lucigenin-enhanced chemiluminescence was significantly elevated in rats with chronic myocardial infarction. Peroxynitrite-mediated nitration of protein tyrosine residues was not different between the 2 groups of rats. These results demonstrated that endothelial dysfunction in ischemic heart failure occurs despite an enhanced vascular eNOS and sGC expression and can be attributed to an increase in vascular O_2^- production by an NADH-dependent oxidase. By inactivation of NO, O_2^- production appears to be an essential mechanism for the endothelial dysfunction observed in heart failure.

Impaired endothelium-dependent NO-mediated vasodilatation has been

reported both in humans and in animal models of hypertension (Ignarro *et al*, 1999), diabetes mellitus (Pieper, 1998) and hypercholesterolemia and decreased bioavailability of NO is thought to be involved in these defects. The influence of hypertension on vascular NOS expression is still controversial. An increase in vascular NOS protein expression has been reported in the aorta of hypertensive animals (Vaziri *et al*, 1998, Kerr *et al*, 1999) whereas Chou *et al*, (1998) have found a reduction in both $_{ec}$NOS expression and activity in aorta of spontaneously hypertensive rats. Briones *et al*, (2000) have reported impaired ACh-induced relaxation in mesenteric arteries of hypertensive rats which did not seem to be due to an alteration of $_{ec}$NOS isoform, because no change in basal cNOS activity or in $_{ec}$NOS expression was observed in their study in mesenteric arteries of hypertensive rats, as was also reported in aorta of spontaneously hypertensive rats (Bauersachs *et al*, 1998). It has been suggested that the endothelial dysfunction observed in hypertension is due to an increase in the endothelial O_2^- production (Kerr *et al*, 1999) which scavenges NO and hence reduces its bioavailability. This may explain the findings that in spontaneously hypertensive rats, a reduction of bioactive NO might occur despite an increased NO generation (Bouloumie *et al*, 1997). However, impairment of NO-dependent vasodilator function may occur either at the level of or downstream from sGC. With regard to low protein diet, no data are available on O_2^- production within the vascular wall or on the potential alterations of the expression of smooth muscle sGC. Endothelial function also appears to decline with advancing age. A decrease of sGC expression at the mRNA and protein level, has been demonstrated in the aortae of old normotensive and old spontaneously hypertensive rats compared with their young counterparts (Klöß *et al*, 2000), which was consistent with the observed reduction of the dilator response to sodium nitroprusside. Furthermore, in the same study the expression of sGC α_1 and β_1 mRNA (detected by reverse transcriptase-polymerase chain reaction) in the aorta from old spontaneously hypertensive rats was found to be 2.5-fold (α_1) and 4.3-fold (β_1) lower than in aortae from old normotensive rats. An attenuation of aortic sGC expression has been recognized as a potential mechanism of reduced dilator response

in aged spontaneously hypertensive rats (Bauersachs *et al*, 1998).

In summary, $_{ec}$NOS protein expression was reduced in the aorta from pregnant low protein-fed rats. With regard to low protein feeding, the present study failed to provide data on the potential alterations of the expression of mRNA of two key enzymes of vasorelaxation: $_{ec}$NOS regulating the synthesis of NO, and its target enzyme in smooth muscle cells, sGC. Further work is needed to clarify whether the reduction in vasodilator potency by dietary protein deficiency was due to changes in $_{ec}$NOS and sGC mRNA levels in the resistance vasculature. The unaltered $_{ec}$NOS protein expression in aortae of protein-deficient virgin rats and the observed attenuation of the ACh- and SPN-induced relaxations in mesenteric arteries of protein-deficient virgin rats suggest an impairment of NO-dependent vasodilator function either at the level of or downstream from sGC. Future studies are necessary to investigate the influence of dietary protein deficiency on sGC expression and to clarify the underlying mechanisms.

CHAPTER 7
FINAL DISCUSSION AND SUMMARY.

7.1 Effect of dietary restriction on the maternal cardiovascular adaptation to pregnancy.

During pregnancy, in order to meet the increasing demands for nutrients by the rapidly growing fetus, a number of maternal cardiovascular adaptations take place. These adaptations include major increases in cardiac output and plasma volume and decreased vascular resistance originating during the first trimester of human pregnancy (Phippard *et al*, 1986; Robson *et al*, 1989). In the rat, blood volume and cardiac output are increased by day 18 of gestation (Gilson *et al*, 1992). Blood volume increase results from enhanced sodium appetite and thirst, augmenting renal sodium and water retention. Increased heart rate and contractility, coupled with increased preload, result in the increase in cardiac output. To accommodate this increase in the cardiac output without an elevation in blood pressure, total peripheral vascular resistance to blood flow must be decreased.

Plasma volume expansion is important for normal fetal growth. Fetal growth retardation is observed in several conditions where plasma volume does not increase normally during gestation: women with pregnancy-induced hypertension (Cabrera and Bohr, 1995), pre-eclampsia (Sibai *et al*, 1983), as well as undernourished women (Smith, 1947) have a lower plasma volume expansion associated with a reduced cardiac output and increased vascular resistance. In the rat, also, it has been shown that fetal weight is reduced when plasma volume is reduced by feeding pregnant rats 50% of their normal daily food intake or a low protein diet (6% casein) throughout gestation (Lederman and Rosso, 1989).

Reduced expansion of plasma volume may exert a detrimental effect on fetal growth by interfering with the normal increase in uteroplacental blood flow. Such an association has been shown in malnourished dams (Rosso and Kava, 1980;

Ahokas *et al*, 1983a; Ahokas *et al*, 1986), where plasma volume and fetal weight are both decreased and the uteroplacental blood flow, the supply line of nutrients available for placental transfer to the fetus, is reduced by 40-50%, thus reducing the supply of nutrients to the fetus and causing the proportional fetal growth restriction. Uterine and preplacental vascular resistance have been shown to be 63% and 55% higher in the 50% diet-restricted dams than in *ad libitum*-fed (Ahokas *et al*, 1986). Myometrial blood flow in the 50% diet-restricted rats is lower than that in *ad libitum*-fed (Ahokas *et al*, 1986). The lower uteroplacental blood flow rate is suggested to be the result of a decreased cardiac output, and a decreased fraction of the cardiac output delivered to the uterus (Ahokas *et al*, 1983a).

7.2. NO and the maternal cardiovascular adaptation to pregnancy.

Data available in the literature strongly suggest that the endothelial cell-derived vasodilator NO, plays a central role in cardiovascular adaptation in pregnancy. Plasma levels and the daily urinary excretion of nitrate, the stable oxidation metabolite of NO, and cyclic guanosine monophosphate (cGMP), a cellular mediator of vascular smooth muscle relaxation are significantly increased in pregnant rats (Conrad *et al*, 1993) (see *Evidence that NO-NOS are increased in rat pregnancy*, Chapter 1, section 1.5.2.2) and pregnant women (Boccardo *et al*, 1996), suggesting that total NO biosynthesis is increased. The increase in urinary excretion of cGMP occurs at the time when adaptation of the maternal cardiovascular system is taking place (Conrad *et al*, 1993; Nathan *et al*, 1995). In rats, inhibition of NO synthesis reduces the normal expansion of maternal plasma volume (Molnár *et al*, 1994; Salas *et al*, 1995). Several studies provide convincing evidence showing that NOS expression and activity increase during pregnancy (see *Nitric oxide synthases during pregnancy*, section 1.5.2.2.2, Chapter 1). Goetz *et al* (1994), reported a twofold increase in mRNA transcripts for $_{ec}$NOS in the aorta of pregnant rats. Uterine artery, but not systemic artery endothelial, $_{ec}$NOS protein (Magness *et al*, 1997; Magness *et al*, 2001; Nelson *et al*, 2000;) and mRNA (Bird *et al*, 2000; Magness *et al*, 2001) are elevated by pregnancy. The mechanisms

responsible for the increased NO production during pregnancy are essentially unknown. Particular attention has been paid to the role of oestradiol and progesterone, concentrations of which increase significantly in early pregnancy. It has been suggested that physiological levels of 17β-oestradiol, increase NO release from endothelial cells, resulting in reduced myogenic tone in small arteries from nonpregnant female rats, as compared to male rats (Wellman *et al*, 1996). Oestrogens have been shown to increase endothelium-dependent vasodilator responses to both ACh (Vedernicov *et al*, 1997) and to flow (Cockell and Poston, 1997). This increase in NO release may involve oestrogen-mediated increase in the expression of NOS (Weiner *et al*, 1994). In pregnant guinea pigs, five days of oestrogen treatment increases calcium-sensitive NOS activity and $_{ec}$NOS mRNA expression in skeletal muscle (Weiner *et al,* 1994), thus supporting the idea that the increase in NOS activity is under oestrogen influence. Besides their influence on the NO system, oestrogens stimulate the activity of prostacyclin (PGI_2) (Mikkola *et al*, 1995).

Although it is recognized that changes in NO production may play a role in some parts of the maternal circulation, there is considerable evidence suggesting additional NO-independent mechanisms (Ylikorkala *et al*, 1986). Other endothelium-derived relaxing factors such as PGI_2 and endothelium-derived hyperpolarizing factor may contribute to the vascular adaptation during pregnancy (Gerber *et al*, 1998).

7.3. Influence of a low protein diet on maternal vascular reactivity

Because an increase in NO production may play a role in the maternal cardiovascular adaptation to pregnancy, one may therefore speculate that a reduction in the production of NO, and thereby reduction of the NO-cGMP vascular relaxation pathway, could significantly affect maternal vascular physiology and thus fetal growth. NO is synthesized from L-Arginine (see *L-Arginine, the substrate for generation of NO*, section 1.8, Chapter 1). Although L-Arginine can be synthesized by most mammals, it is classified as a nutritionally essential amino acid for young mammals and for adults at times of stress and illness (Visek *et al*, 1986). Increasing the bioavailability

of L-arginine can influence NO synthesis and increase NO-mediated events: dietary supplementation with Arg restores the biological activity of vascular NO in cholesterol-fed rabbit aorta (Cooke *et al*, 1991). In humans with heart failure, intravenous infusion of Arg increases the cardiac output (Koifman *et al*, 1995), increases maximum vasodilatation (Kanaya *et al*, 1999) and reduces peripheral resistance after reactive hyperaemia (Hirooka *et al*, 1994). Oral Arg administration enhances endothelium-dependent vasodilatation in patients with heart failure, (Hambrecht *et al*, 2000). Similarly, a restriction in the provision of precursors of NO in rat gestation adversely affected NO-mediated events: pregnant rats fed 50% of their normal daily food intake from day five of gestation had a marked reduction (50%) in cardiac output, reduced placental blood flow and smaller fetuses and placentas, compared with rats fed *ad libitum* a standard diet (Rosso and Kava, 1980).

Physiological levels of NO produced by the endothelial cells are essential for regulating the relaxation of VSMCs. A suboptimal dietary supply of protein may regulate the function of the vascular endothelium through alterations in NO synthesis. In this way, the interaction between a low protein diet and the physiologic effects in the mesenteric artery during pregnancy may constitute a mechanism through which maternal nutrition can influence vascular changes that occur in the maternal circulation during pregnancy.

The studies described in the present thesis have concentrated upon assessment of vascular function in small mesenteric artery preparations from virgin female rats and pregnant rats (gestation day 18-19) fed either 18% or 9% casein. The set-up with wire-mounted vessels in the myograph chamber enabled certain 'isolated' mechanisms (ie. agonist-receptor interaction) to be studied. The major conclusion reached from the studies described in the present thesis is that ACh-mediated dilatation was impaired in resistance arteries from pregnant and virgin rats fed a diet of low protein concentration (Chapters 4 and 5). In the virgin rats, endothelium-independent mechanisms are also involved, as the vasodilator potency of the NO donor SPN was significantly reduced in

vessels of virgin 9% casein-fed rats (Fig. 4.4) and to a greater extent than the upstream endothelial-mediated mechanisms. This additional defect could provide evidence of decreased responsiveness of vascular smooth muscle and/or augmented clearance of NO, and shows that dietary protein restriction causes distinct abnormalities in the endothelium and vascular smooth muscle. Alternatively, one may speculate that the blunted responsiveness to ACh in virgin low protein-fed rats probably shows the combined effects of a decrease in both the NO producing ($_{ec}$NOS) and effector (soluble guanylyl cyclase) enzymes. Pilot data show unaltered $_{ec}$NOS protein expression in aortae of protein-deficient virgin rats (Chapter 6), thereby suggesting that the observed attenuation of the ACh- and SPN-induced relaxations in mesenteric arteries of protein-deficient virgin rats may be due to an impairment of NO-dependent vasodilator function either at the level of or downstream from sGC.

The ACh-induced relaxations in the mesenteric arteries from both virgin and pregnant rats, were only partially blocked by a combined application of indomethacin and L-NAME (Figs. 4.7 and 5.10). Thus, elimination of the synthesis or action of NO and prostanoids only partially suppressed endothelium-dependent dilatation in the resistance vasculature confirming that a factor other than NO or a prostanoid is also involved in the relaxation. One of the factors that can induce relaxation of the mesenteric artery in response to muscarinic agonist ACh and other vasoactive substances is EDHF (Garland and McPherson, 1992; Gerber *et al*, 1998).

The response to another relaxing agent, bradykinin, was also tested in arteries from pregnant rats on the low protein diet to see whether the endothelial defect is faithfully reproduced when responses to alternative endothelium agonists are investigated. Relaxation induced by the endothelium-dependent vasodilator bradykinin was also impaired, thereby suggesting that the abnormality described in the low protein fed pregnant rats in response to dietary protein restriction is not specific to ACh but represents a global defect in relaxation to another endothelium dependent vasodilator (Chapter 5).

The impaired endothelium-dependent relaxation pathway in 9% casein-fed pregnant rats observed in the present study, could be related to possible abnormalities in the production and/or activity of endothelium-derived relaxing factors such as NO, PGI_2, and EDHF. When the EDHF-mediated component of vasorelaxation was unveiled in the absence of NO and prostacyclin, the maximum relaxant response to ACh was greater but not significantly in 18% casein-fed compared with 9% casein-fed rats and the response to the lower, and likely more physiologically relevant, concentrations of ACh was reduced in arteries from pregnant rats on the 9% casein diet (Fig 5.11), suggesting that the defect in response to ACh was due partly to a reduction in the component attributable to EDHF and that NO synthesis may increase in a compensatory manner (Chapter 5). Earlier studies have shown that the endothelium-dependent hyperpolarization to ACh was diminished in mesenteric arteries of spontaneously hypertensive rats compared with Wistar Kyoto rats and the decreased hyperpolarization accounted, at least in part, for the impaired relaxation to ACh. (Fujii *et al*, 1992). Further studies are necessary to elucidate the influence of a low protein diet on EDHF-mediated hyperpolarization and relaxation in mesenteric arteries of pregnant rats.

A pilot study showed that $_{ec}$NOS protein expression was reduced in the aorta from pregnant low protein-fed rats [Fig. 6.5(A), Chapter 6]. Data on urinary nitrite/nitrate excretion in pregnant rats on the low protein diet were inconclusive, showing no alterations in excretion from days 1 to 13, and a decrease on day 18 compared with 18% casein-fed pregnant rats (Chapter 2). But recent data show that the ACh-induced NO levels in mesenteric arteries are reduced in pregnant dams fed low protein (9% casein) compared with 18% casein-fed pregnant dams (Brawley *et al*, 2002a). Assessment of whole body NO production by measurement of urinary nitrite/nitrate excretion may not be reflective of the NO available to produce vascular relaxation. Also, possible tissue-specific differences in the expression of NOS isoforms may explain the dissociation between whole body nitrite/nitrate production and ACh-induced nitrite/nitrate production.

The vascular studies therefore suggested that a low protein diet may interfere with the normal enhancement of endothelium dependent relaxation in pregnancy; in turn, this may contribute to failure to expand the extracellular volume (Lederman and Rosso, 1989), leading in turn to fetal growth restriction a common observation during pregnancy in rat dams fed a low protein diet (Langley and Jackson, 1994; Langley-Evans *et al*, 1996). Indeed, in a previous study it has been found that plasma volume was reduced in pregnant rats that were fed a 9% casein diet (Welham *et al*, 1998).

7.4 Mechanisms of impaired fetal growth in protein-deficient dams

Maternal nutrition is one of the many factors which help to regulate fetal growth, and contribute to the resulting birth weight. A diet containing 9% casein, which has been used in the present thesis, is still able to maintain pregnancy but results in offspring of low birth weight, compared with a diet containing the optimum of 18% casein. Rosso and Cramoy (1979), reviewed a number of studies which examined the effects of dietary restriction on the weight of the mother and her offspring at delivery. In the rat, protein deprivation throughout pregnancy results in net loss of maternal body weight, smaller and thinner placentas, reduced uterine tissue weight, and smaller fetuses (Hastings-Roberts and Zeman, 1977).

