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THE ROLE OF THE RENIN-ANGIOTENSIN SYSTEM IN THE FETAL ORIGINS OF HYPERTENTION

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ABSTRACT

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By Rachel Caroline Sherman

Cardiovascular disease is a major cause of death throughout the world. Hypertension is a risk factor for cardiovascular disease, which is caused by a combination of genetic, environmental and lifestyle factors. It is hypothesised that maternal nutrition during pregnancy may also contribute to the risk of adult hypertension in her offspring. A rat model has been developed which supports this hypothesis. Rats are fed either a 9% casein (low protein) diet or an 18% casein (control) diet throughout pregnancy. The offspring of the low protein fed rats have elevated blood pressure in later life compared with controls. This thesis examines the role of the renin-angiotensin system in the development of the elevated blood pressure observed in this rat model.

Feeding a 9% casein diet during pregnancy led to a decrease in weight gain compared to rats fed a control diet, but did not effect litter size. The offspring were of low to normal birthweight, and had a tendency to gain more weight than control rats in later life. Systolic blood pressure was significantly elevated from 4 weeks of age in the rats exposed to a maternal low protein diet compared, with control rats.

Plasma renin activity, angiotensin II and angiotensinogen concentrations were unchanged in the offspring of rats fed a low protein diet compared with rats fed a control diet. Pulmonary angiotensin converting enzyme (ACE) activity tended to be higher in the low protein exposed rats from early life, compared with control rats. This difference was statistically significant by 12 weeks of age. Urinary prostaglandin E2 (PGE2) excretion was elevated in the offspring of rats fed a low protein diet. Urinary PGE2 excretion was also measured in a group of children whose birth characteristics were known. PGE2 concentration was inversely correlated with head circumference and ponderal index, which are markers of fetal growth.

Intervention with an ACE inhibitor (captopril) reduced the blood pressure in the low protein exposed rats to control levels during the treatment period. Early treatment with captopril or losartan (an angiotensin II receptor antagonist) prevented the onset of hypertension in this model and had no effect upon the blood pressures of the control rats. Treatment with an alternative anti-hypertensive drug (nifedipine), which does not target the renin-angiotensin system, had no effect upon the blood pressures of the low protein exposed rats.

This thesis supports the hypothesis that maternal diet during pregnancy programmes blood pressure in her offspring. The results show that the renin-angiotensin system is altered in this model of maternal-diet-induced hypertension. The results of the intervention studies are suggestive of a role for the renin-angiotensin system in the development of the elevated blood pressure that is observed in the low protein exposed rats. These findings also show that the development of elevated blood pressure in this model is preventable. This may have implications for future public health.

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Publications

Papers:

Langley-Evans SC, Welham SJM, Sherman RC, Jackson AA. Weanling rats exposed to maternal low protein diets during discrete periods of gestation exhibit differing severity of hypertension. *Clinical Science* 1996; **91**: 607-615.

Sherman RC & Langley-Evans SC. Early administration of angiotensin converting enzyme inhibitor captopril, prevents the development of hypertension programmed by intrauterine exposure to a maternal low protein diet in the rat. *Clinical Science* 1998; **94**: 373-381.

Langley-Evans SC, Sherman RC, Welham SJM, Nwagwu Gardner DS, Jackson AA. Intrauterine programming of hypertension: The role of the renin-angiotensin system. *Biochem.Soc.Trans.* 1999; **27**: 88-93.

Sherman RC, Jackson AA, Langley-Evans SC. Long term modification of the excretion of prostaglandin E2 by fetal exposure to a maternal low protein diet in the rat. *Ann. Nutr. Metab*; **43**: 98-106.

Sherman RC, Langley-Evans SC. Antihypertensive treatment in early postnatal life modulates prenatal dietary influences upon blood pressure, in the rat. *Clinical Science.* Submitted.

Abstracts:

Sherman RC, Langley-Evans SC. Elevated urinary excretion of prostaglandins in rats exposed to a maternal low protein diet. *Clinical Science* 1997; **92**: 13p.

Langley-Evans SC, Sherman RC, Welham SJM, Jackson AA. Fetal exposure to maternal low protein diets during discrete periods of pregnancy induces hypertension in the rat. *Clinical Science* 1997; **92**: 13p.

Sherman RC, Jackson AA, Langley-Evans SC. The role of the renin-angiotensin system in the intrauterine programming of hypertension. *The Proceedings of the 16th International Congress of Nutrition.* 1997: PR131.

Sherman RC, Jackson AA, Langley-Evans SC. The role of the renin-angiotensin system in the intrauterine programming of hypertension. *The Proceedings of the Nutrition Society* 1998; **57**: 79A.

Sherman RC, Jackson AA, Langley-Evans. The role of the renin-angiotensin system in the fetal origins of hypertension. *Clinical Science* 1998; **94**: 1p.

Sherman RC, Jackson AA, Langley-Evans SC. 15-Hydroxy-prostaglandin dehydrogenase activity is altered in a rat model of the fetal origins of adult hypertension. *The Proceedings of the Nutrition Society* 1998; **58**: 51A.

Sherman RC, Jackson AA, Langley-Evans SC. Early losartan treatment prevents the onset of hypertension in rats exposed to a maternal low protein diet in utero. *J. Endocrinology* 1999; **160**: P160.

Abreviations

ACE	angiotensin converting enzyme
ACTH	adrenocorticotrophin hormone
ANOVA	analysis of variance
AT	angiotensin II receptor
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
CHD	coronary heart disease
CO_2	carbon dioxide
CoV	coefficient of variance
CRF	corticotrophin releasing factor
CVD	cardiovascular disease
°C	degrees centigrade
D	day
DOCA	deoxycorticosteroid treated
G	gram
GPDH	glycerol-3-phosphate dehydrogenase
GS	glutamine synthase
HCl	hydrochloric acid
HHL	hippuryl-histidyl-leucine
HPA	hypothalmic-pituitary-adrenal
11β-HSD	11β-hydroxysteroid dehydrogenase
IGF	insulin-like growth factor
Kg	kilogram
L	litre
Mg	milligram
MmHg	millimetres of mercury
Mol	moles
MRC	Medical Research Council
N	number
NAD	nicotinomide adenine dinucleotide
NaOH	sodium hydroxide
ng	nanograms
NIDDM	non-insulin dependant diabetes mellitus
NS	not significant
OD	optical density
p	probability value
PDGF	platelet derived growth factor
pg	picagrams
PG	prostaglandin
PGDH	15-OH-prostaglandin dehydrogenase
pmol	picamoles
SDS	Special Diet Services
SEM	standard error of the mean
SHR	spontaneously hypertensive rat
St.dev	standard deviation
TAT	tyrosine aminotransferase

Chapter 1

Introduction

Non-communicable diseases are a major and increasing public health concern globally. They are caused by a combination of genetic, environmental and lifestyle factors. Investigation and determination of the factors that affect these diseases is important in order to provide information to aid strategies which may lead to their treatment and prevention. Epidemiological research has led to the recognition that fetal growth retardation may affect the risk of disease in later life. It is hypothesised that maternal nutrition may permanently programme the physiology of her offspring. The mechanisms, which may link maternal diet during pregnancy to disease in her offspring, are currently being investigated.

Hypertension is a major risk factor for cardiovascular disease, which is the most common cause of death in the western world and an increasing problem in developing countries. Epidemiological studies have shown a relationship between intrauterine growth retardation and an increased risk of hypertension in later life. A rat model has been developed which has provided evidence that supports the hypothesis that maternal diet during pregnancy programmes blood pressure in her offspring. Rats are fed either a 9% casein (low protein) diet or an 18% casein (control) diet during pregnancy. The offspring of rats fed a low protein diet have elevated blood pressures compared with control rats. This thesis contains a series of studies, the first of which aims to reproduce the rat model of maternal-diet-induced hypertension. The aim of the second set of studies is to characterise the renin-angiotensin system in the offspring of the low protein fed rats. The following chapter examines changes in prostaglandins that may relate to alterations of the renin-angiotensin system. Further studies concentrate more specifically on the role of angiotensin II in the development of high blood pressure in this model, by using targeted interventions.