The mechanism responsible for restricted fetal growth in protein-deficient dams remains largely unknown. Plasma insulin-like growth factor levels are lower in the fetuses of protein restricted (5% protein) rat dams (Muaku *et al*, 1995). NO synthesis is an important regulator of uteroplacental blood flow (Thaete *et al*, 1997). Arginine deficiency could lead to a decrease in the production of nitric oxide, which may affect blood flow to the placenta, and therefore the delivery of oxygen and nutrients to the fetus. However, studies by Rees *et al* (1999), showed that dietary protein deficiency (9% casein) had no effect on maternal serum or fetal free amino acid pool concentrations of most amino acids including L-arginine. L-Threonine was the only essential amino acid that decreased significantly both in the maternal serum and fetal

body of protein-deficient rats (Rees *et al,* 1999). This may be due either to an augmented drive to produce glycine, or an increased demand to metabolize methionine, which makes up a higher proportion of the amino acid supply in the low protein diet (Rees *et al,* 1999). Rees *et al* (1999) suggest that an excess of methionine in the diet may influence the early overgrowth of the fetuses exposed to a maternal low protein diet, as it is metabolized to homocysteine, which leads to increased growth when added to embryo cultures in vitro (VanAerts *et al,* 1994). Rees *et al* (1999) concluded that the changes in threonine metabolism may be linked to the abnormal fetal growth. Protein malnutrition also reduced the size of the placenta (Malandro *et al,* 1996), a critical organ for normal growth and development of the fetus (Reynolds and Redmer, 1995). This is consistent with data obtained in the present thesis (Chapter 5). NO plays an important role in controlling angiogenesis, placental-fetal blood flow and therefore nutrient supply from maternal to fetal blood during pregnancy (Sooranna *et al,* 1995). Both constitutive NOS (cNOS, Ca^{2+}-dependent) and inducible NOS (iNOS, Ca^{2+}-independent) have been identified in the placenta (Sladek *et al,* 1997). Wu *et al* (1998), showed that maternal dietary protein restriction decreased arginine and ornithine concentrations, constitutive and inducible NOS activities and NO production in placenta and endometrium of pigs during early pregnancy, thereby contributing to retarded placental and fetal development.

7.5 Fetal programming: effects of maternal dietary manipulation on vascular function of the offspring.

The concept of fetal programming is that of permanent alterations of fetal metabolism brought by the intrauterine environment in which the fetus develops. Evidence now suggests that the intrauterine environment modifies not just the immediate intrauterine wellbeing of the fetus (ie. fetal growth) but also its long term susceptibility to disease as adult. Programming is thought to arise from suboptimal supply of nutrients and oxygen by the mother at times of rapid cell division during intrauterine development, so-called 'critical' periods of fetal growth

(Widdowson and McCance, 1975). The timing of these critical periods varies for different tissues. The fetus adapts to nutritional insults by slowing cell division (especially in those tissues undergoing critical periods at the time) and changing the distribution of cell types, as well as by altering hormonal feedback, metabolic activity and organ structure. In humans, the data supporting the idea has largely come from epidemiological studies which have shown that poor growth during prenatal life predisposes a person to the later development of type-2 diabetes mellitus, hypertension and stroke in adulthood (Hales and Barker, 1992).

Over the last decade confirmation of this hypothesis has been obtained from studies in the offspring of rats who were fed a low protein diet (9% casein) similar to the diet used for the studies in the present thesis. It was found that the offspring became hypertensive during their adult lives (Langley and Jackson, 1994).

In a study by Holemans *et al* (1999), fetal growth restriction was induced by a 50 % reduction of normal dietary intake in pregnant rats during the second half of pregnancy. Small mesenteric arteries from the offspring of these animals showed reduced endothelium-dependent relaxation (to ACh and bradykinin), but enhanced sensitivity to exogenous NO (sodium nitroprusside). No changes in the blood pressure were found. Ozaki *et al* (2001), investigated the effects of 70% global undernutrition throughout gestation in the rat on the blood pressure of male and female offspring, and on the development of systemic vascular function. The offspring were growth restricted at birth, and systolic and/or diastolic and mean arterial blood pressures, were elevated from 60 days onward in male offspring and from 100 days onward in female offspring. In male adult restricted offspring (200 days), maximal vasoconstriction to the thromboxane A2 mimetic, U46619 was enhanced (Ozaki *et al*, 2001). Ozaki *et al* (2001), concluded that maternal undernutrition in rat gestation adversely affected cardiovascular function in the offspring and that these abnormalities increased with age and were more pronounced in males.

Ghosh *et al* (2001), found that the endothelium-dependent relaxation

induced by ACh was blunted in isolated branches of the femoral artery from 160-day-old female offspring of dams fed a saturated fat diet (20 % fat) when compared with female offspring of dams fed a breeding diet (4 % fat). These offspring exhibited elevated plasma triglyceride and reduced plasma high density lipoprotein cholesterol concentrations (Ghosh *et al*, 2001). The fatty acid composition of the aortas was abnormal, with a marked reduction in the content of arachidonic and docosahexaenoic acids. The study by Ghosh *et al* (2001) showed that a high fat diet in pregnant rats produced abnormal vascular function, plasma lipid disturbances and altered vascular fatty acid content in their female offspring during adulthood.

It is not possible to investigate vascular function in the fetal rat, however this can be achieved in the larger fetal lamb. Ozaki *et al* (2000), investigated vasoconstrictive and vasodilatory responses of isolated small arteries from the femoral vascular bed of fetal sheep and from late-gestation pregnant ewes. Ewes were fed either 100% of the nutritional requirement throughout pregnancy or a restricted diet of 85% or 50% of the nutritional requirement for the first 70 days of pregnancy. For the remainder of pregnancy all ewes were fed the complete diet. Endothelium-dependent vasorelaxation responses to ACh and bradykinin were well developed in fetuses at 0.6 and 0.9 gestation and were similar to those in the ewes. In fetuses at 0.9 gestation the 50% nutritional restriction of the ewe led to blunted endothelium-dependent vasodilatation in response to ACh and blunted endothelium-independent vasodilatation in response to sodium nitroprusside (Ozaki *et al*, 2000). Responses in the fetuses at 0.9 gestation, in which the ewes were fed a restricted diet of 85%, were normal. The study by Ozaki and coworkers (2000) showed that the blunted responses to ACh and sodium nitroprusside in the fetuses at 0.9 gestation among the group of dietarily restricted ewes (restricted diet of 50% group) were indicative of impaired vascular smooth muscle sensitivity to NO and that this defect may contribute to the development of hypertension in later life. Protein restriction (9% casein) in pregnancy has been shown to impair endothelium-dependent and -independent relaxation in small arteries of rat male offspring (Brawley *et al*, 2002b).

7.6. Further ways forward

Further work needs to be done to elucidate the precise mechanisms by which a low protein diet produces vascular dysfunction. This should clarify whether the reduction in vasodilator potency by dietary protein deficiency was due to changes in $_{ec}$NOS and sGC mRNA levels in the resistance vasculature. Also, future studies are necessary to investigate the influence of dietary protein deficiency on sGC protein expression and to clarify the underlying mechanisms. Whether the amounts of NOS isoforms are altered in the blood vessels of low protein-fed rats compared with 18% casein-fed, should represent important areas for future investigation. As arginine plays a crucial role in NO production by all isoforms of NOS, there is also a need to fully understand the regulation of arginine metabolism (synthesis and catabolism) at molecular, cellular and whole body levels (Morris, 2002) and to explain the 'arginine paradox' for NO generation (see *The L-arginine paradox*, section 1.8.2 Chapter 1).

Such studies using this experimental model may also provide insights into the mechanisms behind programming.

7.7. Summary

- Assessment of whole body NO production by measurement of the urinary excretion of nitrite and nitrate in pregnant rats fed either 18% or 9% casein, showed no alterations in excretion from days 1 to 13, and a decrease on day 18 in pregnant rats on the 9% casein diet compared with 18% casein-fed (Chapter 2), although overall the data were inconclusive. These findings suggested that whole body nitrite/nitrate production may not be reflective of the NO available to produce vascular relaxation.

- To overcome the shortcomings of investigating NO production through measurements of urinary NOx, the direct effects of a 9% casein diet on the mesenteric vascular function were studied in arteries from virgin and pregnant rats. The responses of the vessels to vasoactive agents, the effects of which are known to be mediated by, or are independent of NO, were investigated using small vessel myography.

- **VIRGIN RATS:** In mesenteric resistance arteries from virgin low protein-fed rats, the endothelium-dependent vascular relaxation to ACh and the response to exogenous NO donor SPN were significantly reduced.

- **PREGNANT RATS:** In arteries from pregnant low protein-fed rats, the ACh- and bradykinin endothelium-dependent vasodilatation was significantly reduced.

- A pilot study showed that $_{ec}$NOS protein expression was reduced in the aorta from pregnant low protein-fed rats (Chapter 6).

- Explanations for these findings are discussed within each chapter and in the concluding discussion (Chapter 7).

BIBLIOGRAPHY

Aalkjaer, C., and Mulvany, M. (1981). Functional and morphological properties of human omental resistance vessels. Blood Vessels *18*, 233-244.

Abraham, W. T., Schrier, R. W. (1997). Cardiac failure, liver disease and nephrotic syndrome. In Diseases of the kidney, R. W. G. Schrier, C., ed. (Boston: Little, Brown and Co.), pp. 2353-2392.

Adams, M. R., McCredie, R., Jessup, W., Robinson, J., Sullivan, D., and Celermajer, D. S. (1997). Oral L-arginine improves endothelium-dependent dilatation and reduces monocyte adhesion to endothelial cells in young men with coronary artery disease. Atherosclerosis *129*, 261-269.

Aellig, W. H. (1981). A new technique for recording compliance of human hand veins. Br. J. Clin. Pharmacol. *11*, 237-43.

Ahokas, R. A., Reynolds, S. L., Anderson, G. D., and Lipshitz, J. (1984). Maternal organ distribution of cardiac output in the diet-restricted pregnant rat. J. Nutr. *114*, 2262-2268.

Ahokas, R. A., Anderson, G. D., and Lipshitz, J. (1983a). Cardiac-output and uteroplacental blood-flow in diet-restricted and diet-repleted pregnant rats. American Journal Of Obstetrics and Gynecology *146*, 6-13.

Ahokas, R. A., Anderson, G. D., and Lipshitz, J. (1983b). Effect of dietary restriction, during the last week only or throughout gestation, on cardiac-output and uteroplacental blood-flow in pregnant rats. Journal Of Nutrition *113*, 1766-1776.

Ahokas, R. A., Friedman, S. A., and Sibai, B. M. (1997). Effect of indomethacin and N-omega-nitro-L-arginine methyl ester on the pressure/flow relation in isolated perfused hindlimbs from pregnant and nonpregnant rats. Journal Of the Society For Gynecologic Investigation *4*, 229-235.

Ahokas, R. A., Reynolds, S. L., Anderson, G. D., and Lipshitz, J. (1986). Catecholamine-mediated reduction in uteroplacental blood-flow in the diet-restricted, term-pregnant rat. Journal Of Nutrition *116*, 412-418.

Ahokas, R. A., and Sibai, B. M. (1992). Endothelium-derived relaxing factor inhibition augments vascular angiotensin-II reactivity in the pregnant rat hind-limb. American Journal Of Obstetrics and Gynecology *167*, 1053-1058.

Alderton, W. K., Cooper, C.E., Knowles, R.G. (2001). Nitric oxide synthases: structure, function, and inhibition. Biochem. J. *357*, 593-615.

Alexander, B. T., Miller, M.T., Kassab, S., Novak, J., Reckelhoff, J.F., Kruckeberg, W.C., Granger, J.P. (1999). Differential expression of renal nitric oxide synthase isoforms during pregnancy in rats. Hypertension *33 [part II]*, 435-439.

Ammann, P., Bourrin, S., Bonjour, J. P., Meyer, J. M., Rizzoli, R. (2000). Protein undernutrition-induced bone loss is associated with decreased IGF-I levels and estrogen deficiency. J. Bone Miner. Res. *15*, 683-690.

Anderson, G. D., Ahokas, R. A., Lipshitz, J., and Dilts, P. V. Jr. (1980). Effect of maternal dietary restriction during pregnancy on maternal weight gain and fetal birth weight in the rat. J. Nutr. *110*, 883-890.

Andersson, D.A., Zygmunt, P.M., Movahed, P., Andersson, T.L., Hogestatt, E.D. (2000). Effects of inhibitors of small- and intermediate-conductance calcium-activated potassium channels, inwardly-rectifying potassium channels and Na(+)/K(+) ATPase on EDHF relaxations in the rat hepatic artery. Br. J. Pharmacol. *129*, 1490-1496.

Arnal, J. F., Munzel, T., Venema, R. C., James, N. L., Bai, C. L., Mitch, W. E., Harrison, D. G. (1995). Interactions between l-arginine and l-glutamine change endothelial no production - an effect independent of no synthase substrate availability. Journal Of Clinical Investigation *95*, 2565-2572.

Arnal, J. F., Yamin, J., Dockery, S., and Harrison, D. G. (1994). Regulation of endothelial nitric-oxide synthase messenger-rna, protein, and activity during cell-growth. American Journal Of Physiology-Cell Physiology *36*, C1381-C1388.

Ashworth, J. R., Warren, A. Y., Baker, P. N., Johnson, I. R. (1996). A comparison of endothelium-dependent relaxation in omental and myometrial resistance arteries in pregnant and nonpregnant women. Am. J. Obstet. Gynecol. *175*, 1307-12.

Ashworth, J. R., Warren, A. Y., Baker, P. N., Johnson, I. R. (1997). Loss of endothelium-dependent relaxation in myometrial resistance arteries in pre-eclampsia. Br J Obstet Gynaecol. *104*, 1152-8.

Assali, N. S. (1989). Dynamics of the uteroplacental circulation in health and disease. Am. J. Perinatol. *6*, 105-9.

Atherton, J. (1983). Glomerular filtration rate and salt and water reabsorption during pregnancy in the conscious rat. J. Physiol *334*, 493-504.

Atherton, J. C., Dark, J. M., Garland, H. O., Morgan, M. R. A., Pidgeon, J., Soni, S. (1982). Changes in water and electrolyte balance, plasma volume and composition during pregnancy in the rat. J. Physiol.(London) *330*, 81-93.

Atherton, J. C., and Pirie, S. C. (1981). The effect of pregnancy on glomerular filtration rate and salt and water reabsorption in the rat. J. Physiol. *319*, 153-164.

Auch-Schwelk, W., Katusic, Z. S., Vanhoutte, P. M. (1990). Thromboxane A2 receptor antagonists inhibit endothelium-dependent contraction. Hypertension *15*, 699-703.

Ballejo, G., Barbosa, T. A., Coelho, E. B., Antoniali, C., Salgado, M.C.O. (2002). Pregnancy-associated increase in rat systemic arteries endothelial nitric oxide production diminishes vasoconstrictor but does not enhance vasodilator responses. Life Sciences *70*, 3131-3142.

Barbagallo, M., Dominguez, L. J., Licata, G., Shan, J., Bing, L., Karpinski, E., Pang, P. K. T., Resnick, L. M. (2001). Vascular effects of progesterone. Role of cellular calcium regulation. Hypertension *37*, 142-147.

Barbul, A. (1986). Arginine: biochemistry, physiology, and therapeutic implications. J. Parenter. Enter. Nutr. *10*, 227-38.

Barker, D. J. P., Osmond, C., Golding, J., Kuh, D., Wadsworth, M.E.J. (1989). Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. BMJ *298*, 564-567.

Barker, D. J. P. (1994). The undernourished baby. In Mothers, Babies, and Health in Later Life, B. D. J. P., ed. (London: BMJ Publishing group), pp. 121-139.

Baron, A., Frieden, M., Beny, J.L. (1997). Epoxyeicosatrienoic acids activate a high-conductance, Ca(2+)-dependent K + channel on pig coronary artery endothelial cells. J. Physiol. *504*, 537-543.

Barron, W. M., Stamoutsos, B. A., Lindheimer, M. D. (1984). Role of blood volume in the regulation of vasopressin secretion during pregnancy in the rat. J. Clin. Invest. *73*, 923-932.

Barrow, S. E., Blair, I. A., Waddell, K. A., Shepherd, G. L., Lewis, P. J., and Dollery, C. T. (1983). Prostacyclin in late pregnancy: analysis of 6-oxo-PGF1alpha in maternal plasma. In Prostacyclin in Pregnancy, P. J. Lewis, Moncada, S., and O'Grady, J., ed. (New York: Raven Press), pp. 79-85.

Bauersachs, J., Heck, M., Fraccarollo, D., Hildemann, S. K., Ertl, G., Wehling, M., Christ, M. (2002). Addition of Spironolactone to angiotensin-converting enzyme inhibition in heart failure improves endothelial vasomotor dysfunction. Role of vascular superoxide anion formation and endothelial nitric oxide synthase expression. J. Am. Coll. Cardiol. *39*, 351-358.

Bauersachs, J., Bouloumie, A., Fraccarollo, D., Hu, K., Busse, R., and Ertl, G. (1999). Endothelial dysfunction in chronic myocardial infarction despite increased vascular endothelial nitric oxide synthase and soluble guanylate cyclase expression - Role of enhanced vascular superoxide production. Circulation *100*, 292-298.

Bauersachs, J., Bouloumie, A., Mulsch, A., Wiemer, G., Fleming, I., and Busse, R. (1998). Vasodilator dysfunction in aged spontaneously hypertensive rats: changes in NO synthase III and soluble guanylyl cyclase expression, and in superoxide anion production. Cardiovascular Research *37*, 772-779.

Baylis, C. (1982). Glomerular ultrafiltration in the pseudopregnant rat. American Journal Of Physiology *243*, F300-F305.

Baylis, C. (1980). The mechanism of the increase in glomerular filtration rate in the twelve-day pregnant rat. J. Physiol. *305*, 405-414.

Baylis, C., Samsell, L. and Deng A. (1992). A new model of systemic hypertension with high glomerular capillary blood pressure (P_{GC}) and proteinuria: Chronic blockade of endogenous EDRF (abstract). J. Am. Soc. Nephrol. *2*, 471.

Baylis, C., and Engels, K. (1992). Adverse interactions between pregnancy and a new model of systemic hypertension produced by chronic blockade of endothelial derived relaxing factor (edrf) in the rat. Clinical and Experimental Hypertension Part B-Hypertension In Pregnancy *11*, 117-129.

Baylis, C., Harton, P., and Engels, K. (1990). Endothelial derived relaxing factor controls renal hemodynamics in the normal rat-kidney. Journal Of the American Society Of Nephrology *1*, 875-881.

Beierwaltes, W. H. (1995). Selective neuronal nitric oxide synthase inhibition blocks furosemide-stimulated renin secretion in vivo. Am. J. Physiol. *269*, F134-F139.

Bell, C. (1973). Estrogen-induced sensitization of the uterine artery of the guinea pig to ACh. Br. J. Pharmacol. *49*, 595-601.

Bellamy, M. F., McDowell, I. F. W., Ramsey, M. W., Brownlee, M., Bones, C., Newcombe, R. G., and Lewis, M. J. (1998). Hyperhomocysteinemia after an oral methionine load acutely impairs endothelial function in healthy adults. Circulation *98*, 1848-1852.

Belo, L., Caslake, M., Gaffney, D., Santos-Silva, A., Pereira-Leite, L., Quintanilha, A., Rebelo, I. (2002). Changes in LDL size and HDL concentration in normal and preeclamptic pregnancies. Atherosclerosis *162*, 425-432.

Bergstrom, J., Ahlberg, M., and Alvestrand, A. (1985). Influence of protein intake on renal hemodynamics and plasma hormone concentrations in normal subjects. Acta. Med. Scand. *217*, 189.

Berridge, M. (1997). Elementary and global aspects of calcium signalling. J. Physiol. *499*, 291-306.

Bevan, J. A., and Osher J.V. (1972). A direct method for recording tension changes in the wall of small blood vessels in vitro. Agents Actions *2*, 257-260.

Bird, I. M., Sullivan, J. A., Di, T., Cale, J. M., Zhang, L., Zheng, J., and Magness, R. R. (2000). Pregnancy-dependent changes in cell signalling underlie changes in differential control of vasodilator production in uterine artery endothelial cells. Endocrinology *141*, 1107-1117.

Boccardo, P., Soregaroli, M., Aiello, S., Noris, M., Donadelli, R., Lojacono, A., Benigni, A. (1996). Systemic and fetal-maternal nitric oxide synthesis in normal pregnancy and pre-eclampsia. Br. J. Obstet. Gynaecol. *103*, 879-886.

Bode-Böger, S. M., Böger, R. H., Kienke, S., Junker, W., and Frölich, J. C. (1996). Elevated L-arginine/dimethylarginine ratio contributes to enhanced systemic NO production by dietary L-arginine in hypercholesterolemic rabbits. Biochem. Biophys. Res. Commun. *219*, 598-603.

Böger, R. H., Bode-Böger, S. M., Gerecke, U., Gutzki, F. M., Tsikas, D., and Frölich, J. C. (1996a). Urinary NO_3^- excretion as an indicator of nitric oxide formation *in vivo* during oral administration of L-Arginine or L-NAME in rats.
Cl. Exp. Pharmacol. Physiol. *23*, 11-15.

Böger, R. H., Bode-Böger, S. M., Szuba, A., Tsao, P. S., Chan, J. R., Tangphao, O., Blaschke, T. F., Cooke, J. P. (1998). Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. Circulation *98*, 1842-1847.

Böger, R. H., Lentz, S. R., Bode-Böger, S. M., Knapp, H. R., and Haynes, W. G. (2001). Elevation of asymmetrical dimethylarginine may mediate endothelial dysfunction during experimental hyperhomocyst(e)inaemia in humans. Clinical Science *100*, 161-167.

Böger, R. H., Sydow, K., Borlak, J., Thum, T., Lenzen, H., Schubert, B., Tsikas, D., Bode-Böger, S. M. (2000). LDL cholesterol upregulates synthesis of asymmetrical dimethylarginine in human endothelial cells. Involvement of S-adenosylmethionine-dependent methyltransferases. Circ. Res. *87*, 99-105.

Bolz, S.S., Fisslthaler, B., Pieperhoff, S., De Wit, C., Fleming, I., Busse, R., Pohl, U. (2000). Antisense oligonucleotides against cytochrome P450 2C8 attenuate EDHF-mediated Ca^{2+} changes and dilation in isolated resistance arteries. FASEB J. *14*, 255-260.

Borell, U., Fernstrom, I., Westman, H. (1953). Hormonal influences in the uterine arteries: an arteriographic study in the human. Acta Obstet. Gynecol. Scand. *32*, 271-284.

Bouloumie, A., Bauersachs, J., Linz, W., Scholkens, B. A., Wiemer, G., Fleming, I., Busse, R. (1997). Endothelial dysfunction coincides with an enhanced nitric oxide synthase expression and superoxide anion production. Hypertension *30*, 934-941.

Brawley, L., Torrens, C., Itoh, S., Barker, A., Poston, L., Clough, G. F., Hanson, M. A. (2002a). Dietary protein restriction attenuates ACh-induced nitric oxide levels in small mesenteric arteries from pregnant rat dams. FASEB JOURNAL *16*, A104.

Brawley, L., Itoh,S., Torrens, C., Barker, A.C., Poston, L., Hanson, M.A. (2002b). Protein restriction in pregnancy impairs endothelium-dependent and -independent relaxation in small arteries of rat male offspring. Journal of Physiology-London *539*, 123P-123P.

Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991). Cloned and expressed nitric-oxide synthase structurally resembles cytochrome-p-450 reductase. Nature *351*, 714-718.

Bredt, D. S., and Snyder, S. H. (1994). Nitric oxide: a physiologic messenger molecule. Annu. Rev. Biochem. *63*, 175-195.

Brink, P. R., Valiunas, V., and Christ, G. R. (2000). Homotypic, heterotypic and heteromeric gap junction channels. Curr. Top. Membr. *49*, 131-142.

Briones, A. M., Alonso, M. J., Marin, J., Balfagon, G., and Salaices, M. (2000). Influence of hypertension on nitric oxide synthase expression and vascular effects of lipopolysaccharide in rat mesenteric arteries. British Journal of Pharmacology *131*, 185-194.

Brown, M. A., Tibben, V. C., Zammit, G. M., Cario, G. M. and Carlton, M. A. (1995). NO excretion in normal and hypertensive pregnancies. Hypertens. Preg. *14*, 319-326.

Bruce, N. W. (1976). The distribution of blood flow to the reproductive organs of rats near term. J. Reprod. Fertil. *46*, 35-.

Buga, G. M., Singh, R., Pervin, S., Rogers, N. E., Schmitz, D. A., Jenkison, C. P., Cederbaum, S. D., and Ignarro, L. J. (1996). Arginase activity in endothelial cells: inhibition by N^G-hydroxy-L-arginine during high output NO production. Am. J. Physiol. *271*, H1988-H1998.

Buhimischi, I., Ali, M., Jain, V. (1996). Differential regulation of nitric oxide in the rat uterus and cervix during pregnancy and labour. Hum. Reprod. *11*, 1755-1766.

Busse, R., Edwards, G., Feletou, M., Fleming, I., Vanhoutte, P. M., and Weston, A. H. (2002). EDHF: bringing the concepts together. Trends Pharmac. Sci. *23*, 374-380.

Cabrera, C., and Bohr, D. (1995). The role of nitric oxide in the central control of blood pressure. Biochem. Biophys. Res. Commun. *206*, 77-81.

Cadnapaphornchai, M. A., Ohara, M., Morris, K. G., Jr.,, Knotek, M., Rogachev, B., Ladtkow, T., Carter, E. P., and Schrier, R. W. (2001). Chronic NOS inhibition reverses systemic vasodilation and glomerular hyperfiltration in pregnancy. Am. J. Physiol Renal Physiol *280*, F592-F598.

Calver, A., Collier, J., Moncada, S., Vallance, P. (1992). Effect of local intra-arterial NG-monomethyl-L-arginine in patients with hypertension: the nitric oxide dilator mechanism appears abnormal. J. Hypertension *10*, 1025-1031.

Campbell, W. B., Halushka, P. V. (1996). Eicosanoids and platelet-activating factor. In The Pharmacologic basis of Therapeutics, J. G. Hardman, Limbird, L. E., Molinoff, P. M., Ruddon, R. W., Gilman, A. G., ed. (New York: McGraw-Hill), pp. 601.

Chapman, A. B., Abraham, W. T., Zamudio, S., Coffin, C., Merouani, A., Young, D., Johnson, A., Osorio, F., Goldberg, C., Moore, L.G., Dahms, T., and Schrier, R. W. (1998). Temporal relationships between hormonal and hemodynamic changes in early human pregnancy. Kidney Int. *54*, 2056-2063.

Chaytor, A. T., Evans, W. H., and Griffith, T. M. (1998). Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries. J. Physiol. *508*, 561-573.

Chaytor, A. T., Taylor, H. J., Griffith, T. M. (2002). Gap junction-dependent and -independent EDHF-type relaxations may involve smooth muscle cAMP accumulation. Am. J. Physiol. *282*, H1548-H1555.

Chen, P. Y., and Sanders, P. W. (1991). L-Arginine abrogates salt-sensitive hypertension in Dahl/Rapp rats. J. Clin. Invest. *88*, 1559-1567.

Chen, G., and Suzuki, H. (1989). Some electrical properties of the endothelium-dependent hyperpolarization recorded from rat arterial smooth muscle cells. J. Physiol. (Lond). *410*, 91-106.

Choi, J. W., Im, M. W., Pai, S. H. (2003). Nitric oxide production increases during normal pregnancy and decreases in preeclampsia. Ann. Clin. Lab. Sci. *32*, 257-263.

Christ, G. J. and Barr, L. (2000). The neuronal control of smooth muscle. In A Functional View of Smooth Muscle Control (Barr, L. and Christ, G. J., eds), pp. 345-396, JAI Press, Greenwich, CT.

Christensen, K. L., and Mulvany, M. J. (1993). Mesenteric arcade arterioles contribute substantially to vascular resistance in conscious rats. J. Vasc. Res. *30*, 73-79.

Chu, Y., Heistad, D.D., Knudtson, K. L., Lamping, K.G., Faraci, F. M. (2002). Quantification of mRNA for endothelial NO synthase in mouse blood vessels by real-time polymerase chain reaction. Arterioscler. Thromb. Vasc. Biol. *22*, 611-616.

Chu, Z. M., and Beilin, L. J. (1993). Mechanisms of vasodilatation in pregnancy: studies of the role of prostaglandins and nitric oxide in changes of vascular reactivity in the in situ blood perfused mesentery of pregnant rats. Br. J. Pharmacol. *109*, 322-329.

Clapp, J. F., Seaward, B. L., Sleamaker, R. H., Hiser, J. (1988). Maternal physiologic adaptations to early human pregnancy. Am. J. Obstet. Gynaecol. *159*, 1456-1460.

Clapp, J. F. I., and Capeless, E. (1997). Cardiovascular function before, during, and after the first and subsequent pregnancies. Am. J. Cardiol. *80*, 1469-1473.

Clarke, H. E., Coates, M. E., Eva, J. K., and al, e. (1978). Dietary Standards for laboratory animals: Report of the Laboratory Animals Centre Diets Advisory Committe. Laboratory Animals *11*, 1-28.

Cockell, A. P., and Poston, L. (1997). Flow-mediated vasodilatation is enhanced in normal pregnancy but reduced in preeclampsia. Hypertension *30*, 247-251.

Cockell, A. P. and Poston, L. (1997a). 17Beta-estradiol stimulates flow-induced vasodilatation in isolated small mesenteric arteries from prepubertal female rats. Am. J. Obstet. Gynecol. *177*, 1432-8.

Comini, L., Bachetti, T., Gaia, G. et al. (1996). Aorta and skeletal muscle NO synthase expression in experimental heart failure. J. Mol. Cell Cardiol. *28*, 2241-2248.

Conrad, K. P., and Colpoys, M. C. (1986). Evidence against the hypothesis that prostaglandins are the vasodepressor agents of pregnancy. J. Clin. Invest. *77*, 236-245.

Conrad, K. P., and Vernier, K. A. (1989). Plasma level, urinary excretion, and metabolic production of cGMP during gestation in rats. Am. J. Physiol. *257*, R847-R853.

Conrad, K. P. (1984). Renal hemodynamics during pregnancy in chronically catheterised, conscious rats. Kidney Int. *26*, 24-29.

Conrad, K. P., and and Lindheimer, M. D. (1999). Renal and cardiovascular alterations. In Chesley's Hypertensive Disorders in Pregnancy, R. J. Lindheimer M.D., and Cunningham F.G., Stamford, C.T., ed.: Appleton and Lange), pp. 263-326.

Conrad, K. P., Kerchner, L. J. and Mosher, M. D. (1999a). Plasma and 24-h NOx and cGMP during normal pregnancy and preeclampsia in women on a reduced NOx diet. Am. J. Physiol. *277*, F48-F57.

Conrad, K. P., Joffe, G. M., Kruszyna, H., Kruszyna, R., Rochelle, L. G., Smith, R. P., Chavez, J. E., and Mosher, M. D. (1993). Identification of increased nitric-oxide biosynthesis during pregnancy in rats. Faseb Journal *7*, 566-571.

Cooke, J. P., Andon, N. A., Girerd, X. J., Hirsch, A. T., and Creager, M. A. (1991). Arginine restores cholinergic relaxation of hypercholesterolemic rabbit thoracic aorta. Circulation *83*, 1057-1062.

Cooke, J. P., Rossitch, E., Anton, N. A., Loscalzo, J., and Dzau, V. J. (1991). Flow activates an endothelial potassium channel to release an endogenous nitrovasodilator. Journal of Clinical Investigation *88*, 1663-71.

Crandall, M. E., Keve, T. M., and McLaughlin, M. K. (1990). Characterization of norepinephrine sensitivity in the maternal splanchic circulation during pregnancy. Am. J. Obstet. Gynecol. *162*, 1296-1301.

Creager, M. A., Gallagher, S. J., Girerd, X. J., Coleman, S. M., Dzau, V. J., and Cooke, J. P. (1992). L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. J. Clin. Invest. *90*, 1248-1253.

Dalle Lucca, J. J., Adeagbo, A. S. O., and Alsip, N. L. (2000). Influence of oestrous cycle and pregnancy on the reactivity of the rat mesenteric vascular bed. Human Reproduction *15*, 961-968.

D'Angelo, G., and Osol, G. (1993). Regional variation in resistance artery diameter responses to alpha-adrenergic stimulation during pregnancy. Am. J. Physiol. *264*, H78-H85.

Danielson, L. A., and Conrad, K. P. (1995). Acute blockade of nitric-oxide synthase inhibits renal vasodilation and hyperfiltration during pregnancy in chronically instrumented conscious rats. Journal Of Clinical Investigation *96*, 482-490.

Davidge, S. T., Baker, P. N., McLaughlin, M. K., Roberts, J. M. (1995). Nitric oxide produced by endothelial cells increases production of eicosanoids through activation of prostaglandin H synthase. Circ. Res. *77*, 274-283.

Davidge, S. T., and McLaughlin, M. K. (1992). Endogenous modulation of the blunted adrenergic response in resistance-sized mesenteric-arteries from the pregnant rat. American Journal Of Obstetrics and Gynecology *167*, 1691-1698.

Davison, J. M., and Dunlop, W. (1980). Renal haemodynamics and tubular function in normal human pregnancy. Kidney Int. *18*, 152-161.

Davison, J. M., and Lindheimer, M. (1980). Changes in renal haemodynamics and kidney weight during pregnancy in the anaesthetised rat. J. Physiol. *301*, 129-136.

De Nucci, G., Gryglewski, R. J., Warner, T. D., and Vane, J. R. (1988). Receptor-mediated release of EDRF and prostacyclin from bovine aortic endothelial cells is coupled. Proc. Natl. Acad. Sci. USA *85*, 2334-2338.

De Weerd, W. F., and Leeb-Lundberg, L. M. (1997). Bradykinin sequesters B2 bradykinin receptors and the receptor-coupled Galpha subunits Gαq and Gαi in caveolae in DDT1 MF-2 smooth muscle cells. J. Biol. Chem. *272*, 17858-17866.

Deng, A. H., Engels, K., and Baylis, C. (1996). Impact of nitric oxide deficiency on blood pressure and glomerular hemodynamic adaptations to pregnancy in the rat. Kidney International *50*, 1132-1138.

Desai, M., Crowther, N. J., Lucas, A., and Hales, C. N. (1996). Organ-selective growth in the offspring of protein-restricted mothers. Br. J. of Nutr. *76*, 591-603.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A. M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature *399*, 601-605.

Don, B. R., Blake, S., Hutchison, F. N., Kaysen, G. A., Schambelan, M. (1989). Dietary protein intake modulates glomerular eicosanoid production in the rat. Am. J. Physiology Renal FLuid Electrolyte Physiol. *256*, F711.

Dora, K. A. and Garland, C. J. (2001). Properties of smooth muscle hyperpolarization and relaxation to K^+ in the rat isolated mesenteric artery. Am J Physiol (Heart Circ Physiol). *280*, H2424-9.

Dora, K. A., Martin, P. E. M., Chaytor, A. T., Evans, H., Garland, C. J., and Griffith, T. M. (1999). Role of heterocellular gap junctional communication in endothelium-dependent smooth muscle hyperpolarization: inhibition by a connexin-mimetic peptide. Biochem. Biophys. Res. Commun. *254*, 27-31.