Literature Review

1.1. Cardiovascular Disease

Cardiovascular disease (CVD) is the most common cause of death in most industrialised countries. In 1990, Coronary Heart Disease accounted for 27% of all deaths in the United Kingdom and stroke for 12%. CHD accounts for 30% of all male deaths in the UK and stroke for 9%, compared with 23% and 15% of deaths, respectively, for women (Department of Health, 1994). There are a number of factors, including genetic, environmental and behavioural factors, which are accepted to affect the risk of cardiovascular disease. One of the major factors which has been found to be directly related to the risk of CVD is blood pressure (Stamler *et al*, 1993). A linear relationship between CVD incidence and diastolic blood pressure was shown by MacMahon *et al* (1990) in an overview analysis of 9 prospective studies, which together followed approximately 420,000 adults for an average of 10 years. These results were found to be independent of sex, age, and ethnicity. MacMahon and colleagues also found that a sustained reduction of raised blood pressures with hypotensive agents significantly reduced the incidence of CVD. A 5-6mm Hg reduction in diastolic pressure was associated with a 14% reduction in acute coronary events. The British regional heart study sampled 7735 men from 24 towns and found strong associations between town mean blood pressure and town cardiovascular mortality rates, particularly for systolic blood pressure (Shaper *et al*, 1981).

1.2. Blood pressure

The circulatory system serves to transport and distribute essential substances to the tissues and to remove by-products of metabolism. It also shares in such homeostatic mechanisms as regulation of body temperature, humoral communication throughout the body and adjustments of oxygen and nutrient supply in different physiological states. The cardiovascular system consists of a pump (the heart), a series of distributing and collecting tubes (arteries and veins) and an extensive system of thin vessels that permit rapid exchange between the tissues and the vascular channels (capillaries). The heart

consists of two pumps in series: one to propel blood through the lungs for exchange of oxygen and carbon dioxide and the other to propel blood to all other tissues of the body. Blood is the vehicle of transportation. It is a suspension of various types of cells in a complex aqueous medium, the plasma. The elements of blood serve multiple functions essential for metabolism and the defence of the body against injury (Berne & Levy, 1988).

Blood pressure is the pulsating pressure of blood on the walls of the main arteries, and it is measured both at its highest pressure (systolic pressure), which occurs when the heart is contracting, and at the lowest pressure (diastolic pressure) when the heart is relaxed. Blood pressure can be measured using a number of methods, including direct methods such as a catheter or needle being introduced into peripheral arteries of patients so that arterial blood pressure can then be measured by means of strain guages. Ordinarily, however, it is estimated indirectly by means of a sphygmomanometer. This involves inflating an inextensible cuff, usually around the upper arm to occlude the artery then listening for the sound of the blood flow returning whilst allowing the cuff to deflate. The pressure at which the first sound is detected represents the systolic blood pressure, and the point at which the sounds disappear indicates the diastolic pressure.

Blood pressure is the relationship between cardiac output and total resistance in the peripheral blood vessels and is regulated by a number of mechanisms. Short term variations in blood pressure are detected by neural mechanisms such as chemoreceptors and baroreceptors which are sensitive to changes in blood gas saturation and pressure or volume in the vasculature (Watson *et al*, 1980). Long term blood pressure is controlled primarily by the kidney which controls blood volume and by local hormonal systems and metabolites (Guyton, 1991).

Blood pressure is a continuous variable for which the distribution differs between populations and is dependent upon age, gender, race and environmental factors. For these reasons it is difficult to classify blood pressure as 'normal' or 'high'. It is

convenient to classify blood pressure for purposes of diagnoses and treatment but important to remember that the classifications are arbitrary (Materson, 1987). Hypertension is considered to be a blood pressure at which the individual is at an increased risk of cardiovascular disease, but it is important to recognise that the risk of cardiovascular disease increases throughout the normotensive range.

1.3. Hormonal control of blood pressure

Blood pressure is controlled by an interaction of multiple systems. The factors that affect blood pressure are cardiac output, blood volume and peripheral resistance. Blood volume may be affected by haemorrhage or salt intake, and therefore water retention, for example. The determinants of peripheral resistance are viscosity of blood and blood vessel diameter. Viscosity is affected by the number of red blood cells in relation to solutes and fluid. Dehydration therefore tends to cause an increase in blood pressure whereas; anaemia or haemorrhage tend to lead to a decrease in blood pressure. Blood vessel diameter can be affected by certain chemicals (hormones). For example adrenalin, antidiuretic hormone, and angiotensin II cause vasoconstriction, while prostaglandins, kinins and histamine cause vasodilation. These hormones also interact with the kidney to control blood pressure. Renin is released from the macula densa in response to either decreased sodium delivery or a decrease in pressure in the afferent arteriole. This results in the production of angiotensin II and then aldosterone, which leads to increased sodium reabsorption in the distal convoluted tubule and expansion of the extracellular fluid volume. Increased extracellular fluid osmolality leads to the release of anti-diuretic hormone, which causes water retention by the kidney in order to increase volume and pressure. Angiotensin II also causes vasoconstriction of the efferent arteriole, which leads to an increase in glomerular filtration rate and therefore an increase in urine excretion, decreasing plasma volume and blood pressure. High blood pressure increases renal blood flow which also leads to increased glomerular filtration rate and therefore a decrease in pressure.

1.3.1. The Renin-angiotensin system

The renin-angiotensin system is a system of hormones and enzymes of renal origin involved in the control of blood pressure (figure 1.2). Renin is an enzyme, which is formed and stored in granules in the juxtaglomerular cells, of the afferent and efferent arterioles of the kidney. It acts on an α_2-globulin (angiotensinogen), carried by the plasma, to cleave off the decapeptide angiotensin I. This is then split to form the octapeptide angiotensin II in the presence of angiotensin converting enzyme (ACE) and chloride ions. Angiotensin II is a potent vasoconstrictor and is converted, in the adrenal cortex, by a peptidase to the heptapeptide angiotensin III, which stimulates the secretion of aldosterone from the adrenal cortex (Campbell, 1992, Bader *et al*, 1994).

Renin release is stimulated by low plasma sodium content and reduced stretch of the juxtaglomerular cells (caused by reduced blood pressure in the afferent arterioles). Salt loading decreases renin activity whilst salt depletion increases it (Laragh, 1992). These changes in renin activity are accompanied by corresponding changes of the content of renin and renin mRNA in the kidney (Barrett *et al*, 1989). Stimulation of renal sympathetic nerves induces renin release via activation of β-adrenergic receptors on the juxtaglomerular cells (Dibona, 1982). A mild increase in renal sympathetic nerve activity, induced by carotid occlusion, stimulated renin secretion in conscious dogs (Kirchheim *et al*, 1987). Renin release also appears to be mediated by local prostaglandins. Prostaglandins have been demonstrated to directly stimulate the release of renin from in vivo and in vitro kidney preparations (Jackson *et al*, 1982, Wagner & Kurtz, 1998). Renin release and subsequent angiotensin II formation affect vascular tone, sympathetic nerve activity, adrenal steroid release and central nervous system control of blood pressure. Generally angiotensin II formation is regulated so as to stabilise blood pressure and extracellular fluid volume (Griendling & Alexander, 1994, Berne & Levy, 1988, Campbell, 1992).

All of the main effects of angiotensin II (shown in figure 1.1) are likely to result in an expansion of extracellular fluid volume and an increase blood pressure.

Figure 1.1. The main effects of angiotensin II

■ Angiotensin II and angiotensin III stimulate the release of aldosterone from the adrenal cortex, thus enhancing distal tubular and collecting duct sodium reabsorption (Laragh *et al*, 1960).

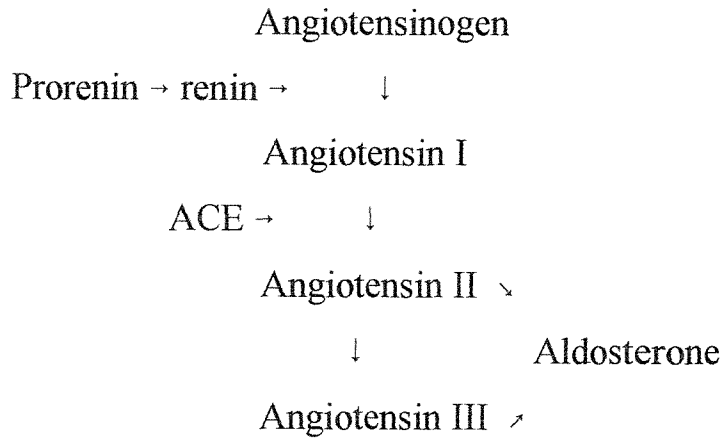
■ Angiotensin II causes systemic vasoconstriction (Griendling & Alexander, 1994).

■ Angiotensin II stimulates the release of anti-diuretic hormone from the posterior pituitary (Dzau & Pratt, 1986).

■ Angiotensin II acts on sympathetic nerve terminals to enhance neurotransmitter release (Levens *et al*, 1981).

■ Angiotensin II acts within the brain to stimulate thirst and thereby increase water intake (Griendling & Alexander, 1994).