Dora, K. A., Sandow, S. L., Gallagher, N. T., Takano, H., Rummery, N. M., Hill, C. E., Garland, C. J. (2003). Myoendothelial Gap Junctions May Provide the Pathway for EDHF in Mouse Mesenteric Artery. J. Vasc. Res. [published online ahead of print].

Doughty, J. M., Boyle, J. P., Langton, P. D. (2000). Potassium does not mimic EDHF in rat mesenteric arteries. Br. J. Pharmacol. *130*, 1174-1182.

Drexler, H., and Hornig, B. (1999). Endothelial dysfunction in human disease. J. Mol. Cell. Cardiol. *31*, 51-60.

Durante, W., Schafer, A. I. (1998). Carbon monoxide and vascular cell function. Int. J. Mol. Med. *2*, 255-262.

Duvekot, J. J., Cheriex, E. C., Pieters, F. A. A., Menheere, P. C. A., and Peeters, L. L. H. (1993). Early pregnancy changes in hemodynamic and volume homeostasis are consecutive adjustments triggered by a primary fall in systemic vascular tone. Am. J. Obstet. Gynecol. *169*, 1382-1392.

Duvekot, J. J., and Peeters, L. L. (1994). Renal haemodynamics and volume homeostasis in pregnancy. Obstet. Gynecol. Surv. *49*, 830-839.

Edwards, G., Dora, K. A., Gardener, M. J., Garland, C. J., and Weston, A. H. (1998). K+ is an EDHF in rat arteries. Nature *396*, 269-272.

Edwards, G., Gardener, M.J., Feletou, M., Brady, G., Vanhoutte, P.M., Weston, A.H. (1999). Further investigation of endothelium-derived hyperpolarizing factor (EDHF) in rat hepatic artery: studies using 1-EBIO and ouabain. Br J Pharmacol. *128*, 1064-70.

Edwards, G., Feletou, M., Gardener, M. J., and Thollon, C. (1999a). Role of gap junctions in the responses to EDHF in rat and guinea-pig small arteries. Br. J. Pharmacol. *128*, 1788-1794.

Edwards, G. and Weston, A. H. (2001). EDHF -- are there gaps in the pathway? J. Physiol. (London) *531*, 299.

El Sayed, A. A., Haylor, J., and El Nahas, A. M. (1991). Mediators of the direct effects of amino acids on the rat kidney. Clinical Science *81*, 427.

Espiner, E. A., Richards, A. M., Yandle, T. G., Nicholls, M. G. (1995). Natriuretic hormones. Endocrinol. Metab. Clin. North Am. *24*, 481-509.

Felder, C. C. (1995). Muscarinic acetylcholine receptors: signal transduction through multiple effectors. FASEB J. *9*, 619-625.

Feron, O., Smith, T. W., Michel, T., and Kelly, R. A. (1997). Dynamic targeting of the agonist-stimulated m2 muscarinic acetylcholine receptor to caveolae in cardiac myocytes. J. Biol. Chem. *272*, 17744-17748.

Fine, L. (1986). The biology of renal hypertrophy. Kidney International *29*, 619.

Fisslthaler, B., Popp, R., Kiss, L., Potente, M., Harder, D. R., Fleming, I., and Busse, R. (1999). Cytochrome P450 2C is an EDHF synthase in coronary arteries. Nature *401*, 493-497.

Ford, G. A., Robson, S. C., Mahdy, Z. A. (1996). Superficial hand vein responses to NG-monomethyl-L-arginine in post-partum and non-pregnant women. Clin. Sci. *90*, 493-7.

Förstermann, U., Hertting, G., and Neufang, B. (1986). The role of endothelial and non-endothelial prostaglandins in the relaxation of isolated blood vessels of the rabbit induced by acetylcholine and bradykinin. Br. J. Pharmacol. *87*, 521-532.

Fujii, K., Tominaga, M., Ohmori, S., Kobayashi, K., Koga, T., Takata, Y., and Fujishima, M. (1992). Decreased endothelium-dependent hyperpolarization to acetylcholine in smooth muscle of the mesenteric artery of spontaneously hypertensive rats. Circulation Research *70*, 660-669.

Fulep, E. E., Vedernikov, Y. P., Saade, G. R., and Garfield, R. E. (2001). The role of endothelium-derived hyperpolarizing factor in the regulation of the uterine circulation in pregnant rats. Am. J. Obstet. Gynecol. *185*, 638-642.

Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature *399*, 597-601.

Furchgott, R. F., and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by ACh. Nature *288*, 373-376.

Gabbai, F. B., Blantz, R.C. (1999). Role of Nitric Oxide in Renal Hemodynamics. Seminars in Nephrology *19*, 242-250.

Gandley, R. E., Conrad, K. P., and and McLaughlin, M. K. (2001). Endothelin and nitric oxide mediate reduced myogenic reactivity of small renal arteries from pregnant rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. *280*, R1-R7.

Gant, N. F., Whalley, PJ., Everett, R. B., Worley, R. J., and MacDonald, P.C. (1987). Control of vascular reactivity in pregnancy. Am. J. Kidney Dis. *9*, 303-307.

Garg, U. C., and Hassid, A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. J. Clin. Invest. *83*, 1774-1777.

Garland, C. J., Plane, F., Kemp, B. K., Cocks, T. M. (1995). Endothelium-dependent hyperpolarization: A role in the control of vascular tone. Trends Pharmacol. Sci. *16*, 23-30.

Garland, C. J., and McPherson, G. A. (1992). Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. British Journal of Pharmacology *105*, 429-435.

Garland, H. O., Atherton, J. C., Baylis, C., Morgan, M. R. A., and Milne, C. M. (1987). Hormone profiles for progesterone, estradiol, prolactin, plasma-renin activity, aldosterone and corticosterone during pregnancy and pseudopregnancy in 2 strains of rat - correlation with renal studies. J. Of Endocrinology *113*, 435-444.

Garland, H. O., and Green, R. (1982). Micropuncture study of changes in glomerular filtration and ion and water handling by the rat kidney during pregnancy. J. Physiol. *329*, 389-409.

Gary, J. D., and Clarke, S. (1998). RNA and protein interactions modulated by protein arginine methylation. Prog. Nucleic Acid Res. Mol. Biol. *61*, 65-131.

Gebremedhin, D., Harder, D.R., Pratt, P.F., Campbell, W.B. (1998). Bioassay of an endothelium-derived hyperpolarizing factor from bovine coronary arteries: role of a cytochrome P450 metabolite. J. Vasc. Res. *35*, 274-284.

Gerber, R. T., Holemans, K., ObrienCoker, I., Mallet, A. I., VanBree, R., VanAssche, F. A., Poston, L. (1999). Cholesterol-independent endothelial dysfunction in virgin and pregnant rats fed a diet high in saturated fat. Journal Of Physiology-London *517*, 607-616.

Gerber, R. T., Anwar, M. A., and Poston, L. (1998). Enhanced ACh-induced relaxation in small mesenteric arteries from pregnant rats: an important role for endothelium-derived hyperpolarizing factor (EDHF). Br. J. Pharmacol. *125*, 455-460.

Ghosh, P., Bitsanis, D., Ghebremeskel, K., Crawford, M., and Poston, L. (2001). Abnormal aortic fatty acid composition and small artery function in offspring of rats fed a high fat diet in pregnancy. Journal of Physiology-London *533*, 815-822.

Gillham, J. C., Kenny, L. C., Baker, P. N. (2003). An overview of EDHF in normal and compromised pregnancies. Eur. J. Obstet. Gynec. Reprod. Biol. *109*, 2-7.

Gilliam, M. B., Sherman, M. P., Griscavage, J. M., Ignarro, L. J. (1993). A spectrophotometric assay for nitrate using NADPH oxidation by Aspergillus nitrate reductase. Anal. Biochem. *212*, 359-365.

Gilson, G. C., Mosher, M. D., and Conrad, K. P. (1992). Systemic haemodynamics and oxygen transport during pregnancy in chronically instrumented rats. Am. J. Physiol. *263*, H1911-H1918.

Gladwell, S. J. and Jefferys, J. G. (2001). Second messenger modulation of electrotonic coupling between region CA3 pyramidal cell axons in the rat hippocampus. Neurosci. Lett. *300*, 1-4.

Godfrey, K. M., Barker, D. J. P., Robinson, S., and Osmond, C. (1997). Maternal birthweight and diet in pregnancy in relation to the infant's thinness at birth. British Journal Of Obstetrics and Gynaecology *104*, 663-667.

Goetz, R. M., Morano, I., Calovini, T., Studer, R., and Holtz, J. (1994). Increased expression of endothelial constitutive nitric oxide synthase in rat aorta during pregnancy. Biochem. Biophys. Res. Commun. *205*, 905-910.

Gonzalez, C. G., Garcia, F. D., Fernandez, S. F., and Patterson, A. M. (1997). Role of 17-β-estradiol and progesterone on glucose homeostasis: Effects of food restriction (50%) in pregnant and nonpregnant rats. J. Endocri. Invest. *20*, 397-403.

Govers, R., and Rabelink, T. J. (2001). Cellular regulation of endothelial nitric oxide synthase. Am. J. of Physiol. *280*, F193-F206.

Graf, K., Grafe, M., Auch-Schwelk, W., Baumgarten, C.R., Scheffer, H., Hildebrandt, A., and Fleck, E. (1994). Tissue kallikrein activity and kinin release in human endothelial cells. Eur. J. Clin. Chem. Clin. Biochem. *32*, 495-500.

Gratton, J. P., Fontana, J., O'Connor, D. S., Garcia-Gardena, G., McCabe, T. J., and Sessa, W. C. (2000). Reconstitution of an endothelial NO synthase, hsp90 and caveolin-1 complex in vitro: evidence that hsp90 facilitates calmodulin stimulated displacement of eNOS from caveolin-1. J. Biol. Chem. *275*, 22268-22272.

Green, L. C., Tannenbaum, S. R., Goldman, P. (1981a). Nitrate synthesis in the germfree and conventional rat. Science *212*, 56-58.

Green, L. C., Ruiz de Luzuriaga, K., Wagner, D. A., Rand, W., Istfan, N., Young, V. R., Tannenbaum, S. R. (1981b). Nitrate biosynthesis in man. Proc. Natl. Acad. Sci. USA *78*, 7764-7768.

Griffith, T. M. and Taylor, H. J. (1999). Cyclic AMP mediates EDHF-type relaxations of rabbit jugular vein. Biochem Biophys Res Commun. *263*, 52-57.

Griffith, T.M., Chaytor, A.T., Taylor, H.J., Giddings, B.D., Edwards, D.H. (2002). cAMP facilitates EDHF-type relaxations in conduit arteries by enhancing electrotonic conduction via gap junctions. Proc. Natl. Acad. Sci. *99* :6392-7.

Gross, K. L., Hartman, W. J., Ronnenberg, A., and Prior, R. L. (1991). Arginine-deficient diets alter plasma and tissue amino acids in young and aged rats. J. Nutr. *121*, 1591-1599.

Guyton, A. C., Hall, J. E. (2000). The Body Fluid Compartments: Extracellular and intracellular Fluids; Interstitial Fluid and Edema. In Textbook of Medical Physiology, W. Schmitt, Gruliow, R., ed. (New York: W.B. Saunders Company), pp. 264-277.

Hales, C. N., Desai, M., Ozanne, S.E., Crowther, N.J. (1996). Fishing in the stream of diabetes:from measuring insulin to the control of fetal organogenesis. Biochemical Society Transactions *24*, 341-350.

Hales, C. N., and Barker, D. J. P. (1992). Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia *35*, 595-602.

Halliwell, B. (1997). What nitrates tyrosine? is nitrotyrosine specific as a biomarker of peroxynitrite formation in vivo? FEBS Lett. *411*, 157-160.

Hambrecht, R., Hilbrich, L., Erbs, S., Gielen, S., Fiehn, E., Schoene, N., and Schiler, G. (2000). Correction of endothelial dysfunction in heart failure: additional effects of exercise training and oral L-arginine supplementation. J. Am. Coll. Cardiol. *35*, 706-713.

Hamet, P., Pang, S. C., Tremblay, J. (1989). Atrial natriuretic factor-induced egression of cyclic guanosine 3':5'-monophosphate in cultured vascular smooth muscle and endothelial cells. J. Biol. Chem. *264*, 12364-12369.

Harding, H. R., Rosen, F., and Nichol, C. A. (1961). Am. J. Physiol. *201*, 271.

Harding, J. E., and Johnston, B.M. (1995). Nutrition and fetal growth. Reprod. Fertil. Dev. *7*, 539-547.

Harris, D., Martin, P. E. M., Evans, H. W., Kendall, D. A., Griffith, T. M., Randall, M. D. (2000). Role of gap junctions in endothelium-derived hyperpolarizing factor responses and mechanisms of K^+ relaxation. Eur. J. Pharmacol. *402*, 119-28.

Harris, M. B., Ju, H., Venema, V. J., Liang, H., Zou, R., Michell, B.J., Chen, Z. P., Kemp, B. E., and Venema, R. C. (2001). Reciprocal phosphorylation and regulation of endothelial nitric-oxide synthase in response to bradykinin stimulation. J. Biol. Chem. *276*, 16587-16591.

Harrison, D. G. (1997). Cellular and molecular mechanisms of endothelial cell dysfunction. J. Clin. Invest. *100*, 2153-2157.

Hart, J. L., Freas, W., and Muldoon, S. M. (1986). Neurovascular function in rat during pregnancy. Am. J. Physiol. *251*, H1000-H1008.

Haynes, M. P., Sinha, D., Russell, K. S., Collinge, M., Fulton, D., Morales-Ruiz, M., Sessa, W. C., and Bender, J. R. (2000). Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. Circ. Res. *87*, 677-682.

Hecker, M., Sessa, W. C., Harris, H. J., Anggard, E. E., Vane, J. R. (1990). The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. Proc. Natl. Acad. Sci. (USA) *87*, 8612-16.

Hecker, M., Bara, A. T., Bauersachs, J., and Busse, R. (1994). Characterization of endothelium-derived hyperpolarizing factor as a cytochrome p450-derived arachidonic-acid metabolite in mammals. Journal Of Physiology-London *481*, 407-414.

Hecker, M., Mulsch, A., Bassenge, E., and Busse, R. (1993). Vasoconstriction and increased flow - 2 principal mechanisms of shear stress-dependent endothelial autacoid release. American Journal Of Physiology *265*, H828-H833.

Henning, B., Toborek, M., and Cader, A. A. (1994). Nutrition, endothelial cell metabolism, and atherosclerosis. Critical Reviews in Food Science and Nutrition *34*, 253-282.

Hermsteiner, M., Zoltan, D. R., Künzel, W. (2001). The vasoconstrictor response of uterine and mesenteric resistance arteries is differentially altered in the course of pregnancy. European Journal of Obstetrics and Gynaecology and Reproductive Biology *100*, 29-35.

Hirooka, Y., Imaizumi, T., Tagawa, T., Shiramoto, M., Endo, T., Ando, S., Takeshita, A. (1994). Effects of L-arginine on impaired acetylcholine-induced and ischemic vasodilatation of the forearm in patients with heart failure. Circulation *90*, 658-668.

Hirschberg, R., and Kopple, J.D. (1987). Role of growth hormone in the amino acid-induced acute rise in renal function in man. Kidney Int. *32*, 382.

Hirschberg, R. R., Zipser, R. D., Slomowitz, L. A., Kopple, J. D. (1988). Glucagon and prostaglandins are mediators of amino-acid induced rise in renal haemodynamics. Kidney Int. *33*, 1147-55.

Hirst, G. D., Edwards, F. R., Gould, D. J., Sandow, S. L., Hill, C. E. (1997). Electrical properties of iridial arterioles of the rat. Am. J. Physiol. *273*, H2465-H2472.

Hokanson, D. E., Sumner, D. S., and Strandness D. E. Jr (1975). An electrically calibrated plethysmograph for direct measurement of limb blood flow. IEEE Trans. Biomed. Eng. *22*, 25-9.

Holemans, K., Gerber, R., Meurrens, K., DeClerck, F., Poston, L., and VanAssche, F. A. (1999). Maternal food restriction in the second half of pregnancy affects vascular function but not blood pressure of rat female offspring. British Journal Of Nutrition *81*, 73-79.

Hostetter, T. H. (1986). Human renal response to meat meal. Am. J. Physiol. *250*, F613-F618.

Huang, P. L., Huang, Z., Mashimo, H., Bloch, K. D., Moskowitz, M. A., Bevan, J. A., and Fishman, M. C. (1995). Hypertension in mice lacking the gene for endothelial nitric oxide synthase. Nature *377*, 239-242.

Hull, S. S. J., Kaiser, L., Jaffe, M. D., and Sparks, H. V. Jr. (1986). Endothelium-dependent flow induced dilation of canine femoral and saphenous arteries. Blood vessels *23*, 183-198.

Hwa, J. J., Ghibaudi, L., Williams, P., and Chatterjee, M. (1994). Comparison of acetylcholine-dependent relaxation in large and small arteries of rat mesenteric vascular bed. Am. J. Physiol. *266*, H952-H958.

Hytten, F. E., and Paintin D. B. (1963). Increase in plasma volume during normal pregnancy. J. Obstet. Gynaecol. Br. Commonw. *70*, 402-407.

Ignarro, L. J., Cirino, G., Casinin, A., and Napoli, C. (1999). Nitric oxide as a signalling molecule in the vascular system: an overview. J. Cardiovasc. Pharmacol. *34*, 879-886.

Imig, J.D., Falck, J.R., Wei, S., Capdevila, J.H. (2001). Epoxygenase metabolites contribute to nitric oxide-independent afferent arteriolar vasodilation in response to bradykinin. J. Vasc. Res. *38*, 247-255.