Figure 1.2. The renin-angiotensin system



Angiotensin II apparently exerts a direct feedback inhibition of renin release (Hackenthal *et al*, 1990) and stimulates the release of local prostaglandins (Currie & Needleman, 1984, Wagner & Kurtz, 1998), which should reduce the effect of angiotensin II on blood pressure.

Angiotensin II reacts with specific receptors on the cell surface. There are two major sub-types of angiotensin II receptor known as the AT_1 and AT_2 receptors. AT_1 receptors have been shown to mediate contraction of blood vessels, aldosterone secretion, water drinking and hypertension in renal artery stenosis (Griendling & Alexander, 1994). They are abundant in the liver, kidney, aorta, adrenal and lung (Murphy *et al*, 1991). The physiological effects of the AT_2 receptor are unknown, however they are more abundant in embryonic and neonatal tissues than in adult (Grady *et al*, 1991), suggesting that they may have a role in development.

In the studies of the renin-angiotensin system in patients with hypertension, plasma renin activity has been found to show an inverse association with systolic blood pressure (Meade *et al*, 1983). A small proportion of patients with essential hypertension show plasma renin levels above the range found for normotensive controls (Thurston *et al*, 1978). In rats polymorphisms of the renin gene have been shown to co-segregate and be associated with raised blood pressure (Rapp *et al*, 1989). However, studies in humans have found no associations between the renin polymorphisms and hypertension (Soubrier *et al*, 1990). Association of polymorphisms at the angiotensinogen locus and hypertension have been observed (Jeunemaitre *et al*, 1992). Genetic studies of the angiotensin converting enzyme have again shown discrepancies between rat models and humans. Co-segregation of blood pressure with a marker for the ACE locus has been found in rats (Hilbert *et al*, 1991) but not in humans (Jeunemaitre *et al*, 1992). The therapeutic value of drugs which interfere with the renin-angiotensin system, such as ACE inhibitors, angiotensin receptor antagonists and renin inhibitors, have shown the importance of the system in blood pressure regulation (Swales, 1994).

It now apparent that as well as the circulating renin-angiotensin system there are also several local tissue angiotensin II producing systems. These have been recognised in the brain, kidney, adrenal and the arterial wall (Campbell, 1987). Angiotensinogen mRNA has been detected in the kidney, brain, lung, adrenal and stomach (Campbell, 1987) and renin mRNA has been detected in the adrenal, heart and testes (Campbell, 1987).

The renin-angiotensin system has also been shown to be active during intrauterine life (Lumbers 1995) which may be important in the programming of hypertension in later life. Angiotensin II receptors are widely distributed in the fetal mouse (Lumbers, 1985). The most commonly expressed angiotensin II receptor in the mouse and rat embryo is the AT_2 receptor, for which there is no known action (Timmermans *et al*, 1993). The distribution of renin in the fetal kidney is widespread in comparison to the adult kidney (Gomez *et al*, 1988). The placenta has levels of ACE that are equivalent to the adult lung and is probably a major site of conversion of angiotensin I to angiotensin II (Raimbach & Thomas, 1990). Angiotensin II has a pressor effect in the fetus but large doses are required (Lumbers, 1995). This is probably due to the removal of angiotensin II by the placenta (Lumbers & Reid, 1979). Blocking the renin-angiotensin system with an ACE inhibitor leads to a fall in arterial pressure in the fetus (Lumbers *et al*, 1992). Treatment with ACE inhibitors during pregnancy has been associated with human neonatal renal failure (Shubiger *et al*, 1988), suggesting that the fetal renin-angiotensin system plays an essential role in the maintenance of fetal renal function.

Glucocorticoids have been well-characterised as having a programming effect upon the developing fetus (Langley-Evans, 1997) and they influence the actions of and are influenced by the renin-angiotensin system. Glucocorticoids have been shown to induce the expression of the angiotensin II type 1 receptor in vascular smooth muscle cells (Sato *et al*, 1994). A local renin-angiotensin system is found in the adrenal cortex (Robertson & Nicolls, 1993), where its exact functions are unknown, but it is possible that it influences the release of adrenal hormones.

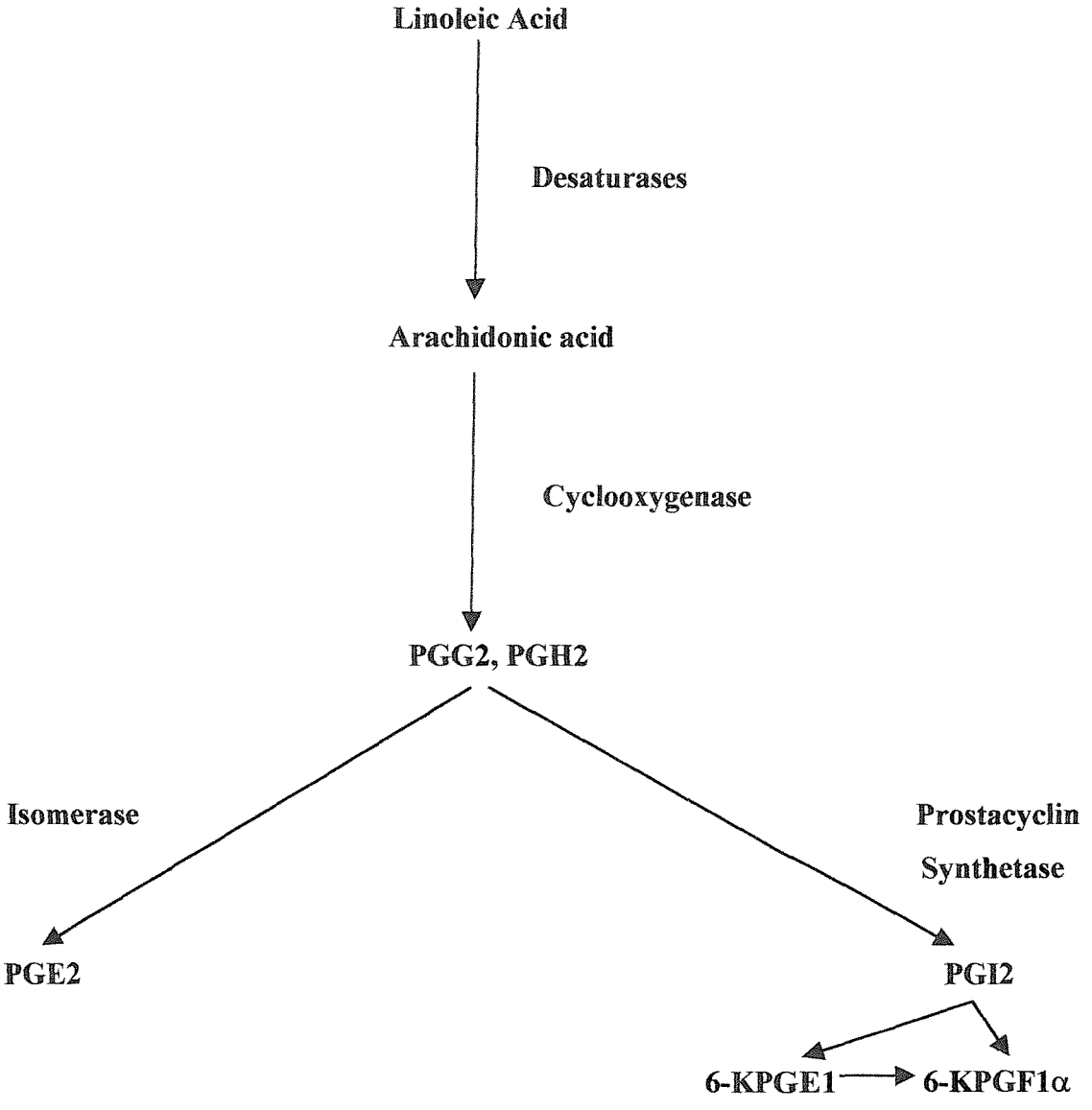
1.3.2. Prostaglandins

Prostaglandins are local hormones that are derived from polyunsaturated fatty acids in cell membranes (figure 1.3) and effect a wide variety of physiological processes. Renal prostaglandins are known to mediate blood flow and its distribution within the kidney and their interactions with the renin-angiotensin system are of particular interest in hypertension. Prostaglandins PGE_2 and PGI_2 are potent renal vasodilators and help to maintain renal blood flow, specifically in the presence of vasoconstrictor hormones such as angiotensin II (Moncada & Vane, 1978, Farrario & Flack, 1996). Angiotensin II stimulates the release of PGE_2 and PGI_2 by the kidney, which subsequently blunts the peptide-induced vasoconstriction (Farrario & Flack, 1996). PGE_2, PGI_2 and 6-keto-PGE_1 have a stimulatory effect on the release of renin, which is thought to be mediated by cyclic AMP (Jackson *et al*, 1982, Wagner & Kurtz, 1998).