Inoue, N., Venema, R. C., Sayegh, H. S., Ohara, Y., Murphy, T. J., and Harrison, D. G. (1995). Molecular regulation of the bovine endothelial cell NO synthase by transforming growth factor-β 1. Arterioscler. Thromb. Vasc. Biol. *15*, 1255-1261.

Ito, S., Juncos, L. A., Nushiro, N., Johnson, C. S., Carretero, O. A. (1991). Endothelium-derived relaxing factor modulates endothelin action in afferent arterioles. Hypertension *17*, 1052-6.

Ito, S., Carretero, O. A., and Abe, K. (1996). Nitric oxide in the juxtaglomerular apparatus. Kidney International *49*, S-6-S-8.

Itoh, H., Bird, I. M., Nakao, K., and Magness, R.R. (1998). Pregnancy increases soluble and particulate guanylate cyclases and decreases the clearance receptor of natriuretic peptides in ovine uterine, but not systemic arteries. Endocrinology *138*, 3329-3341.

Itoh, S., Brawley, L., Wheeler, T., Anthony, F., Poston, L., and Hanson, M. A. (2002). Vasodilatation to vascular endothelial growth factor in the uterine artery of the pregnant rat is blunted by low dietary protein intake. Pediatric Research *51*, 485-491.

Johnson, R. A., and Freeman, R. H. (1994). Renin release in rats during blockade of nitric oxide synthesis. Am. J. Physiol. *266*, R1723-R1729.

Joyce, J. M., Phernetton, T. M., Shaw, C. E., Modrick, M. L., and Magness, R. R. (2002). Endothelial vasodilator production by uterine and systemic arteries. IX. eNOS gradients in cycling and pregnant ewes. Am. J. Physiol. Heart Circ. Physiol. *282*, H342-H348.

Kamada, Y., Nagaretani, H., Tamura, S., Ohama, T., Maruyama, T., Hiraoka, H., Yamashita, S., Yamada, A., Kiso, S., Inui, Y., Ito, N., Kayanoki, Y., Kawata, S., Matsuzawa, Y. (2001). Vascular endothelial dysfunction resulting from L-arginine deficiency in a patient with lysinuric protein intolerance. J. Clin. Invest. *108*, 717-724.

Kanaya, Y., Nakamura, M., Kobayashi, N., Hiramori, K. (1999). Effects of L-arginine on lower limb vasodilator reserve and exercise capacity in patients with chronic heart failure. Heart *81*, 512-517.

Kenny, L. C., Baker, P. N., Kendall, D. A., Randall, M. D., Dunn, W. R. (2002). The role of gap junctions in mediating endothelium-dependent responses to bradykinin in myometrial small arteries isolated from pregnant women. Br. J. Pharmacol. *136*, 1085-1088.

Kenny, L. C., Baker, P. N., Kendall, D. A., Randall, M. D., Dunn, W. R. (2002a). Differential mechanisms of endothelium-dependent vasodilator responses in human myometrial small arteries in normal pregnancy and pre-eclampsia. Clin Sci (Lond). *103*, 67-73.

Kerr, S., Brosnan, M. J., McIntyre, M., Reid, M., Dominiczack, A. F., and Hamilton, C. A. (1999). Superoxide anion production is increased in a model of genetic hypertension. Role of the endothelium. Hypertension *33*, 1353-1358.

Khalil, R. A., Crews, J. K., Novak, J., Kassab, S., Granger, J. P. (1998). Enhanced vascular reactivity during inhibition of nitric oxide synthesis in pregnant rats. Hypertension *31*, 1065-1069.

Kim, H. P., Lee, J. Y., Jeong, J. K., Bae, S. W., Lee, H. K., Jo, I. (1999). Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor alpha localized in caveolae. Biochem. Biophys. Res. Commun. *263*, 257-262.

Kim, T. H., Weiner, C. P., and Thomson, L. P. (1994). Effect of pregnancy on contraction and endothelium-mediated relaxation of renal and mesenteric arteries. Am. J. Physiol. *267*, H41-H47.

King, A. J. (1995). Nitric oxide and the renal hemodynamic response to proteins. Semin. Nephrol. *15*, 405-14.

King, A. J., Troy, J. L., Anderson, J., Neuringer, J. R., Gunning, M., and Brenner, B. M. (1991). Nitric Oxide: A potential mediator of amino-acid-induced renal hyperemia and hyperfiltration. Journal of the American Society of Nephrology *1*, 1271-1277.

Klöß, S., Bouloumie, A., Mulsch, A. (2000). Aging and chronic hypertension decrease expression of rat aortic soluble guanylate cyclase. Hypertension *35*, 43-47.

Knock, G. A., and Poston, L. (1996). Bradykinin-mediated relaxation of isolated maternal resistance arteries in normal pregnancy and preeclampsia. American Journal Of Obstetrics and Gynecology *175*, 1668-1674.

Knopp, R. H., Boroush, M. A., O'Sullivan, J. B. (1975). Lipid metabolism in pregnancy II. Postheparin lipolytic activity and hypertriglyceridemia in the pregnant rat. Metabolism *24*, 481-493.

Koifman, B., Wollman, Y., Bogomolny, N., Chernichowsky, T., Finkelstein, A., Peer, G., Scherez, J., Blum, M., Laniado, S., Iaina, A., Keren, G. (1995). Improvement of cardiac performance by intravenous infusion of L-Arg in patients with moderate congestive heart failure. J. Am. Coll. Cardiol. *26*, 1251-1256.

Koller, A., Sun, D., Huang, A., and Kaley, G. (1994). Corelease of nitric oxide and prostaglandins mediate flow-dependent dilation of rat gracilis muscle arterioles. American Journal of Physiology *267*, H326-H332.

Koller, A., and Kaley, G. (1990). Prostaglandins mediate arteriolar dilation to increased blood flow velocity in skeletal muscle microcirculation. Circ. Res. *67*, 529-534.

Koumentaki, A., Anthony, F. W., Poston, L., and Wheeler, T. (2000). Effects of protein restriction on isolated small artery function in female rats. J. Physiol. *525P*, 20P.

Koumentaki, A., Anthony, F. W., Poston, L., and Wheeler, T. (2001). Impairment of endothelium-dependent relaxation in isolated resistance arteries in pregnant rats fed a low protein diet. J. Physiol. *531P*, 26P-27P.

Kurtz, A., Gotz, K.H., Hamann, M. et al. (1998a). Stimulation of renin secretion by NO donors is related to the cAMP pathway. Am. J. Physiol. *274*, F709-F717.

Kurtz, A., Gotz, K.H., Hamann, M. (1998b). Stimulation of renin secretion by NO is mediated by phosphodiesterase 3. Proc. Natl. Acad. Sci. USA *95*, 4743-4747.

Lacy, P.S., Pilkington, G., Hanvesakul, R., Fish, H.J., Boyle, J.P., Thurston, H. (2000). Evidence against potassium as an endothelium-derived hyperpolarizing factor in rat mesenteric small arteries. Br. J. Pharmacol. *129*, 605-611.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head bacteriophage. Nature *227*, 680-685.

Langley, S. C., and Jackson, A. A. (1994). Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. Clin. Sci. *86*, 217-222

Langley-Evans, S. C., Gardner, D. S., and Jackson, A. A. (1996). Association of disproportionate growth of fetal rats in late gestation with raised systolic blood pressure in later life. J. Reprod. Fertil. *106*, 307-312.

Langley-Evans, S. C. (2000). Critical differences between the two low protein diet protocols in the programming of hypertension in the rat. International Journal of Food Sciences and Nutrition *51*, 11-17.

Law, C. M., Swiet, M., Osmond, C., Fayers, P.M., Barker, D.J.P., Cruddas, A.M., Fall, C.H.D. (1993). Initiation of hypertension in utero and its amplification throughout life. BMJ *306*, 24-27.

Law, C. M., Shiell, A.W. (1996). Is blood pressure inversely related to birth weight? The strength of evidence from systematic review of the literature. J. Hypertension *14*, 935-941.

Leaf, C. D., Wishnok, J. S., Tannenbaum, S. R. (1989). L-Arg is a precursor for nitrate biosynthesis in humans. Biochem. Biophys. Res. Commun. *163*, 1032-7.
Learmont, J. G., Cockell, A. P., Knock, G. A., and Poston, L. (1996). Myogenic and flow-mediated responses in isolated mesenteric small arteries from pregnant and nonpregnant rats. American Journal Of Obstetrics and Gynecology *174*, 1631-1636.

Learmont, J. G., and Poston, L. (1996). Nitric oxide is involved in flow-induced dilation of isolated human small fetoplacental arteries. American Journal Of Obstetrics and Gynecology *174*, 583-588.

Lederman, S. A., and Rosso, P. (1980). Effects of food restriction on maternal weight and body composition in pregnant and nonpregnant rats. Growth *44*, 77-78.

Lederman, S. A., and Rosso, P. (1981). Effects of fasting during pregnancy on maternal and fetal weight and body composition in well-nourished and undernourished rats. Journal Of Nutrition *111*, 1823-1832.

Lederman, S. A., and Rosso, P. (1989). The pattern of plasma-volume changes in well-nourished and in food- restricted or protein-restricted pregnant rats. Journal Of the American College Of Nutrition *8*, 215-224.

Lee, K. E., and Summerill, R.A. (1982). Glomerular filtration rate following administration of individual amino acids in conscious dogs. Q. J. Exp. Physiol. *67*, 459.

Lee, R. M. K. W., Forrest, J. B., Garfield, R. E., and Daniel, E. E. (1983). Ultrastructural changes in mesenteric arteries from spontaneously hypertensive rats. Blood vessels *20*, 72-91.

Lefroy, D. C., Tousoulis, D., Crake, T. (1993). Inhibition of nitric oxide synthesis. Lancet *342*, 1487-8.

Leiper, J., and Vallance, P. (1999). Biological significance of endogenous methylarginines that inhibit nitric oxide synthases. Cardiovasc. Res. *43*, 542-548.

Lentz, S. R., Sobey, C. G., Piegors, D. J., Bhopatkar, M. Y., Faraci, F. M., Malinow, R. M., Heistad, D. D. (1996). Vascular dysfunction in monkeys with diet-induced hyperhomocysteinemia. J. Clin. Invest. *98*, 24-29.

Lerman, A., Burnett, J. C., Higano, S. T., McKinley, L. J., and Holmes, D. R., Jr. (1998). Long-term L-arginine supplementation improves small vessel coronary endothelial function in humans. Circulation *97*, 2123-2128.

Li, P. L., and Campbell, W. B. (1997). Epoxyeicosatrienoic acids activate K+ channels in coronary smooth muscle through a guanine nucleotide binding protein. Circ. Res. *80*, 877-884.

Liao, J. K., and Homey, C. J. (1993). The G proteins of the Gi-alpha ang Gq-alpha family couple the bradykinin receptor to the release of endothelium-derived relaxing factor. J. Clin. Invest. *92*, 2168-2172.

Linder, L., Kiowski, W., Buhler, F. R., and Luscher, T. F. (1990). Indirect evidence for release of EDRF in human forearm microcirculation in vivo. Blunted response in essential hypertension. Circulation *81*, 1762-1767.

Liu, J., Hughes, T. E., Sessa, W. C. (1997). The first 35 amino acids and fatty acylation sites determine the molecular targeting of endothelial nitric oxide synthase into the Golgi region of cells: a green fluorescent protein study. J. Cell Biol. *137*, 1525-1535.

Loscalzo, J. (1996). The oxidative stress of hyperhomocysteinemia. J. Clin. Invest. *98*, 5-7.

Lucas, A., Baker, B. A., Desai, M., and Hales, C. N. (1996). Nutrition in pregnant or lactating rats programs lipid metabolism in the offspring. Br. J. Nutr. *76*, 605-612.

Mackey, K., Meyer, M. C., Stirewalt, W. S., Starcher, B. C., McLaughlin, M. K. (1992). Composition and mechanics of mesenteric resistance arteries from pregnant rats. Am. J. Physiol. *263*, R2-8.

Magness, R., Rosenfeld, C. R., Faucher, D. J., and Mitchell, M. D. (1992). Uterine prostaglandin production in ovine pregnancy: effects of angiotensin II and indomethacin. Am. J. Physiol. *263*, H188-H197.

Magness, R. R., and Rosenfeld, C. R. (1993). Calcium modulation of endothelium-derived prostacyclin production in ovine pregnancy. Endocrinology *132*, 2445-2452.

Magness, R. R., Shaw, C. E., Phernetton, T. M., Zheng, J., and Bird, I. M. (1997). Endothelial vasodilator production by uterine and systemic arteries. II. Pregnancy effects on NO synthase expression. Am. J. Physiol. (Heart Circ. Physiol. 41) *272*, H1730-H1740.

Magness, R. R., Sullivan, J. A., Li, Y., Phernetton, T. M., and Bird I. M. (2001). Endothelial vasodilator production by uterine and systemic arteries. VI. Ovarian and pregnancy effects on eNOS and NOx. Am. J. Physiol. (Heart Circ. Physiol.) *280*, H1692-H1698.

Magness, R. R., Rosenfeld, C. R., Hassan, A. and Shaul, P. W. (1996). Endothelial vasodilator production by uterine and systemic arteries: I. Effects of ANG II on PGI_2 and NO in pregnancy. Am. J. Physiol. (Heart Circ. Physiol. 39) *270*, H1914-H1923.

Magness, R. R. (1991). Endothelium-derived vasoactive substances and uterine blood vessels. Sem. Perinatol. *15*, 68-78.

Magness, R. R., Zheng, J. (1996b). Maternal cardiovascular alterations during pregnancy. In Pediatrics and Perinatology: The Scientific Basis, P. D. Gluckman, Heymann, M.A., ed. (London: Arnold), pp. 762-772.

Malandro, M. S., Beveridge, M. J., Kilberg, M. S., and Novak, D. A. (1996). Effect of a low-protein diet-induced intrauterine growth retardation on rat placental amino acid transport. Am. J. Physiol. *271*, C295-C303.

Malek, A. M., and Izumo, S. (1994). Molecular aspects of signal transduction of shear stress in the endothelial cell. Journal of Hypertension. *12*, 989-99.

Manning, J., Vehaskari, V.M. (2001). Low birth weight-associated adult hypertension in the rat. Pediatr. Nephrol *16*, 417-422.

Marsden, P. A., Heng, H. H., Scherer, S. W., Stewart, R. J., Hall, A. V., Shi, X. M., Tsui, L. C., and Schappert, K. T. (1993). Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. J. Biol. Chem. *268*, 17478-17488.

Massicotte, G., St.Louis, J., Parent, A., Schiffrin, E. L. (1987). Decreased in vitro responses to vasoconstrictors during pregnancy in normotensive and spontaneously hypertensive rats. Can. J. Physiol. Pharmacol. *65*, 2466-2471.

Matoba, T., and Shimokawa, H. (2003). Hydrogen Peroxide is an EDHF in animals and humans. J. Pharmacol. Sci. *92*, 1-6.

Matoba, T., Shimokawa, H., Morikawa, K., Kubota, H., Kunihiro, I., Urakami-Harasawa, L., Mukai, Y., Hirakawa, Y., Akaike, T., Takeshita, A. (2003a). Electron spin resonance detection of hydrogen peroxide as an endothelium-derived hyperpolarizing factor in porcine coronary microvessels. Arterioscler. Thromb. Vasc. Biol. *23*, 1224-1230.

Maxwell, A. J., and Cooke, J. P. (2001). . In Nitric Oxide and the Cardiovascular System, J. A. V. J. Loscalzo, ed.: Totowa, NJ:Humana), pp. 547-585.

Maxwell, A. J., and Cooke, J. P. (1998). Cardiovascular effects of L-arginine. Curr. Opin. Nephrol. Hypertens. *7*, 63-70.

McCarthy, A. L., Taylor, P., Graves, J., Raju, S., and Poston, L. (1994). Endothelium-dependent relaxation of human resistance arteries in pregnancy. Am. J. Obstet. Gynecol. *171*, 1309-15.

McCulloch, A. I., Bottrill, F. E., Randall, M. D., and Hiley, C. R. (1997). Characterisation and modulation of EDHF-mediated relaxations in the rat isolated superior mesenteric arterial bed. Br. J. Pharmacol. *120*, 1431-1438.

McCulloch, A. I., and Randall, M. D. (1998). Sex differences in the relative contributions of nitric oxide and EDHF to agonist-stimulated endothelium-dependent relaxations in the rat isolated mesenteric arterial bed. Br. J. Pharmacol. *123*, 1700-1706.

McLaughlin, M. K., and Conrad, K. P. (1995). Nitric oxide biosynthesis during pregnancy: implications for circulatory changes. Clinical Experimental, Pharmacology and Physiology *22*, 164-171.

Meade, E. A., Jones, D. A., Zimmerman, G. A., McIntyre, T. M., Prescott, S. M. (1996). Prostaglandins and related compounds. Lipid messengers with many actions. Handbook Lipid Res. *8*, 285.

Meredith, I. T., Currie, K. E., Anderson, T. J., Roddy, M. A., Ganz, P., and Creager, M. A. (1996). Post-ischaemic vasodilation in human forearm is dependent on endothelium-derived nitric oxide. American Journal of Physiology *270*, H1435-H1440.

Metzger, B. E., Hare, J. W., Freinkel, N. (1971). Carbohydrate metabolism during pregnancy. IX. Plasma levels of gluconeogenic fuels during fasting in the rat. J. Clin.Endocrinol. *33*, 869.