The pathway leading to the conversion of polyunsaturated fatty acids to prostaglandins is dependent upon the activity of desaturases. The activities of desaturases involved in the conversion of linoleic acid to PGE_2 have been shown to be reduced in animals subjected to a maternal low protein diet during pregnancy and lactation (De Thomas *et al*, 1980 & 1983, Narce *et al* 1991). Protein restriction during pregnancy and lactation similarly exerts a marked effect on the fatty acid composition of liver membrane phospholipids. A significant decrease in both arachidonic acid and docosahexaenoic acid was found. This fatty acid pattern was reversed when protein deficient animals were placed on a control diet at birth (De Thomas *et al*, 1980 & 1983, Narce *et al* 1991).

Spontaneously hypertensive rats exhibit elevated desaturase activity and apparent changes in overall hepatocyte fatty acid profiles. Six month old SHRs have been found to have particularly high desaturase activities and the hepatocyte fatty acid composition indicated an impairment of n-6 polyunsaturated fatty acid metabolism (Narce *et al*, 1994). 15-Hydroxyprostaglandin dehydrogenase is considered to be the key enzyme in the degradation of prostaglandins (Tai *et al*, 1979, Nagai *et al*, 1991). Decreased activity of

Figure 1.3. Prostaglandin metabolism



this enzyme has been found in the kidneys of spontaneously hypertensive rats (Tai *et al*, 1979), which may suggest an overall decreased flow through the pathway.

Many studies indicate an association between high blood pressure and abnormalities in urinary prostanoid excretion (Nasjletti & Baer, 1988). The two-kidney one-clip rat model of hypertension exhibits increased urinary PGE_2 excretion (Lahera *et al*, 1986), whilst in spontaneously hypertensive rats excretion is low (Martineau *et al*, 1984). Urinary excretion of 6-keto-PGF1α is increased in the lyon strain of genetically hypertensive rat (Benzoni *et al*, 1984) and in rats with angiotensin II induced hypertension (Diz *et al*, 1983) but not in rats with two-kidney one-clip hypertension (Lahera *et al*, 1986). Urinary excretion of PGE_2 is increased in patients with renovascular hypertension (Zia *et al*, 1978).

1.4. Hypertension

The Joint National Commission report (1993) defines hypertension as 140 mmHg and above for systolic blood pressure and 90 mmHg and above for diastolic blood pressure. The report also categorises hypertension in to 4 groups of severity, as shown in table 1.1.

Hypertension is a disorder of regulatory mechanisms which leads to abnormalities of the heart and vascular system and damage to other organs. Essential hypertension, or hypertension of an unknown origin accounts for the majority of cases of elevated blood pressure in humans and is considered to be a multifactorial disease (Krakoff, 1994). Considering the complexity and the number of systems involved in the control of blood pressure it is not surprising that it is difficult to pin-point the causes of hypertension. The major contributing factors are those which underlie predisposition such as genetics and structure, those that operate to expose predisposition, such as sodium and alcohol intake, obesity, exercise, smoking and psychosocial stress, and those that operate to maintain exposure such as renal disease and defects in steroid metabolism e.g. aldosteronism (Swales, 1994).

Table 1.1. Categories of Human Hypertension

Category of hypertension	Systolic Pressure (mmHg)	Diastolic Pressure (mmHg)
Stage 1	140-159	90-99
Stage 2	160-179	100-109
Stage 3	180-209	110-119
Stage 4	210-	120-

1.4.1. Genetic factors relating to hypertension

A family history of hypertension has been shown to strongly predict the future risk of developing hypertension in family members, suggestive of a genetic or hereditary component (Hunt *et al*, 1986). It is important to remember that immediate family members are also exposed to similar environmental conditions to one another and that family histories are often difficult to acquire, making it difficult to differentiate genetic effects from environmental effects. Taking into account these influences, people with a positive family history for increased blood pressure have been used to study possible genetic abnormalities. Animal models are also being used. In rats polymorphisms of the renin gene and the angiotensin-converting enzyme gene have been shown to be associated with higher blood pressure (Rapp *et al*, 1989, Hilbert *et al*, 1991), however it has not been possible to reproduce these findings in humans (Morris & Griffiths, 1988, Jeunemaitre *et al*, 1992). Evidence for an effect of both the angiotensinogen gene and the aldosterone gene has been recognised in human studies (Jeunemaitre *et al*, 1992, Manatunga *et al*, 1992). There are many forms of hypertension associated with disorders of mineralocorticoid metabolism that are reported to be familial (Gordon *et al*, 1994). The genes encoding many of the steroidogenic enzymes have been characterised. The syndrome of apparent mineralocorticoid excess is due to a defect of renal 11β-hydroxysteriod dehydrogenase, which leads to an excess of cortisol at the distal nephron (Stewart *et al*, 1988). It is likely that there are several different gene mutations, which

could contribute towards hypertension and may interact with each other and it is important also to consider interactions with environmental factors.

1.4.2. Environmental and lifestyle factors relating to hypertension

Environmental and lifestyle factors, particularly dietary intake, are potentially the easiest determinants of hypertension to control or change. Epidemiological studies in 27 different populations have shown correlations between salt intake and blood pressure (Glieberman, 1973). This has been supported by evidence from intervention studies, where restriction of salt intake has led to a decrease in blood pressure (McGregor *et al*, 1987). Alcohol consumption has also been shown to be related to blood pressure with high intakes being associated with elevated blood pressure, but in groups consuming moderate levels intakes are associated with a lower blood pressure than those who consume no alcohol at all (Gordon & Kannel, 1983). Blood pressure is associated with body weight, and obesity is considered to be one of the best predictors of risk of developing hypertension (Gordon & Kannel, 1976). Non-insulin dependant diabetes, obesity and hypertension are all strongly associated with each other (Pi-Sunyer, 1991) and it is suggested that abnormalities of insulin metabolism may cause an elevation of blood pressure either through stimulation of the sympathetic nervous system or increases renal sodium retention (Reaven & Hoffman, 1987).

1.4.3. Renal disease and hypertension

The majority of patients suffering from renal disease will in time develop hypertension (Wilkinson, 1994). This is due to the kidneys intimate involvement with the control of blood pressure through its control of fluid volume and through the renin-angiotensin system. When hypertension co-exists with renal disease or renal artery stenosis, the renal defect is usually considered to cause the hypertension (Wilkinson, 1994). Renal artery stenosis is usually caused by atherosclerosis, or fibromuscular dysplasia in young people (Bookstein *et al*, 1972). It results in reduced blood flow to one or both of the kidneys, which leads to hypertension and then causes further damage to the kidneys. It is suggested that hypertension causes glomerulosclerosis, however the mechanisms are not

clearly understood. Ketteler *et al* (1995) suggest that transforming growth factor-β (TGF-β) may be the "missing link" between altered haemodynamics and the development of glomerulosclerosis. TGF-β is a fibrogenic cytokine, which has been associated with the development of glomerulosclerosis. It has been demonstrated that TGF-β gene expression is induced by angiotensin II and its over-expression leads to chronic tissue fibrosis (Ketteler *et al*, 1995). Over-expression of TGF-β has been associated with the progression of kidney disease in both humans and experimental models (Yamamoto *et al*, 1993). Treatment with ACE inhibitors shows preventative effects on the course of many renal diseases (Brunner, 1992).

1.4.4. Maintenance of hypertension

Folkow (1978) proposed that following exposure to an initial condition, which results in hypertension, the raised blood pressure may be maintained and amplified through a positive feedback mechanism involving structural vascular adaptation induced by a change in pressure. The positive adaptation suggested was that, following initial exposure to high blood pressure, hypertrophy of vascular smooth muscle occurs which results in further elevation of blood pressure. This is supported by evidence from spontaneously hypertensive rats, which do not develop hypertension long term if they receive a brief period of early treatment (Harrap *et al*, 1990). Lever and Harrap (1992) have proposed that changes in vascular cell structure may be initiated in early development by angiotensin II, growth hormone, cortisol or insulin-like growth factors. In a study by Anderson *et al* (1993) angiotensin II induced TGF-β and collagen synthesis. TGF-β has been associated with cell growth that leads to smooth muscle cell hypertrophy (Gibbons *et al*, 1992). Angiotensin II has been shown to stimulate hypertrophic growth of vascular smooth muscle cells (Itoh *et al*, 1993). In studies of vessel structure and blood pressure the vessel radius was found to increase rapidly with small pressure increments until a point where it becomes less distensible (Berry 1978). At low pressures the elastic arteries act like a tube of elastin, but as pressures increase there is an increased stiffness which presumably reflects an increased recruitment of collagen (Berry 1978). Martyn and Greenwald (1997) hypothesised that an impairment

of elastin synthesis during a critical period of blood vessel development may underlie the association between intrauterine growth retardation and an increased risk of developing higher blood pressure in later life.

1.5. Treatment of hypertension

Hypertension can be treated by both pharmacological and non-pharmacological methods, and a combination is usually recommended. Non-pharmacological treatments include weight reduction, dietary salt and alcohol restriction, smoking cessation and increasing activity. Dietary supplements of potassium, calcium, magnesium or fish oil may also have small effects on reducing blood pressure. Anti-hypertensive drugs are classified in Table 1.2 (Krakoff, 1994).

Table 1.2. Classification of anti-hypertensive agents.

Classification	Anti-hypertensive agents
A	ACE inhibitors, antiadrenergic agents, α-receptor blockers, angiotensin II receptor antagonists.
B	β-blockers
C	Calcium antagonists, central active agents
D	Diuretics, dilating agents

Drug selection is dependent on several different factors such as the age, ethnicity and blood pressure of the patient. Any evidence of glucose intolerance or allergies are taken into account as well as any clinical signs which may give clues as to the mechanism of hypertension (Krakoff, 1994). Anti-hypertensive drugs are used either on their own or in combination.

ACE inhibitors

ACE inhibitors are commonly used in the treatment of hypertension. Their action is not specific and they have other effects that may also influence blood pressure, though their main action is thought to be the inhibition of ACE, causing a reduction in the production

of the vasoconstrictor angiotensin II which leads to a reduction of the effects of the peptide on vascular tone, aldosterone release and renal sodium handling.

The mechanism of action of ACE inhibitors is by competitive inhibition of ACE which are directed to the active site of the enzyme where they complex with the zinc ion (Figure 1.4) (Shapiro & Riordan 1984). The difference in potency and duration of action of different ACE inhibitors may be explained by the differences in binding characteristics (Figure 1.4) (Gohlke & Unger 1994). The different ACE inhibitors are classified into three general chemical groups as shown below in Table 1.3 and Figure 1.4.

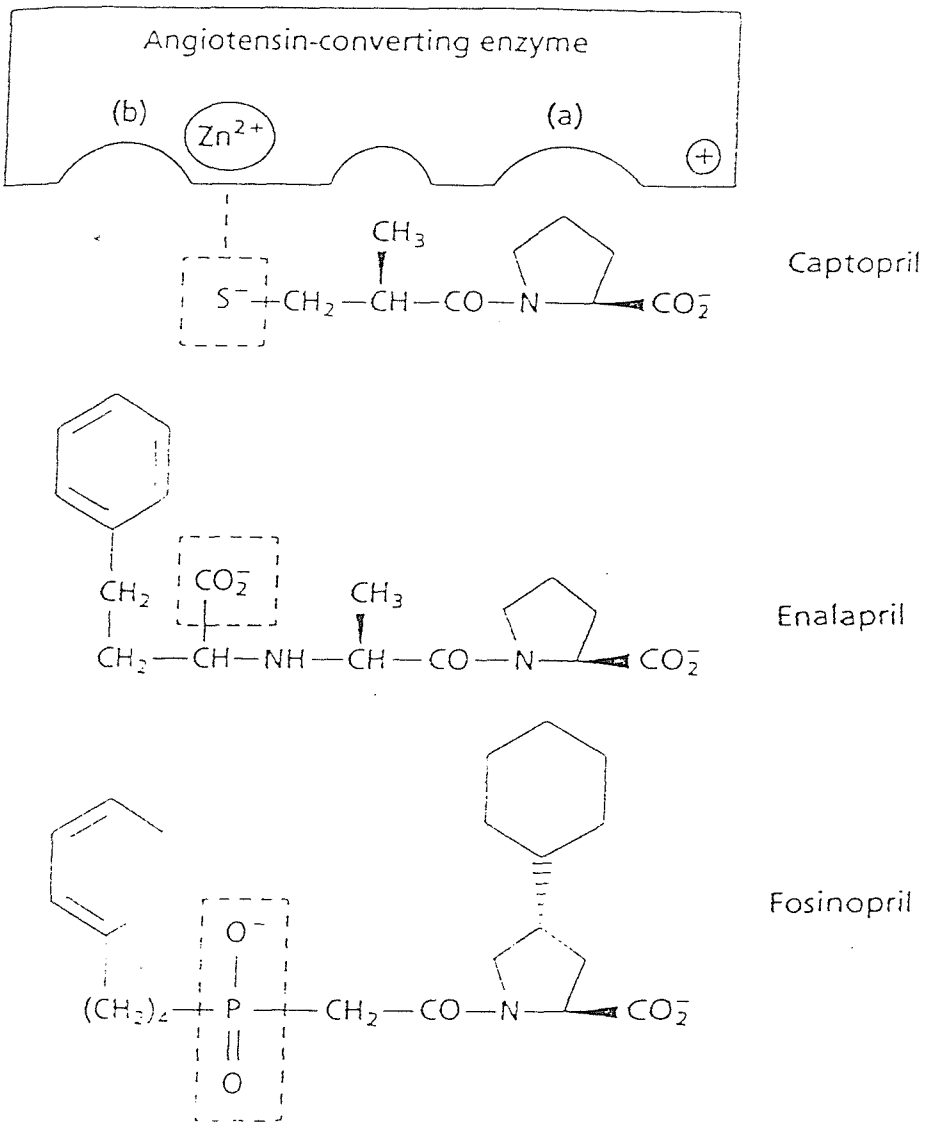
Table 1.3. Classification of ACE inhibitors

Chemical classification	Example
Sulphydryl-containing	Captopril
Carboxyl-containing	Enalapril
Phosphorus-containing	Fosinopril

The action of ACE (also known as kinase II) is not specific to the renin-angiotensin system and the enzyme also has a role in the inactivation of bradykinin as well as of other biologically active peptides. Treatment with ACE inhibitors therefore may lead to increased bradykinin levels which may result in the direct effect of vasodilation or the stimulation of prostaglandin production (Brunner *et al*, 1987). Captopril has been shown to significantly increase prostaglandin levels, for example urinary PGE_2 levels are elevated with captopril treatment as are plasma 6-keto-$PGF_{1\alpha}$ concentrations (Swartz *et al*, 1980). Captopril is also a thiol containing compound and a free radical scavenger (Sunman *et al*, 1993) which are reported to combine with nitric oxide to produce nitrosothiols which are potent relaxants of smooth muscle (Henry *et al*, 1989). The above actions may all lead to a reduction in blood pressure.

Figure 1.4 Hypothetical model of ACE inhibition

Reproduced from Gohlke & Unger (1984)



Spontaneously hypertensive rats do not develop hypertension if treated with an ACE inhibitor in early life (Harrap *et al*, 1990). This is thought to be because hypertension and/or high angiotensin II concentrations at an early age cause structural vascular changes. These changes are thought to be prevented by early captopril treatment given at the time (or before the time) when hypertension usually begins to be observed (Harrap *et al*, 1990). This effect is also reproduced with losartan, which is an angiotensin II receptor antagonist (Morton, 1992). It is thought that angiotensin II may function as a growth factor or modulator which may play a role in the development or maintenance of vascular smooth muscle hypertrophy (Schelling *et al*, 1991). Early captopril treatment has been shown to inhibit vessel growth in rats (Marjorie *et al*, 1996)

ACE inhibitors are established therapeutic agents in the treatment of hypertension and have many advantages over other anti-hypertensive agents. They can be used long term without the development of tolerance and they do not negatively effect the metabolism of glucose and lipids or induce reflex sympathetic activation (Gohlke & Unger, 1994). The common side effects observed with ACE inhibitor treatment are headache, cough and hypotension (Gohlke & Unger, 1994, Semple, 1995).

Captopril also possesses some beneficial side effects such as reducing arthritis, diabetic retinopathy, atherosclerosis and cancers which are angiogenesis dependent (Kleinman & Ponce, 1996).

Other anti-hypertensive drugs

There are other classes of anti-hypertensive drug which act primarily on the renin-angiotensin system, such as renin inhibitors and angiotensin II antagonists. These are not yet as widely used as ACE inhibitors but interest is growing in other methods of blocking the renin-angiotensin system due to the lack of specificity of the ACE inhibitors and the associated side effects. Losartan is a non-peptide angiotensin II receptor antagonist which has been shown to be effective in the treatment of hypertension in both animal and human studies (Burnier *et al*, 1996).