Meyer, M. C., Brayden, J. E., McLaughlin, M. K. (1993). Characteristics of the vascular smooth muscle in the maternal resistance circulation during pregnancy in the rat. Am. J. Obstet. Gynecol. *169*, 1510-1516.

Meyer, M. C., Cummings, K., and Osol, G. (1997). Estrogen replacement attenuates resistance artery adrenergic sensitivity via endothelial vasodilators. Am. J. Physiol. *272*, H2264-H2270.

Michell, B. J., Chen, Z., Tiganis, T., Stapleton, D., Katsis, F., Power, D.A., Sim, A. T., and Kemp, B. E. (2001). Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. J. Biol. Chem. *276*, 17625-17628.

Mikkola, T., Turunen, P., Avela, K., Orpana, A., Viinikka, L., Ylikorkala, O. (1995). 17-beta oestradiol stimulates prostacyclin, but not endothelin-1, production in human vascular endothelial cells. J. Clin. Endocrinol. Metab. *80*, 1832-6.

Miles, A. M., Wink, D. A., Cook, J. C., Grisham, M. B. (1996). Determination of nitric oxide using fluorescence spectroscopy. Methods in Enzymology *268*, 105-120.

Miller, V. M., and Vanhoutte, P. M. (1985). Endothelial alpha2-adrenoreceptors in canine pulmonary and systemic blood vessels. Eur. J. Pharmacol. *118*, 123-129.

Miller, V. M. (2000). Vascular control during pregnancy. Circ. Res. *87*, 344-345.

Mitchell, J. A., Hecker, M., Änggård, E. E., and Vane, J. R. (1990). Cultured endothelial cells maintain their L-arginine level despite the continuous release of EDRF. Eur. J. Pharmacol. *182*, 573-576.

Miyazaki, H., Matsuaoka, H., Cooke, J. P., Usui, M., Ueda, S., Okuda, S., Imaizumi, T. (1999). Endogenous nitric oxide synthase inhibitor: a novel marker of atherosclerosis. Circulation *99*, 1141-1146.

Mohaupt, M., Madsen, K.M., Wilcox, C. S., and Kone, B. C. (1994). Molecular characterization and localisation of three nitric oxide synthase isoforms in rat kidney. In Biology of Nitric Oxide, M. F. S. Moncada, R. Busse and E. A. Higgs, ed. (New York: Portland), pp. 137-141.

Molnar, M., Hertelendy, F. (1992). N-nitro-L-arginine, an inhibitor of nitric oxide synthesis, increases blood pressure in rats and reverses the pregnancy-induced refractoriness to vasopressor agents. Am. J. Obstet. Gynecol. *166*, 1560-1567.

Molnar, M., Suto, T., Toth,T., and Hertelendy, F. (1994). Prolonged blockade of nitric oxide synthesis in gravid rats produces sustained hypertension, proteinuria, thrombocytopenia, and intrauterine growth retardation. Am. J. Obstet. Gynecol. *170*, 1458-1466.

Mombouli, J. V., and Vanhoutte, P. M. (1999). Endothelial dysfunction: from physiology to therapy. J. Mol. Cell. Cardiol. *31*, 61-74.

Moncada, S., Palmer, R.M.J., Higgs, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. *43*, 109-142.

Morgan, B. L., Naismith, D.J. (1977). Effects on the products of conception of protein supplementation of the diets of rats. J. Nutr. *107*, 1590-1594.

Morris, S. M. J. (2002). Regulation of enzymes of the urea cycle and arginine metabolism. Annu. Rev. Nutr. *22*, 87-105.

Morriss, F. H., Rosenfeld, C. R., Crandell, S. S., Adcock, E. W. (1980). Effects of fasting on uterine blood flow and substrate uptake in sheep. J. Nutr. *110*, 2433.

Muaku, S. M., Underwood, L. E., Selvais, P. L., Ketelslegers, J. M., and Maiter, D. (1995). Maternal protein restriction early or late in rat pregnancy has differential effects on fetal growth, plasma insulin-like growth factor I (IGF-I) and liver IGF-I gene expression. Growth Reg. *5*, 125-132.

Mulvany, M. J., and Halpern, W. (1977). Contractile properties of small arterial resistance arteries in spontaneously hypertensive and normotensive rats. Circulation Research *41*, 19-26.

Mulvany, M. J., and Aalkjaer, C. (1990). Structure and function of small arteries. Physiological Reviews *70*, 921-961.

Mutus, B., Rabini, R. A., Staffolani, R., Ricciotti, R., Fumelli, P., Moretti, N., Martarelli, D., and Mazzanti, L. (2001). Homocysteine-induced inhibition of nitric oxide production in platelets: a study on healthy and diabetic subjects. Diabetologia *44*, 979-982.

Myatt, L., Brewer,A. S., Langdon, G., Brockman, D. E. (1992). Attenuation of the vasoconstrictor effects of thromboxane and endothelin by nitric oxide in the human fetal-placental circulation. Am. J. Obstet. Gynecol. *166*, 224-230.

Naismith, D. J. (1973). Adaptations in the metabolism of protein during pregnancy and their nutritional implications. Nutr. Rep. Int. *7*, 383-390.

Naismith, D. J., and Fears, R.B. (1971). Adaptations in the metabolism of protein during pregnancy in the rat. Proc. Nutr. Soc. *31*, 8A-9A.

Naismith, D. J., Morgan, B.L. (1976). The biphasic nature of protein metabolism during pregnancy in the rat. British Journal of Nutrition *36*, 563-566.

Naismith, D. J. (1969). The foetus as a parasite. Proc. Nutr. Soc. *28*, 25-31.

Naismith, D. J. (1966). The requirement for protein, and the utilization of protein and calcium during pregnancy. Metabolism *15*, 582-95.

Naismith, N. J., and Morgan B.L.G. (1975). The regulation of protein metabolism during pregnancy in the rat. Proc. Nutr. Soc. *34*, 27A-28A.

Nakane, M., Arai, K., Saheki, S., Kuno, T., Buechler, W., Murad, F. (1990). Molecular cloning and expression of cDNAs coding for soluble guanylate cyclase. J. Biol. Chem. *265*, 16841-16845.

Nathan, C., and Xie, Q. W. (1994). NOS: roles, tolls, and controls. Cell *78*, 915-918.

Nathan, L., Cuevas, J., Chaudhuri, G. (1995). The role of nitric oxide in the altered vascular reactivity of pregnancy in the rat. Br. J. Pharmacol. *114*, 955-960.

Natuzzi, E. S., Ursell, P. C., Harrison, M. Buscher, C., Riemer, R. K. (1993). Nitric oxide synthase activity in the pregnant rat uterus decreases at parturition. Biochem. Biophys. Res. Commun. *194*, 1-8.

Nelson, S. H., Steinsland, O. D., Wang, Y., Yallampalli, C., Dong, Y. L., and Sanchez, J. M. (2000). Increased nitric oxide synthase activity and expression in the human uterine artery during pregnancy. Circ. Res. *87*, 406-411.

Nelson, S. H., Steinsland, O. S., Suresh, M. S., and Lee, N. M. (1998). Pregnancy augments nitric oxide-dependent dilator response to acetylcholine in the human uterine artery. Human Reproduction *13*, 1361-1367.

Ni, Y. J., Meyer, M., and Osol, G. (1997). Gestation increases nitric oxide-mediated vasodilation in rat uterine arteries. American Journal Of Obstetrics and Gynecology *176*, 856-864.

Nobunaga, T., Tokugawa, Y., Hashimoto, K., Kimura, T., Matsuzaki, N., Nitta, Y., Fujita, T., Kidoguchi, K. I., Azuma, C., Saji, F. (1996). Plasma nitric oxide levels in pregnant patients with preeclampsia and essential hypertension. Gynecol. Obstet. Invest. *41*, 189-193.

Noris, M., Morigi, M., Dondelli, R., Aiello, S., Foppolo, M., Todeschini, M., Orisio, S., Remuzzi, G., and Remuzzi, A. (1995). Nitric oxide synthesis by cultured endothelial cells is modulated by flow conditions. Circulation Research *76*, 536-543.

Ohara, Y., Sayegh, H.S., Yamin, J. J., and Harrison D. G. (1995). Regulation of endothelial constitutive nitric oxide synthase by protein kinase C. Hypertension *25*, 415-420.

Ohashi, Y., Kawashima, S., Hirata, K., Yamashita, T., Ishida, T., Inoue, N., Sakoda, T., Kurihara, H., Yazaki, Y., and Yokoyama, M. (1998). Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. J. Clin. Invest. *102*, 2061-2071.

Osol, G., and Cipolla, M. (1993). Pregnancy-induced changes in the three-dimensional mechanical properties of pressurized rat uteroplacental (radial) arteries. Am. J. Obstet. Gynecol. *168*, 268-274.

Ozaki, T., Nishina, H., Hanson, M.A., Poston, L. (2001). Dietary restriction in pregnant rats causes gender-related hypertension and vascular dysfunction in offspring. J. Physiol. *530*, 141-152.

Ozaki, T., Hawkins, P., Nishina, H., Steyn, C., Poston, L., and Hanson, M. (2000). Effects of undernutrition in early pregnancy on systemic small artery function in late gestation sheep. Am. J. Obstet. Gynecol. *183*, 1301-1307.

Ozcelikay, A. T., Tay, A., Guner, S., Tasyaran, V., Yildizoglou-Ari, N., Dincer, U. D., and Altan, V. M. (2000). Reversal effects of L-arginine treatment on blood pressure and vascular responsiveness of streptozotocin-diabetic rats. Pharmacol. Res. *41*, 201-209.

Paller, M. S., and Hostetter, T.H. (1986). Dietary protein increases plasma renin and reduces pressor reactivity to angiotensin II. Am. J. Physiol (Renal Fluid Electrolyte Physiol. 20) *251*, F34.

Paller, M. S. (1984). Mechanism of decreased pressure responsiveness to Ang II, NE, and vasopressin in pregnant rats. Am. J. Physiol. *247*, H100-8.

Palmer, R. M. J., Ferrige, A. G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature *327*, 524-526.

Palmer, R. M. J., Ferrige, A.G., and Moncada, S. (1989). NO release accounts for the biological activity of endothelium-derived relaxing factor. Nature *327*, 524.

Palmer, R. M. J., and Moncada, S. (1989). A novel citrulline-forming enzyme is implicated in the formation of nitric oxide by vascular endothelial cells. Biochem. Biophys. Res. Commun. *158*, 348-352.

Panza, J. A., Quyyumi, A. A., Brush, J. E. J., and Epstein, S. E. (1990). Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. N. Engl. J. Med. *323*, 22-27.

Parent, A., Schiffrin, E. L., and St-Louis, J. (1990). Role of the endothelium in adrenergic responses of mesenteric artery rings of pregnant rats. Am. J. Obstet. Gynecol. *163*, 229-234.

Pascoal, I. F., Lindheimer, M. D., Nalbantian-Brandt, C., and Umans, J. G. (1995). Contraction and endothelium-dependent relaxation in mesenteric microvessels from pregnant rats. Am. J. Physiol. *269*, H1899-H1904.

Pascoal, I. F., Umans, J. G. (1996). Effect of pregnancy on mechanisms of relaxation in human omental microvessels. Hypertension. *28*, 183-187.

Pascoal, I. F., Lindheimer, M. D., Nalbantian-Brandt, C., Umans, J. G. (1998). Preeclampsia selectively impairs endothelium-dependent relaxation and leads to oscillatory activity in small omental arteries. J. Clin. Invest. *101*, 464-70.

Peters, H., Border, W. A., Noble, N. A. (1999). L-Arginine supplementation increases mesangial cell injury and subsequent tissue fibrosis in experimental glomerulonephritis. Kidney Int. *55*, 2264-2273.

Phippard, A. F., Horvath, J. S., and Glynn, E. M. (1986). Circulation adaptation to pregnancy: Serial studies of haemodynamics, blood volume, renin and aldosterone in the baboon. J. Hypertension *4*, 733.

Piechota, W., and Staszewski, A. (1992). Reference ranges of lipids and apolipoproteins in pregnancy. Eur. J. Obstet. Gynecol. Reprod. Biol. *45*, 27-35.

Pieper, G. M. (1998). Review of alteration in endothelial nitric oxide production in diabetes. Hypertension *31*, 1047-1060.

Pirani, B. B. K., Campbell, D. M., MacGillivray I. (1973). Plasma volume in normal first pregnancy. J. Obstet. Gynaecol. Br. Commonw. *80*, 884-887.

Plane, F., Holland, M., Waldron, G. J., Garland, C. J., and Boyle, J. P. (1997). Evidence that anandamide and EDHF act via different mechanisms in rat isolated mesenteric arteries. Br. J. Pharmacol. *121*, 1509-1511.

Pollock, J. S., Forstermann, U., Mitchell, J. A., Warner, T. D., Schmidt H. H. et al. (1991). Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. Proc. Natl. Acad. Sci. *88*, 10480-10484.

Popp, R., Bauersachs, J., Hecker, M., Fleming, I., Busse, R. (1996). A transferable, beta-naphthoflavone-inducible, hyperpolarizing factor is synthesized by native and cultured porcine coronary endothelial cells. J. Physiol. *497*, 699-709.

Popp, R., Fleming, I., Busse, R. (1998). Pulsatile stretch in coronary arteries elicits release of EDHF: a modulator of arterial compliance. Circ. Res. *82*, 696-703.

Popp, R., Brandes, R. P., Ott, G., Busse, R., Fleming, I. (2002). Dynamic modulation of interendothelial gap junctional communication by 11,12-Epoxyeicosatrienoic acid. Circ. Res. *90*, 800-806.

Premen A.J., H., J.E., and Smith, M. J. (1985). Postprandial regulation of renal hemodynamics: Role of pancreatic glucagon. Am. J. Physiol. (Renal Fluid Electrolyte Physiol.17) *248*, F656.

Premen, A. J. (1988). Potential mechanisms mediating postprandial renal hyperemia and hyperfiltration. FASEB J. *2*, 131.

Premen, A. J., et al. (1990). Renal vascular response to amino acids: Effect of pancreatectomy. Am. J. Physiol.258 (Renal Fluid Electrolyte Physiol.27) *258*, F1154.

Pritchard, J. A., and Adams, R. H. (1960). Erythrocyte production and destruction during pregnancy. Am. J. Obstet. Gynec. *79*, 750-757.

Purcell, T. L., Buhimschi, I. A., Given, R., Chwalisz, K., Garfield, R. E. (1997). Inducible nitric oxide synthase is present in the rat placenta at the fetal-maternal interface and decreases prior to labour. Mol. Hum. Reprod. *3*, 485-491.

Quilley, J., and McGiff, J. C. (2000). Is EDHF an epoxyeicosatrienoic acid? Trends Pharmacol. Sci. *21*, 121-124.

Raij, L., and Baylis, C. (1995). Glomerular actions of nitric-oxide. Kidney International *48*, 20-32.

Ralevic, V., and Burnstock, G. (1996). Mesenteric arterial function in the rat in pregnancy: role of sympathetic and sensory-motor perivascular nerves, endothelium, smooth muscle, nitric oxide and prostaglandins. Br. J. Pharmacol. *117*, 1463-1470.

Ramamurthi, A., and Lewis, R. S. (1997). Measurement and modelling of nitric oxide release rates for nitric oxide donors. Chem. Res. Toxicol. *10*, 408-413.

Ramsay, M. M., Broughton Pipkin, F., Rubin, P. C. (1993). Pressor, heart rate and plasma catecholamine responses to noradrenaline in pregnant and nonpregnant women. Br. J. Obstet. Gynaecol. *100*, 170-176.

Randall, M. D., Alexander, S. P. H., Bennett, T., Boyd, E. A., Fry, J. R., Gardiner, S. M., Kemp, P. A., McCulloch, A. I., and Kendall, D. A. (1996). An endogenous cannabinoid as an endothelium-derived vasorelaxant. Biochem. Biophys. Res. Commun. *229*, 114-120.

Rang, H. P., Dale, M. M., Ritter, J. M. (1999). Nitric oxide. In Pharmacology (Edinburgh: Churchill Livingstone), pp. 188-197.

Ranjan, V., Xiao, Z., and Diamond. S. L. (1995). Constitutive NOS expression in cultured endothelial cells is elevated by fluid shear stress. Am. J. Physiol. *269*, H550-H555.

Rees, D. D., Palmer, R. M., Schulz, R., Hodson, H. F., and Moncada, S. (1990). Characterization of three inhibitors of nitric oxide synthase in vitro and in vivo. Br. J. Pharmacol. *101*, 746-752.

Rees, D. D., Palmer, R. M., and Moncada, S. (1989). Role of the endothelium-derived nitric oxide in the regulation of blood pressure. Proc. Natl. Acad. Sci. (USA) *86*, 3375-3378.

Rees, W. D., Hay, S. M., Brown, D. S., Antipatis, C., and Palmer, R. M. (2000). Maternal protein deficiency causes hypermethylation of DNA in the livers of rat fetuses. J. Nutr. *130*, 1821-1826.

Rees, W. D., Hay, S. M., Buchan, V., Antipatis, C., and Palmer, R. M. (1999). The effects of maternal protein restriction on the growth of the rat fetus and its amino acid supply. British Journal Of Nutrition *81*, 243-250.

Reynolds, L. P., and Redmer, D. A. (1995). Utero-placental vascular development and placental function. J. Anim. Sci. *73*, 1839-1851.

Riemer, R. K., Buscher, C., Bansal, R. K., Black, S. M., He, Y., Natuzzi, E. S. (1997). Increased expression of nitric oxide synthase in the myometrium of the pregnant rat uterus. Am. J. Physiol. *272*, E1008-E1015.

Robinson, L. J., Busconi, L., and Michel, T. (1995). Agonist-modulated palmitoylation of endothelial nitric oxide synthase. J. Biol. Chem. *270*, 995-998.

Robson, S. C., Hunter, S., Boys, R. J., and Dunlop, W. (1989). Serial study of factors influencing changes in cardiac output during human pregnancy. American Journal Of Physiology *256*, H1060-H1065.

Rosso, P., and Streeter, M. R. (1979). Effects of food or protein restriction on plasma volume expansion in pregnant rats. J. Nutr. *109*, 1887-1892.

Rosso, P., and Kava, R. (1980). Effects of food restriction on cardiac output and blood flow to the uterus and placenta in the pregnant rat. J. Nutr. *110*, 2350-2354.

Rosso, P. (1977a). Maternal nutrition, nutrient exchange and fetal growth. In Nutritional disorders of American women, M. Winick, ed. (New York: John Wiley and Sons), pp. 3-25.

Rosso, P. (1977b). Maternal-fetal exchange during protein malnutrition in the rat. Placental transfer of alpha-amino isobutyric acid. J. Nutr. *107*, 2002-2005.

Rosso, P. (1977c). Maternal-fetal exchange during protein malnutrition in the rat. Placental transfer of glucose and a non-metabolizable glucose analog. J. Nutr. *107*, 2006-2010.

Rosso, P., and Cramoy, C. (1979). Nutrition and pregnancy. In Human Nutrition: A comprehensive Treatise, Volume 1, Nutrition: Pre- and Postnatal development, M. Winick, ed. (New York: Plenum Press), pp. 133-228.

Rovinsky, J. J., and Jaffin, H. (1965). Cardiovascular haemodynamics in pregnancy. I. Blood and plasma volumes in multiple pregnancy. Am. J. Obstet. Gynec. *93*, 1-15.

Rubanyi, G. M., Romero, J. C., and Vanhoutte, P. M. (1986). Flow-induced release of endothelium-derived relaxing factor. American Journal of Physiology *250*, H1145-H1149.

Sakuma, I., Liu, M.Y., Sato, A., Hayashi, T., Iguchi, A., Kitabatake, A., and Hattori, Y. (2002). Endothelium-dependent hyperpolarization and relaxation in mesenteric arteries of middle-aged rats: influence of oestrogen. Br. J. Pharmacol. *135*, 48-54.

Salas, S. P., Altermatt, F., Campos, M., Giacaman, A., and Rosso, P. (1995). Effects of long-term nitric-oxide synthesis inhibition on plasma- volume expansion and fetal growth in the pregnant rat. Hypertension *26*, 1019-1023.

Salas, S. P., Giacaman, A., Altermatt, F., Campos, M., and Rosso, P. (1997). Does long-term nitric oxide synthase inhibition in the pregnant rat produce a preeclampsia like syndrome? Hypertension *29*, 250.

Schieffer, B., Drexler, H., Ling, B. N., Marrero, M. B. (1997). G protein-coupled receptors control vascular smooth muscle cell proliferation via pp60c-src and $p21^{ras}$. Am. J. Physiol. *272*, 2019-2030.

Schmidt, H. H., Nau, H., Wittfoht, W., Gerlach, J., Prescher, K. E., Klein, M. M., Niroomand, F., and Bohme, E. (1988). Arginine is a physiological precursor of endothelium-derived nitric oxide. Eur. J. Pharmacol. *154*, 213-216.

Schrier, R. W., and Briner, V.A. (1991). Peripheral arterial vasodilatation hypothesis of sodium and water retention in pregnancy: implications for pathogenesis of preeclampsia-eclampsia. Obstet Gynecol *77*, 632-639.

Selhub, J. (1999). Homocysteine metabolism. Annu. Rev. Nutr. *19*, 217-246.

Sessa, W. C. (1994). The nitric oxide synthase family of proteins. J. Vasc. Res. *31*, 131-143.

Shaamash, A. H., Elsnosy, E. D., Makhlouf, A. M., Zakhari, M. M., Ibrahim, O. A., EL-dien, H. M. (2000). Maternal and fetal serum nitric oxide (NO) concentrations in normal pregnancy, pre-eclampsia and eclampsia. Int J Gynaecol Obstet. *68*, 207-14.

Shan, J., Resnick, L. M., Qin-Yue, L., Xi-Chen, W., Barbagallo, M., and Pang, P. K. T. (1994). Vascular effects of 17-beta estradiol in male Sprague-Dawley rats. American Journal of Physiology *266*, H967-H973.

Shaul, P. W., J., N. A., Brannon, T. S., Ujiie, K., Wells, L. B., Nisen, P. A., Lowenstein, C. J., Snyder, S. H., and Star, R. A. (1995). Prolonged in vivo hypoxia enhances nitric oxide synthase type I and type III gene expression in adult rat lung. Am. J. Respir. Cell Mol. Biol. *13*, 167-174.

Shimokawa, H., Yatsutake, H., Fujii, K., Owada, M. K., Nakaike, R., Fukumoto, Y., Takayanagi, T., Nagao, T., Egashira, K., Fujishima, M., Takeshita, A. (1996). The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in the rat mesenteric circulation. J. Cardiovasc. Pharmacol. *28*, 703-11.

Shinozaki, K., Nishio, Y., Okamura, T., Yoshida, Y., Maegawa, H., Kojima, H., Masada, M., Toda, N., Kikkawa, R., Kashiwagi, A. (2000). Oral administration of tetrahydrobiopterin prevents endothelial dysfunction and vascular oxidative stress in the aortas of insulin-resistant rats. Circ. Res. *87*, 566-573.

Sibai, B. M., Anderson, G. D., Spinnato, J. A., Shaver, D. C. (1983). Plasma volume findings in patients with mild pregnancy-induced hypertension. Am. J. Obstet. Gynecol. *147*, 16-19.

Sladek, S. M., and Roberts, J. M., (1996). Nitric oxide synthase activity in the gravid rat uterus decreases a day before the onset of parturition. Am. J. Obstet. Gynecol. *175*, 1661-1667.

Slim, R. M., Toborek, M., Watkins, B. A., Boissonneault, G. A., Henning, B. (1996). Susceptibility to hepatic oxidative stress in rabbits fed different animal and plant fats. J. Am. Coll. Nutr. *15*, 289-294.

Smarason, A. K., Allman, K. G., Young, D., Redman, C. W., (1997). Elevated levels of serum nitrate, a stable end product of nitric oxide, in women with preeclampsia. Br. J. Obstet. Gynaecol. *104*, 538-543.

Smith, C. A. (1947). Effects of maternal undernutrition upon the newborn infant in Holland 1944-1945. J. Pediatr. *30*, 229-243.

Smith, C. J., Sun, D., Hoegler, C., et al. (1996). Reduced gene expression of vascular endothelial NO synthase and cyclooxygenase-1 in heart failure. Circ. Res. *78*, 58-64.

Sooranna, S. R., Morris, N. H., and Steer, P. J. (1995). Placental nitric oxide metabolism. Reprod. Fertil. Dev. *7*, 1525-1531.

Stamler, J. S., Osborne, J. A., Jaraki, O., Rabbani, L. E., Mullins, M., Singel, D., Loscalzo, J. (1993). Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. J. Clin. Invest. *91*, 308-318.

Steele, S.C., Warren, A. Y., Johnson, I. R. (1993). Effect of the vascular endothelium on norepinephrine-induced contractions in uterine radial arteries from the nonpregnant and pregnant human uterus. Am. J. Obstet. Gynecol. *168*, 1623-28.

Stephensen, C. B. (2001). Vitamin A, infection, and immune function. Annu. Rev. Nutr. *21*, 167-192.

St-Louis, J., and Sicotte, B. (1992). Prostaglandin- or endothelium-mediated vasodilation is not involved in the blunted responses of blood vessels to vasoconstrictors in pregnant rats. Am. J. Obstet. Gynecol. *166*, 684-692.

Stuehr, D., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L., and Wiseman, J. (1991). N omega-Hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. J. of Biol. Chem. *266*, 6259-6263.

Stuhlinger, M. C., Tsao, P. S., Her, J. H., Kimoto, M., Balint, R. F., and Cooke, J. P. (2001). Homocysteine impairs the nitric oxide synthase pathway. Role of asymmetric dimethylarginine. Circulation *104*, 2569-2575.

Susano, S., Watanabe, H., Tanaka, S., Sekiguchi, F., Shimamura, K. (1999). Endothelium-derived relaxing, contracting and hyperpolarizing factors of mesenteric arteries of hypertensive rats. Br. J. Pharmacol. *126*, 709-716.

Swierkosz, T. A., Mitchell, J. A., Sessa, W. C., Hecker, M., Vane, J. R. (1990). L-glutamine inhibits the release of endothelium-derived relaxing factor from the rabbit aorta. Biochem. Biophys. Res. Commun. *172*, 143-148.

Tawakol, A., Omland, T., Gerhard, M., Wu, J. T., and Creager, M. A. (1997). Hyperhomocysteinemia is associated with impaired endothelium-dependent vasodilatation in humans. Circulation *95*, 1119-1121.

Taylor, H. J., Chaytor, A. T., Edwards, D. H., Griffith, T. M. (2001). Gap junction-dependent increases in smooth muscle cAMP underpin the EDHF phenomenon in rabbit arteries. Biochem. Biophys. Res. Commun. *283*, 583-589.

Thaete, I. G., Neerhof, M. G., Gosnell, E. R., Kushner, D. M. (1997). Placental perfusion is decreased in a dose-dependent fashion in chronic NOS-inhibition-induced IUGR. Am. J. Obstet. Gynecol. *176*, 569.

Thomas, S. R., Chen, K., and Keaney, J. F., Jr. (2002). Hydrogen Peroxide activates endothelial nitric oxide synthase through coordinated phosphorylation and dephosphorylation via a Phosphoinositide 3-Kinase -dependent signaling pathway. The J. of Biol. Chem. *277*, 6017-6024.

Tolins, J. P., and Raij, L. (1991). Effects of amino acid infusion on renal hemodynamics. Hypertension *17*, 1045-51.

Tolins, J. P. (1992). Mechanisms of glucagon-induced renal vasodilatation: role of prostaglandins and EDRF. J. Lab. Clin. Med. *120*, 941-948.

Tolins, J. P., Shultz, P. J., Westberg, G., and Raij, L. (1995). Renal haemodynamic effects of dietary protein in the rat: Role of NO. J. Lab. Clin. Med. *125*, 228-236.

Tolins, J. P., Palmer, R.M., Moncada, S., Raih, L. (1990). Role of endothelium-derived relaxing factor in regulation of renal haemodynamic responses. Am. J. Physiol. *258 (3 Pt 2)*, H655-H662.

Topper, J. N., Cai, J., Falb, D., Gimbrone, M. A. Jr. (1996). Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: Cyclooxygenase-3, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively upregulated by steady laminar shear stress. Proc. Natl. Acad. Sci. *93*, 10417.

Tsikas, D., Böger, R. H., Sandmann, J., Bode-Böger, S. M., Frölich, J. C. (2000). Endogenous nitric oxide synthase inhibitors are responsible for the L-arginine paradox. FEBS Letters *478*, 1-3.

Upchurch, G. R., Welch, G. N., Fabian, A. J., Freedman, J. E., Johnson, J. L., Keaney, J. F., Loscalzo, J. (1997). Homocysteine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J. Biol. Chem. *272*, 17012-17017.

Vagnoni, K. E., Shaw, C. E., Phernetton, T. M., Meglin, B. M., Bird, I. M., and Magness, R. R. (1998). Endothelial vasodilator production by uterine and systemic arteries. III. Ovarian and estrogen effects on NO synthase. Am. J. Physiol. (Heart Circ. Physiol. 44) *275*, H1845-H1856.

Vallance, P. (2001). The asymmetrical dimethylarginine/dimethylarginine dimethylaminohydrolase pathway in the regulation of nitric oxide generation. Clinical Science *100*, 159-160.

Vallance, P., Leone, A., Calver, A., Collier, J., and Moncada, S. (1992). Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis. J. Cardiovasc. Pharmacol. *20 (Suppl. 12)*, S60-S62.

VanAerts, L. A. G. J. M., Blom, H. J., DeAbreu, R. A., Trijbels, F. J. M., Eskes, T. K. A. B., Copius Peereboom-Stegeman, J. H. J. C., and Noordhoek, J. (1994). Prevention of neural tube defects by and toxicity of L-homocysteine in cultured postimplantation rat embryos. Teratology *50*, 348-360.

Vander, A., Sherman, J. H., Luciano, D. S. (1998). Human Physiology: the mechanisms of body function, 7th Edition (Boston, Massachusetts: WCB/McGraw-Hill).

Varga, I., Rigo, J. Jr., Somos, P., Joo, J. G., Nagy, B. (2000). Analysis of maternal circulation and renal function in physiologic pregnancies; parallel examinations of the changes in the cardiac output and the GFR. J. Matern. Fetal Med. *9*, 97-104.

Vedernikov, Y. P., Liao, Q. P., Jain, V., Saade, G. R., Chwalisz, K., and Garfield, R. E. (1997). Effect of chronic treatment with 17beta-estradiol and progesterone on endothelium-dependent and endothelium-independent relaxation in isolated aortic rings from ovariectomized rats. Am. J. Obstet. Gynecol. *176*, 603-608.

Vehaskari, V. M., Aviles, D.H., Manning, J. (2001). Prenatal programming of adult hypertension in the rat. Kidney International *59*, 238-245.

Venema, R. C., Sayegh, H. S., Kent, J. D., Harrison, D. G. (1996). Identification, characterization and comparison of the calmodulin binding domains of the endothelial and inducible nitric oxide synthases. J. Bio. Chem. *271*, 6435-6440.

Visek, W. J. (1986). Arginine needs, physiological state and usual diets. A reevaluation. J. Nutr. *116*, 36-46.

Vishwanathan, K., Tackett, R. L., Stewart, J. T., Bartlett, M. G. (2000). Determination of arginine and methylated arginines in human plasma by liquid chromatography-tandem mass spectrometry. J. Chromatogr. B. Biomed. Sci. Appl *748*, 157-66.

Wada, L., Don, B. R., and Schambelan, M. (1991). Hormonal mediators of amino-acid induced glomerular hyperfiltration in humans. Am. J. Physiol. (Renal Fluid Electrolyte Physiol. 29) *260*, F797.

Wang, S. Y., Datta, S., Segal, S. (2002). Pregnancy alters adrenergic mechanisms in uterine arterioles of rats. Anesth. Analg. *94*, 1304-9.

Weiner, C., Liu, K. Z., Thompson, L., Herrig, J., and Chestnut, D. (1991). Effect of pregnancy on endothelium and smooth muscle: their role in reduced adrenergic sensitivity. Am. J. Physiol. *261*, H1275-H1283.

Weiner, C., Martinez, E., Zhu, L.K., Ghodsi, A., and Chestnut, D. (1989b). In vitro release of EDRF by ACh is increased during the guinea-pig pregnancy. Am. J. Obstet. Gynecol. *161*, 1599-1605.

Weiner, C. P., Martinez, E., Chestnut, D. H., and Ghodsi, A. (1989). Effect of pregnancy on uterine and carotid artery response to norepinephrine, epinephrine, and phenylephrine in vessels with documented functional endothelium. Am. J. Obstet. Gynecol. *161*, 1605-1610.

Weiner, C. P., Lizasoain, I. K., Baylis, S. A., Knowles, R. G., Charles, I. G., and Moncada, S. (1994). Induction of calcium-dependent nitric oxide synthases by sex hormones. Proc. Natl. Acad. Sci. USA *91*, 5212-5216.

Welham, S. J. M., Wheeler, T., and Langley-Evans, S. C. (1998). Maternal plasma volume expansion is modulated in early pregnancy by a low protein diet in the rat. Clinical Science *94*, M15.

Wellman, G. C., Bonev, A. D., Nelson, M. T., and Brayden, J. E. (1996). Gender differences in coronary artery diameter involve estrogen, nitric oxide and calcium-dependent potassium channels. Circ. Res. *79*, 1024-1030.

Wess, J. (1996). Molecular biology of muscarinic acetylcholine receptors. Crit. Rev. Neurobiol. *10*, 69-99.

Wheeler, T. (1999). Factors influencing maternal cardiovascular adaptation during pregnancy: their relevance to fetal growth and programming. In Fetal Programming: Influences on Development and Disease in Later Life., P. M. S. O' Brien, Wheeler, T., and Barker, D. J. P., ed. (London: RCOG Press), pp. 299-312.

White, R., and Hiley, C. R. (1997). A comparison of EDHF-mediated and anandamide-induced relaxations in the rat isolated mesenteric artery. Br. J. Pharmacol. *122*, 1573-1584.

Widdowson, E., and McCance, R. (1975). A review: new thought on growth. Ped. Res. *9*, 154-156.

Widmann, M.D., Weintraub, N.L., Fudge. J.L., Brooks, L.A., Dellsperger, K.C. (1998). Cytochrome P-450 pathway in acetylcholine-induced canine coronary microvascular vasodilation in vivo. Am. J. Physiol. *43*, H283-H289.

Wilcox, C. S., Welch, W.J., Murad, F., Gross, S.T., Taylor, G., Levi, R., Schmidt, HHHW. (1992). NOS in macula densa regulates glomerular capillary pressure. Proc. Natl. Acad. Sci. USA *89*, 11993-11997.

Wilkins, M. R., Redondo, J., Brown, L. A. (1997). The natriuretic-peptide family. Lancet *349*, 1307-1310.