Diuretics have been used in the treatment of hypertension for several years. The main type used are the thiazide-like diuretics which work by acting on the distal renal tubule to cause a net loss of sodium and water. The reduction in arterial pressure is limited as they cause activation of the renin-angiotensin system (Krakoff, 1994).

Furosemide is a loop diuretic, which reduces arterial pressure by causing a loss of salt and water, but like the thiazides they activate the renin-angiotensin system. Loop diuretics appear to be more effective in treating patients with renal insufficiency and are less likely to impair glucose tolerance than the thiazides (Krakoff, 1994).

Reserpine is an example of a central antiadrenergic agent which reduce arterial pressure by diminishing sympathetic tone. Its side effects include sedation and severe depression as well as nasal congestion (Krakoff, 1994). The central antiadrenergic drugs are less popular than other antihypertensives due to the adverse symptoms and reactions that they cause.

Peripheral antiadrenergic drugs include neuron depletors, which deplete peripheral adrenergic neurons of noradrenalin, β-receptor blockers, which reduce sympathetic activity by competitive inhibition, and α-receptor blockers, which inhibit the action of neuronally released noradrenalin (Krakoff, 1994).

Calcium receptor antagonists e.g. nifedipine, nicardipine decrease blood pressure primarily by a reduction in peripheral resistance (Anderson *et al*, 1989) as well as causing a mild diuresis.

Due to the multi-factorial nature of hypertension some drugs will only work in certain individuals and antihypertensives are often used in combination. Additive effects are often observed when using a combination of drugs in the treatment of hypertension.

1.6. Fetal origins of hypertension

A number of epidemiological studies carried out by Barker and colleagues led to the hypothesis that the nourishment a fetus receives from its mother determines its susceptibility to disease in later life. A review of 34 studies by Law & Sheill (1996) concluded that birthweight is inversely related to blood pressure in both adults and children.

The first group of studies carried out by Barker's group (Barker & Osmond, 1986) related deaths from CVD and chronic bronchitis to infant and maternal death rates around the time of birth or infancy, of the deceased people studied. These studies were geographically based, and the assumptions made were that the people who had recently died would have been exposed to the same risk of adverse maternal or perinatal factors that determined the neonatal death rate in these areas at a particular time. A study carried out by Barker and Osmond (1986) showed a high correlation between infant mortality and death from ischaemic heart disease. Correlations were also found with other diseases such as chronic bronchitis and some cancers (Barker & Osmond, 1986).

Subsequent retrospective analysis of populations in a later study by Barker *et al* (1989a) found mortality from ischaemic heart disease to be related to both neonatal and postnatal mortality in England and Wales, suggesting that intrauterine and early postnatal environments affect the risk of CVD in later life. In further studies a relationship between birth weight and infant weight and death from ischaemic heart disease was observed (Barker *et* al, 1989b). This led to research into the relationship between growth *in utero*, blood pressure in childhood and adult life and mortality from CVD. Data from two different cohort studies was used.

The first cohort was a follow up study of 5362 people born in 1946, in England, Wales or Scotland. Birth weights and mothers heights were taken from maternity records, and in the last follow up study, when the population were aged 36 years, heights, weights, blood pressures and pulse rates were measured in the subjects who were contacted (86% of the

sample) (Barker *et al*, 1989a). The second cohort study used, followed subjects born in 1970 and included measurements of birth weight, gestational period and mothers blood pressure. When the subjects were ten years old measurements of heights, weights, blood pressure and pulse rates were made. An inverse relationship was found between systolic blood pressure at 10 years old, and birth weight. At 36 years old this relationship was stronger (Barker *et al*, 1989b).

In 1990, Barker *et al* used records from a Lancashire hospital and a follow up study to determine the relationship between fetal and placental size and the risk of hypertension in adult life. The hospital records contained details of the birth weights, placental weights, crown to heel length and head circumference of all babies born after 1932. In the follow up study details were taken on medical history, current medication, smoking habits, alcohol intake, socioeconomic group and family history of CVD. Blood pressure measurements were also made at this time. The findings of this study were that systolic and diastolic blood pressures were strongly related to placental weight. For any given birth weight, blood pressures rose with placental weight and the highest blood pressures were found in those people who had been small babies with large placentas. These results were independent of current BMI's and alcohol consumption which were also related to blood pressure, although this relationship was not as strong. Large placentas at any birth weight were related to a decrease in the ratio of length to head circumference. This disproportionate growth is considered to be consistent with a diversion of blood away from the trunk to the brain and this could therefore cause irreversible damage to the fetus, which could affect the arterial structure and consequently the blood pressure in later life. Disproportionate growth and large placentas were later linked to low social class, low haemoglobin levels (Barker *et al*, 1992) and nutritional deficiencies in the mother (Margetts *et al*, 1991).

The findings by Barker have been supported by studies carried out by many different groups. The results have been reproduced in other populations such as Jamaica, India, USA and Sweden (Forrester *et al*, 1996, Stein *et al*, 1996, Rich-Edwards *et al*, 1995,

Curhan *et al*, 1996, Gennser *et al*, 1988) and also supported by studies in children (Law *et al*, 1991, Whincup *et al*, 1989). Godfrey *et al* (1994) have shown a relationship between maternal nutritional status during pregnancy (assessed by measuring triceps skinfold thickness and weight gain during pregnancy) and blood pressure in childhood.

More recent studies have shown relationships between carbohydrate and protein intake during pregnancy and placental and fetal growth (Godfrey *et al*, 1996, Godfrey *et al*, 1997). High carbohydrate intakes in early pregnancy and low dairy protein intakes in late pregnancy were found to be associated with lower placental and birth weights and lower ponderal index (Godfrey *et al*, 1996, Godfrey *et al*, 1997). In another study Campbell *et al* (1996) found a complex biphasic relationship between maternal protein and carbohydrate intake and blood pressure in the offspring. A low animal protein intake (less than 50g daily) along with a high carbohydrate intake during pregnancy was associated with a higher blood pressure in the offspring some 45 years later. An increased blood pressure in the offspring was also associated with a combination of a higher protein intake (above 50g per day) and a lower carbohydrate intake. These increases in blood pressure were associated with a decreased placental size. Both studies showed small changes in relation to complex variations in dietary intake.

The above work led to the development of the 'fetal origins' hypothesis, which states that the nutritional environment of the mother may permanently 'program' the structure and physiology of her offspring.

1.7. Programming

Programming is the process whereby a particular stimulus in the fetus or neonate, which acts during a sensitive period of development, establishes a long term or permanent biological response (Lucas 1991). Evidence for programming, outside the field of nutrition, has been available for some considerable time. For example the development of the hypothalamus in the rat brain is initially established in a female pattern i.e. to secrete gonadotrophins in a cyclical pattern. The release of testosterone from the male

rats developing testes at a specific point in development (day 5), programmes the rat brain so that gonadotrophins are secreted in a male (acyclic) fashion and the animals behaviour is male orientated (Harris & Levine, 1962). If female rats are treated with testosterone during this period they develop as genetic females but do not ovulate or show female sexual behaviour (Barraclough 1961). Treatment with testosterone after this 'sensitive period' does not have the same masculinizing effect on the female rat (Barraclough 1961).

Injections of androgens into the neonatal rat affect the androgen responsiveness of hepatic enzyme activities and steroid binding proteins, and this effect is permanent (Gustafsson & Stenburg, 1974, Sloop *et al*, 1983). Other hormones such as thyroxine are known to affect early brain development and the subsequent function of the hypothalamus (Brasal & Boyd, 1975). There are other examples of programming which are not primarily hormonal but involve other stimulus which are environmental or sensory, such as a duckling learning to follow the moving object it sees after hatching.

In all of the above examples, the timing of the stimulus is very important. This brief period of time in which the animal is sensitive to the stimulus was described as a 'critical period of development' by Widdowson & McCance (1975). Other terms used have been the 'vulnerable' period (Dobbing 1981) and the 'sensitive' period (Smart 1991).