Williams, D. J., Vallance, P. J. T., Neild, G. H., Spencer, J. A. D., Imms, F. J. (1997). NO-mediated vasodilatation in human pregnancy. Am. J. Physiol. *272*, H748-H752.

Wu, G., and Morris, S. M. Jr. (1998). Arginine metabolism: nitric oxide and beyond. Biochem. J. *336*, 1-17.

Wu, G., Meininger, C. J. (2000). Arginine nutrition and cardiovascular function. J. Nutr. *130*, 2626-29.

Wu, G., Flynn, N. E., Flynn, S. P., Jolly, C. A., and Davis, P. K. (1999). Dietary protein or arginine deficiency impairs constitutive and inducible nitric oxide synthesis by young rats. J. Nutr. *129*, 1347-1354.

Wu, G., Haynes, T. E., Li, H., Yan, W., Meininger, C. J. (2001). Glutamine metabolism to glucosamine is necessary for glutamine inhibition of endothelial nitric oxide synthesis. Biochem. J. *353*, 245-252.

Wu, G., Pond, W. G., Flynn, S. P., Ott, T. L., and Bazer, F. W. (1998). Maternal dietary protein deficiency decreases nitric oxide synthase and ornithine decarboxylase activities in placenta and endometrium of pigs during early gestation. J. Nutr. *128*, 2395-2402.

Xie, L., Hattori, Y., Tume, N., Gross, S. S. (2000). The preferred source of arginine for high-output nitric oxide synthesis in blood vessels. Semin. Perinatol. *24*, 42-45.

Yamasaki, M., Lindheimer, M. D., and Umans, J. G. (1996). Effects of pregnancy on femoral microvascular responses in the rat. Am. J. Obstet. Gynecol. *175*, 730-36.

Yashiro, Y., and Ohhashi, T. (1997). Flow- and agonist-mediated NO-and prostaglandin-dependent dilation in spinal arteries. Am. J. Physiol. *273*, H2217-23.

Yiu, V., Bluka, S., Zurakowski, D., McCormick, M., Brenner, B., Jabs, K. (1999). Relationship between birthweight and blood pressure in childhood. Am.J. Kidney Diseases *33*, 253-260.

Ylikorkala, O., Pekonen, F., and Viinikka, L. (1986). Renal prostacyclin and thromboxane in normotensive and preeclamptic pregnant women and their infants. J. Clin. Endocrinol. Metab. *63*, 1307-1312.

Young, J. B., and Landsberg, L. (1979). Sympathoadrenal activity in fasting pregnant rats. Dissociation of adrenal medullary and sympathetic nervous responses. J. Clin. Invest. *64*, 109-116.

Zhang, X. H., Li, H., Jin, H. L., Ebin, Z., Brodsky, S., Goligorsky, M. S. (2000). Effects of homocysteine on endothelial NO production. Am. J. Physiol. *279*, F671-F678.

Zhang, Y., Stewart, K. G., and Davidge, S. T. (2001). Endogenous estrogen mediates vascular reactivity and distensibility in pregnant rat mesenteric arteries. Am. J. Physiol. *280*, H956-H961.

APPENDICES

APPENDIX 1.

Composition of rat CRMX laboratory chow diet

NUTRIENT	PERCENTAGE COMPOSITION
Protein	18.8
Total starch	44.8
Total fibre	12.3
Sucrose	4.7
Choline	0.95
Mineral mix	3.63
Vitamin mix	2.84
Trace elements	8.6
Corn Oil	3.4

Supplied by SDS.

APPENDIX 2.

Composition of synthetic diets.

INGREDIENT	DIETARY CONSTITUTION (g/kg of diet)	
	18% CASEIN	9% CASEIN
Casein	180	90
Cornstarch	425	485
Cellulose fibre	50	50
Sucrose	213	243
Choline chloride	2	2
DL-methionine	5	5
AIN-76™ Mineral mix	20	20
AIN-76™ Vitamin mix	5	5
Corn oil	100	100

APPENDIX 3.

Creatinine assay

A commercially available creatinine kit, validated for measurement of creatinine in serum plasma or urine, was used to assay creatinine concentrations (Code 555-A, Sigma Diagnostics, Poole, UK).

Principle of the assay

Most methods for creatinine measurement are based on the Jaffe' reaction where yellow/orange colour forms when the metabolite is treated with alkaline picrate. The Jaffe reaction is not specific and a number of substances, including proteins in body fluids interfere. A method with improved specificity was developed by Slot, who noted that under acid conditions the creatinine-picrate colour faded faster than the interfering chromogens. The work of these authors serves as the basis for the assay, where colour derived from creatinine is destroyed at acid pH. The difference in colour intensity measured at 500 nm before and after acidification is proportional to the concentration of creatinine present.

Method of the assay

The assay was performed at room temperature in 10 mm cuvettes. Each urine sample was diluted 15X with distilled water. 300 µl of standards, blank and unknown urine samples were assayed in duplicate. An alkaline picrate solution was prepared by mixing 5 volumes of creatinine colour reagent (picric acid, approximately 0.6%, sodium borate and surfactant, Catalog No 555-1) with 1 volume of sodium hydroxide solution (1.0 N) (Catalog No 930-65). 3 ml of alkaline picrate solution was dispensed into each cuvette. Each cuvette was then mixed and incubated at room temperature for 12 minutes. After a 12-min incubation at room temperature, initial absorbance (A) was determined at a wavelength of 500 nm with a spectrophotometer. 100 µl of acid reagent (mixture of sulfuric acid and acetic acid, Catalogue No 555-2) was then added into each cuvete. Each cuvette was then mixed immediately and thoroughly, and incubated for a further 5 minutes at room temperature. Final absorbance of standards and unknown samples versus urine blank as reference, was determined at the same wavelength used for the initial readings. Creatinine concentrations in the urine samples under investigation were calculated from the absorbance of 3 mg/dL creatinine standard included in each series of tests (Catalog No 925-3).

APPENDIX 4

The data show example data obtained from mesenteric resistance vessels taken from a virgin Wistar rat (body weight = 246 g).

MYOGRAPHY EXPERIMENTAL RECORD SHEET			
EXPT #:1		DATE: 21/05/1999	
SEX: Female		BODY WT: 246 g	
NEAR		FAR	
alpha = 1.74	delta = 0.3	alpha = 1.72	delta = 0.3
a_1	0.47	a_1	0.69
a_2	6.4	a_2	7.7
X_0	400	X_0	470
Y_0	1.72	Y_0	1.29

X_1	650	Y_1	2.13	X_1	680	Y_1	2.04
X_2	720	Y_2	3.25	X_2	730	Y_2	2.51
X_3	750	Y_3	4.09	X_3	775	Y_3	3.70
X_4	765	Y_4	4.65	X_4	800	Y_4	4.85
X_5	785	Y_5	5.56	X_5	820	Y_5	5.72

	NEAR	FAR
R^2	0.997	0.996
Vessel Diameter (l_{100})	311.273	291.336
Microreading	737.309	779.120

		FORCE	TENSION			FORCE	TENSION
NAK	Y_1	1.92	3.71 mN/mm	NAK	Y_1	1.53	3.62 mN/mm
	Y_2	9.51	23.84 kPa		Y_2	10.38	24.84 kPa
NA	Y_1	1.92	3.68 mN/mm	NA	Y_1	1.53	3.61 mN/mm
	Y_2	9.45	23.66 kPa		Y_2	10.35	24.76 kPa
K	Y_1	1.63	3.90 mN/mm	K	Y_1	1.03	3.85 mN/mm
	Y_2	9.60	25.04 kPa		Y_2	10.44	26.42 kPa
NAK	Y_1	1.63	4.48 mN/mm	NAK	Y_1	1.03	4.78 mN/mm
	Y_2	10.80	28.81 kPa		Y_2	12.71	32.85 kPa

APPENDIX 5

BASIC PROGRAMME

```
1 ' Normalization program for small vessel myograph /MJM 28/8-87
3 '
4 ' Constants
5 PI=3.142 : P100=13.3 ' p100=13.3 kPa (100 mmHg)
8 '
9 ' First set of parameters
10 PRINT "Enter l1/l100"; : INPUT RATIO : LPRINT RATIO
20 PRINT "Enter 2 delta"; : INPUT DELTA2 : LPRINT DELTA2
30 PRINT "Enter x0"; : INPUT X0 : LPRINT X0
40 PRINT "Enter L0"; : INPUT L0 : LPRINT L0
45 PRINT : LPRINT
48 '
49 ' Second set of parameters
50 PRINT "Enter alpha"; : INPUT ALPHA : LPRINT ALPHA
60 PRINT "Enter a1"; : INPUT A1 : LPRINT A1
70 PRINT "Enter a2"; : INPUT A2 : LPRINT A2
80 PRINT "Enter y0"; : INPUT Y0 : LPRINT Y0
88 '
89 ' Calculate twice segment length
90 AA=ABS(A1-A2)*DELTA2 : PRINT : PRINT "2a="; AA : LPRINT AA
96 LPRINT
98 '
99 ' Enter points on resting tension - i.c. determination
100 I=0
110 I=I+1
120 PRINT : LPRINT
130 PRINT "Enter y(";I;")"; : INPUT Y(I) : LPRINT Y(I)
140 PRINT "Enter x(";I;")"; : INPUT X(I) : LPRINT X(I)
150 T(I)=(Y(I)-Y0)*(ALPHA/(AA)) ' Wall tension (N/m)
160 L(I)=((X(I)-X0)*2+L0) ' Internal circumference (um)
170 P(I)=T(I)/(L(I)/(2000*PI)) ' Effective pressure (kPa)
180 PRINT "p(";I;")=";P(I) : LPRINT P(I)
190 IF P(I)<13.3 THEN GOTO 110 ELSE N=I
198 '
199 ' Regression of points to T=A exp(B L)
200 XSUM=0 : X2SUM=0 : YSUM=0 : Y2SUM=0 : XYSUM=0
210 FOR I=1 TO N
220 XSUM=XSUM+L(I)
230 X2SUM=X2SUM+L(I)*L(I)
240 YSUM=YSUM+LOG(T(I))
250 Y2SUM=Y2SUM+LOG(T(I))*LOG(T(I))
260 XYSUM=XYSUM+L(I)*LOG(T(I))
270 NEXT I
280 X=X2SUM-(1/N)*(XSUM*XSUM)
290 Y=Y2SUM-(1/N)*(YSUM*YSUM)
300 Z=XYSUM-(1/N)*(XSUM*YSUM)
310 R2=Z*Z/(X*Y)
320 B=Z/X
330 A=EXP(YSUM/N-B*XSUM/N)
340 PRINT : LPRINT
350 PRINT "a=";A
360 PRINT "b=";B
370 PRINT "r2=";R2 : LPRINT R2 ' coefficient of determination
380 PRINT

390 '
499 ' Iteration to solve L100 = (2 pi A exp(B L100))/p100
500 LEST=L(N) ' seed estimate of L100
510 FL=2000*PI*A*EXP(B*LEST)/P100 ' Value of L with this estimate
530 LEST=LEST+(LEST-FL)/20 ' new estimate of L100
540 'PRINT LEST optional control for convergence
550 IF ABS(FL-LEST)>.1 THEN GOTO 510 ' reiterate until equation
560 '
599 ' Final calculations                                approx solved
600 L100=LEST
610 L1=RATIO*L100
620 PRINT "l(100)=";L100/PI : LPRINT L100/PI   Normalized lumen
630 X1=X0+(L1-L0)/2
640 PRINT "x1=";X1 : LPRINT X1 ' Setting for i.c. = 0.9 L100
650 END
```

APPENDIX 6

FORCE CALIBRATION RECORD SHEET

X(gm)	Y							
	NEAR				FAR			
Wt (g)	FORCE READING		RECORDER		FORCE READING		RECORDER	
0	1.92 2.01 2.10 2.15	2.05			1.83 2.41 2.49 2.55	2.31		
1	4.47 4.53 4.65 4.78	4.61			5.00 5.15 4.85 4.84	4.96		
2	7.05 7.13 7.62 7.37	7.29			7.29 8.59 8.65 7.83	8.09		
5	14.51 14.95 14.05 14.19	14.43			17.06 16.85 15.90 16.98	16.69		
R^2	0.9993				0.9997			
b	2.470				2.892			
alpha*	1.99				1.70			

***alpha = 1/b •9.81 • 0.5**

where *b* = slope of the regression line (units: reading/ gram)

***g* = gravitational acceleration (9.81 m • s^{-2} = 9.81 mN/ gram)**

(see *Force calibration*).

APPENDIX 7

Protein Assay

Standard: 1 mg/ml BSA made up in buffer. Range 0.2 mg/ml to 1 mg/ml protein.

Reagent package (Catalog No. 500-0116, Bio-Rad Laboratories Inc., Hercules, CA).

Reagent A: alkaline copper tartrate solution

Reagent B: dilute Folin Reagent

Reagent S.

Assay Protocol

1. Preparation of working reagent

Add 20 µl of reagent S to each ml of reagent A that will be needed for the run. This working reagent A′ is stable for one week. If samples do not contain detergent, step # 1 may be omitted and reagent A can be used as supplied.

2. Prepare 5 dilutions of protein standard containing from 0.2 to 1 mg/ml protein. A standard curve should be prepared each time the assay is performed. Always prepare the standards in the same buffer as the sample.

3. Pipette 20 µl of standards and samples into clean, dry plastic cuvettes with 1 cm path length matched to the laboratory spectrophotometer.

4. Add 100 µl of reagent A′ into each cuvette.

5. Add 800 µl reagent B into each cuvette and mix immediately.

6. After 15 minutes, absorbances can be read at 750 nm. The absorbances will be stable at least 1 hour.

APPENDIX 8

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

- **Reagents**

1. Acrylamide, electrophoresis grade
2. Bis-acrylamide (N, N´-methylenebisacrylamide)
3. Tris (2-hydroxymethyl-2-methyl-1, 3-propanediol)
4. SDS (sodium dodecyl sulfate or sodium lauryl sulfate)
5. TEMED (N, N, N´, N´-tetramethylene-ethylenediamine)
6. Ammonium persulfate
7. 2-mercaptoethanol
8. Glycerol
9. Bromophenol blue
10. Glycine
11. Hydrochloric acid (HCl)

- **Stock Solutions**

1. 2M Tris-HCl (pH 8.8), 100 ml

   Dissolve 24.2 g Tris base in 50 ml dH_2O. Adjust pH to 8.8 with 1 N HCl.

   Add dH_2O to a total volume of 100 ml.

2. 1M Tris-HCl (pH 6.8), 100 ml

   Dissolve 12.1 g Tris base in 50 ml dH_2O. Adjust pH to 6.8 with 1 N HCl.

   Add dH_2O to a total volume of 100 ml.

3. 10% (w/v) SDS, 100 ml, store at room temperature

   Dissolve 10 g SDS in 100 ml dH_2O.

4. 50% (v/v) glycerol, 100 ml

   Pour 50 ml 100% glycerol. Add 50 ml dH_2O.

- **Working solutions**

1. 30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide, 200 ml
   Mix 60.0 g acrylamide and 1.6 g N, N´-methylenebisacrylamide in a total volume of 200 ml dH_2O. Filter solution through a 0.45 µm filter and store at 4°C protected from light. Discard after 30 days.
2. 4X Tris-HCl/SDS (1.5M Tris-HCl/0.4% SDS), 100 ml
   Add 4 ml of 10% SDS to 75 ml 2M Tris-HCl (pH 8.8). Add dH_2O to 100 ml total volume.
3. 4X Tris-HCl/SDS (0.5M Tris-HCl/0.4% SDS), 100 ml
   Add 4 ml of 10% SDS to 50 ml 1M Tris-HCl (pH 6.8). Add dH_2O to 100 ml total volume.
4. 10% ammonium persulfate, 5 ml
   Dissolve 0.5 g ammonium persulfate in 5 ml dH_2O. Stable for months in a capped tube in the refrigerator.
5. Tris-glycine electrophoresis buffer, 1 litre
   25mM Tris-HCl/250mM glycine/0.1% SDS
   A 5X stock can be made by dissolving 15.1 g of Tris base and 94 g of glycine in 900 ml of dH_2O. Then, 50 ml of a 10% (w/v) stock solution of electrophoresis-grade SDS is added, and the volume is adjusted to 1000 ml with dH_2O.

6. 5x SDS gel-loading buffer, 10 ml
   60 mM Tris-HCl (pH 6.8) / 25% glycerol / 2% SDS / 0.1% bromophenol blue / 14.4mM 2-mercaptoethanol.
   0.6 ml 1 M Tris-HCl (pH 6.8), 5 ml 50% glycerol, 2 ml 10% SDS, 1 ml 1% bromophenol blue, 0.5 ml 2-mercaptoethanol. The volume is adjusted to 10 ml with dH_2O.

- **Preparation of the 7.5 % separating gel**

In a 50 ml conical tube, mix 7.5 ml of 30% acrylamide / 0.8% bis-acrylamide solution, 7.5 ml of 4X Tris-HCl/SDS, pH 8.8, and 15 ml of dH_2O. Add 100 µl of 10% ammonium persulfate and 20 µl of TEMED. Swirl gently to mix. Use immediately.

- **Preparation of the 4% stacking gel**

In a 50 ml conical tube, mix 2.6 ml of 30% acrylamide / 0.8% bis-acrylamide solution, 5 ml of 4X Tris-HCl/SDS, pH 6.8, and 12.2 ml of dH_2O. Add 100 µl of 10% ammonium persulfate and 20 µl of TEMED. Swirl gently to mix. Use immediately. The stacking gel is prepared in this way regardless of the acrylamide concentration used in the separating gel.