It is considered that in the fetal origins of adult disease, the timing, duration and type of nutritional insult will all have an effect on the outcome of disease. It has been suggested that generally undernutrition in early gestation produces proportionately small babies, which suggests an adaptation during early development causing a decreased growth rate to avoid disproportionate growth (Barker 1994). Nutritional restriction during mid-gestation produces a baby which is relatively thin in proportion to its length and is at risk of diabetes and hypertension in later life (Barker 1994). In late gestation the growth of the brain is maintained at the expense of other growth, resulting in a baby that is short in relation to its head circumference and has an increased risk of defects of liver metabolism

and hypertension in later life (Barker 1994). Undernutrition at different times during gestation will affect the development of different organs. The nutritional insult will, most likely, have its greatest effect on the organ that is in its period of rapid growth and development at the time of the insult. For example late gestational undernutrition primarily effects the growth and development of the kidney and the lung, which experience a large proportion of their growth at this stage (Hinchliffe *et al*, 1992).

1.8. Possible criticisms of the 'fetal origins' hypothesis

The 'fetal origins' hypothesis has received criticism in the literature. The major criticism is that people who have a poor prenatal environment are likely to also be at a disadvantage in their postnatal environment and that it is the postnatal environment that has an effect upon the risk of disease. A study by Bartley *et al* (1994) found that low birth weight babies were more likely to experience socioeconomic disadvantage subsequently and suggested that this should be taken into account when studying the early development of adult diseases. However, Barker (1994) has shown that the associations between fetal growth and adult disease persist when corrected statistically for socioeconomic group and are also independent of factors which may affect disease in postnatal life such as smoking, alcohol consumption and obesity. Experiments in animals also support the hypothesis that undernutrition in the mother leads to persistent elevation of blood pressure in the offspring. A rat model has been used to show that maternal dietary manipulation during pregnancy only, leads to elevated blood pressure in the offspring. These offspring are exposed to similar conditions in postnatal life as animals with normal blood pressure which haven't received prenatal exposure to altered maternal diet (Langley & Jackson 1994).

Lucas & Morley (1994) reported an absence of correlations between maternal nutrition and growth performance and blood pressure in the offspring at 7.5-8 years of age. All of the children studied, however, were born preterm. Studies in twins have also shown no relationship between birth weight and adult cardiovascular disease (Christensen *et al*, 1995). In both of these studies size at birth may have been restricted by factors other than

maternal nutrition and Barker has emphasised that maternal nutrition is considered to be the important factor in determining the risk of adult disease. The results from these studies support the theory that catch-up growth, against a background of growth constraint, may be important in the development of later disease.

During adolescence it appears more difficult to identify associations between fetal growth and blood pressure (Matthes *et al*, 1994). It is thought that this may be due to puberty and the associated hormonally induced growth spurt (Tanner, 1989). In 1996 Law and Sheill reviewed the literature on the relationship between birthweight and blood pressure in later life. They concluded that the published literature from 34 studies across an age range of 0 to 71 years supported a negative relationship between blood pressure and birth weight and that this could not be accounted for by the predominance of a single academic group, country or method of analysis.

1.9. Fetal Development

Fetal growth and therefore birth weight, is determined by major factors such as genetics, maternal size, hormones and nutrition. The fetus has a genetic potential which is influenced by its environment for example if a male shire horse and a female shetland are mated the offspring have the genetic potential to be large but the foal is born small due to the size of its mother (Walton & Hammond, 1938). In studies of the fetal origins of adult disease, maternal nutrition is considered to be the important factor in determining the risk of adult disease (Barker 1994).

The embryonic period of human development extends to 8 weeks after fertilisation when the conceptus becomes known as the fetus. At this stage it is recognisably human, with arms and legs, a heart that beats and a nervous system which is beginning to respond to tactile stimuli (Tanner, 1989). The high rate of growth of the fetus is largely due to the fact that the cells are still multiplying. The proportion of the cells undergoing division in any tissue becomes progressively less as the fetus gets older (Tanner, 1989). It is this high rate of growth which make the fetus particularly 'vulnerable' to fluctuations in its

environment. If a period of undernutrition continues until after cell division of a particular organ has stopped then the effect will be irreversible and the organ will develop with fewer cells (Winick & Noble, 1966). The cells of that organ can then only increase in size and not number resulting in disproportionate growth (Widdowson & McCance, 1963).

1.10. Animal models of the fetal origins of disease

There are many benefits of using animal models to study diseases such as hypertension. Hypertension is strongly associated with several environmental and social factors, which are much easier to control or remove in studies of animals than in the human population. Secondly it is possible to carry out more invasive studies that are neither ethical nor possible in humans. Animals with a short life span can be used to study the progression of disease throughout life and through successive generations. There are various animal models of hypertension used mainly to study specific aspects of what in humans is a multi-factorial disease. There are examples of genetic models such as the spontaneously hypertensive rat and the renovascular induced models such as Goldblatts (renal artery constriction), and DOCA which are deoxycorticosteroid (DOC) treated as a model for hormonally induced high blood pressure.

The development of suitable animal models was necessary to study the mechanisms underlying the associations between maternal nutrition, fetal growth and hypertension in adult life. Several animal models have been used to study different aspects of intra-uterine growth and development in an effort to elucidate possible mechanisms of programming. Sheep have been used to study insulin and insulin-like growth factors and their relationships to fetal growth (Owens *et al*, 1994, Harding *et al*, 1994). IGF-1 and IGF-2 concentrations have both been positively associated with fetal weight and fetal liver weight, and with blood PO_2 and glucose. These observations are consistent with the hypothesis that both IGF-1 and IGF-2 are chronically regulated by oxygen and nutrition *in utero* and hence these factors influence fetal growth (Owens *et al*, 1994, Harding *et al*, 1994).

Guinea pigs have been used to study the relationship between blood pressure and intra-uterine growth retardation (Persson & Jansson, 1992). Intrauterine growth retardation was induced by performing a unilateral uterine artery ligation at 32 - 37 days gestation. This was found to be associated with elevated adult blood pressure.

There are also various models of nutritionally induced disease being used to study the mechanisms of programming. Rats subjected to a maternal low protein diet (8% protein) have been shown to have altered endocrine pancreas function (Snoeck *et al*, 1990). In neonates exposed to a low protein diet *in utero*, β-cell proliferation and islet size were found to be reduced in the head of the pancreas. Insulin secretion of these fetal islets is also impaired (Dahri *et al*, 1991). Nutritional anaemia has been induced in female rats prior to mating and throughout pregnancy, by feeding an iron-free diet. The offspring were of lower birth weight and were found to have larger hearts, which supports the suggestion that there is an alteration in the cardiovascular development of the pups. However, systolic blood pressure was found to be significantly lower in the anaemic animals than in the controls until 40 days of age, when hypertension was observed (Crowe *et al*, 1993).

Rats exposed to low protein diets during pregnancy produced offspring that were hypertensive in later life (Langley & Jackson, 1994). A range of maternal diets with differing levels of protein restriction have been studied (6-12% protein). An inverse relationship between maternal protein intake and the systolic blood pressure of the offspring was observed. In subsequent work a standard experimental diet of 9% protein compared to a control diet of 18% protein was chosen as this is considered to be a mild protein restriction and sufficient to meet the recommended requirements of a non-pregnant rat (Clarke *et al*, 1978). In several studies pregnant rats have been fed either an 18% protein (control) diet or a 9% protein diet throughout gestation. The offspring of the 9% protein fed mothers were of similar birth weight, but were consistently found to have elevated systolic blood pressures of between 15-30mmHg higher than the control rats (Langley-Evans *et al*, 1996g). The elevation of systolic blood pressure was observed

from 4 weeks of age and was independent of maternal blood pressure changes (Langley-Evans *et al*, 1994). The offspring of the low protein exposed mothers were of low to normal birthweight and had increased placental weights (Langley & Jackson 1994, Langley-Evans *et al*, 1996b). These rats also exhibited disproportionate fetal growth (Langley-Evans *et al*, 1996b). At days 14 and 20 of gestation the low protein exposed rats showed accelerated growth which appeared to falter in late gestation (Langley-Evans *et al*, 1996b). Day 20 fetuses were longer in proportion to body mass but by day 22 (full term) they were short in relation to body mass indicating that the growth of the brain was spared at the expense of the trunk (Langley-Evans *et al*, 1996b).

This model has been used to study a number of possible mechanisms in the programming of hypertension. These include hypothalmic-pituitary-adrenal (HPA) axis, the renin-angiotensin system, renal structure changes and the interactions between the above.

1.11. Mechanisms of programming

Excessive exposure to glucocorticoids *in utero,* induced by maternal injections of dexamethasone (a glucocorticoid analogue) produces offspring, which become hypertensive (Benediktsson *et al*, 1993). Fetal protection from maternal glucocorticoids is normally effected by placental 11β-hydroxysteroid dehydrogenase, which converts physiological glucocorticoids to inactive products. It has been found that rat placental 11β-hydroxysteroid dehydrogenase activity is positively correlated with term fetal weight and negatively correlated with placental weight (Edwards *et al*, 1993). Offspring of rats treated during pregnancy with dexamethasone (which is not metabolised by 11β-hydroxysteroid dehydrogenase) had lower birth weights and higher blood pressure when adult than did offspring of control rats (Benediktsson *et al*, 1993).

In the study of the role of glucocorticoids in the nutritional programming of hypertension placental activity of 11β-hydroxysteroid dehydrogenase has been shown to be lowered by the maternal low protein diet, however, the general placental protein concentration was unaltered (Phillips *et al*, 1996). This leads to an increased exposure of the fetus to

glucocorticoids which may contribute to the late gestational growth retardation observed in these rats (Langley-Evans, 1997). It is suggested that the excess glucocorticoids in the fetus lead to a resetting of the hypothalamic-pituitary-adrenal (HPA) axis. High glucocorticoid levels from the adrenal gland have a negative feedback on the pituitary and the hypothalamus (Dallman *et al*, 1993). The low protein exposed animals show increased sensitivity to corticosterone (Langley-Evans *et al*, 1996f). Langley-Evans *et al* (1996f) suggest that this hypersensitivity to glucocorticoids may be mediated by upregulation of receptor populations. It is suggested that glucocorticoid receptor numbers are greater in the hippocampus and vascular tissue of the low protein exposed rats (Langley-Evans *et al*, 1996c). Greater receptor numbers in the vascular tissue may enable direct or indirect stimulation of blood pressure increases by corticosterone (Langley-Evans *et al*, 1996c).

Intrauterine growth retardation has been associated with poor kidney development, specifically deficits in growth and nephron number (Merlet-Benichou *et al*, 1994). In the rat model of maternal diet induced hypertension the low protein exposed rats exhibit normal glomerular filtration rates but significantly reduced renal blood flow, demonstrating that renal function is programmed *in utero* (Langley-Evans *et al*, 1998). Studies of the renin-angiotensin system have indicated that animals exposed to a low protein diet *in utero* had elevated ACE activity. In a later study captopril, which is an ACE inhibitor, was given to the adult offspring. The offspring that had been exposed to a low protein diet *in utero* showed significantly lowered blood pressure with captopril treatment. Captopril treatment had no effect on the rats born to mothers fed 18% protein diets (Langley-Evans & Jackson, 1995). These results suggest that the renin-angiotensin system has a role in the maintenance of hypertension in this model. In the human population Konje *et al* (1996) have shown that low birth weight babies have elevated cord blood renin concentrations at birth. In 50-53 year old adults, concentrations of both active and inactive renin in plasma were found to be higher in individuals who had been larger at birth (Martyn *et al*, 1996).

The renin-angiotensin system is influenced by glucocorticoids so it is likely that these two mechanisms interact. Glucocorticoids increase the expression of angiotensin II receptors (Sato *et al*, 1994), the synthesis of angiotensinogen and ACE activity (Mendelsohn *et al*, 1982).

1.12. Aims

Epidemiological evidence has shown an association between maternal nutrition and the risk of hypertension in later life. This is an important public health concern as hypertension is linked to cardiovascular disease which is the biggest cause of death in industrialised countries and the incidence is increasing in the developing world (Department of Health, 1994). Investigation and determination of the causes and mechanisms of the disease will aid the development of prevention strategies as well as facilitating early detection and treatment of the disease. A rat model of maternal diet induced hypertension has been developed which has been used to study the possible mechanisms which underlie the pathophysiological processes which lead to the disease.

Rats were fed either a 9% casein (low protein) diet or an 18% casein (control) diet throughout pregnancy. The effect of feeding a maternal low protein diet on both the mother and the offspring in terms of maternal weight gain and growth of the offspring and also the effects on the renin-angiotensin system have been studied. Previous studies had suggested a role for the renin-angiotensin system in the maintenance of high blood pressure in the model of maternal diet induced hypertension. Initial studies sought to characterise any changes that might have been induced in the renin-angiotensin system in this model. The aim of later studies was to determine the effect of treatment with an ACE inhibitor on the blood pressure, renin-angiotensin system, and growth and development of the low protein exposed rats. The long term effects of early intervention with an ACE inhibitor, an angiotensin II receptor antagonist or a calcium receptor antagonist were also studied.

The aim of a further study was to assess whether the rat model of maternal-diet-induced hypertension is an appropriate model in which to study the pathophysiological process that may be involved in the programming of human disease. Biochemical markers, which were altered in the rat model, were also measured in a human population.

Chapter 2

Methods

2.1. Animals

All experimental work was carried out in accordance with the Home Office Animals Act 1986, under license from the Home Office. All animals used were Wistar rats bred in the Southampton University animal facility. Rats were kept in wire mesh cages at a temperature of 22^{0}C on a 12 hour light-dark cycle with free access to food and water at all times. All non-pregnant animals were offered a standard non-purified laboratory chow diet *ad libitum* (CRMX, Special Diet Services, Cambridge, UK.) (Appendix 1).

2.2. Dietary protocol during pregnancy

Virgin female Wistar rats were mated and then housed individually. Mating occurred within one to four days of housing with males. Conception was detected by the appearance of a vaginal plug on the cage floor. The mated females were then given free access to either an 18% casein (control) or a 9% casein (low protein) diet throughout pregnancy, as reported by Langley & Jackson (1994). In order to assess the effects of feeding the 9% casein diet during different periods of gestation experiments in Chapter 3 included rats that were fed for week 1 and/or week 2, and/or week 3 of pregnancy only. All experimental diets were made in the University animal facility and contained equal levels of energy, fibre, fat, vitamins and minerals. The reduction in energy attributable to the lower level of protein in the 9% casein diet, was replaced with increased amounts of cornstarch and sucrose. Both diets were supplemented with D,L methionine to avoid sulphur deficiency, as casein is a poor source of cysteine and methionine. For a full list of ingredients see Appendix 2. All ingredients were mixed and bound together with water. The mixture was rolled into small balls (approximately 50g dry weight) and oven dried at 60^{0}C for 24 hours. Diets were then stored in a cold room at -10^{0}C for up to 3 months. Mothers were returned to a standard laboratory chow (CRMX) diet immediately after giving birth.

2.3. Offspring

All offspring were weighed and had sex determined shortly after birth. Each litter was reduced to a total of eight offspring (4 males and 4 females where possible). Excess offspring were selected at random and sacrificed by decapitation. Litters were weaned from their mothers at 4 weeks of age and housed in pairs of the same sex, with free access to a diet of standard laboratory chow and water.

2.4. Blood pressure determination

Measurements of systolic blood pressure were made on the rats using the tail cuff method (IITC model 229 BP monitor; Linton Instrumentation, Diss, Norfolk, U.K.) as reported by Langley-Evans & Jackson (1994). All animals were acclimatised to a room at 28^0C for 2 hours prior to blood pressure measurement. Conscious animals were placed into a perspex restraint tube maintained at 28^0C, and allowed to rest for 5 minutes prior to testing. An inflatable cuff was placed over the tail and the pressure rapidly raised to 300mmHg. The tail artery pulses were recorded during deflation of the cuff using an IITC software system, with a preset algorithm to reduce subjectivity. The operator was unaware of the experimental group to which each animal was assigned at the time of measurement. Systolic blood pressure was measured 3-5 times for each animal and the average pressure recorded. The instrument used does not require excessive heat to detect a pulse, unlike other tail cuff systems, and care was taken to use appropriate sized cuffs for each animal to avoid an effect of weight upon the pressure recorded. This method has been validated by Bunag (1973) and Pfeffer *et al* (1971) who both found that the correlation between this method and direct cannulation methods was highly significant, and concluded that the tail cuff method was a reliable method of blood pressure measurement.

The effects of training and time spent in the perspex restraint tube were assessed by repeatedly measuring the blood pressures of five 12-week-old male rats every day for a week (Table 2.1), and by allowing three animals to settle in the restraint tube for different lengths of time prior to testing (2, 5 and 10 minutes, Table 2.2). It has previously been shown that the rats take between 0 and 2 minutes to habituate to the restraint tube and

that the reading is then stable if taken within 7 minutes (Langley-Evans *et al*, 1996g). Day to day and within day variation of systolic blood pressure was less than 5%, showing that training has no significant effect upon the blood pressures of the rats (Table 2.1). Time spent in the restraint tube had no significant effect on blood pressure (coefficient of variation < 5% for each rat, Table 2.2). The effect of training was the same in rats exposed to a maternal low protein diet as in the control rats (Sherman & Langley-Evans, 1998).

2.5. Drug treatments

2.5.1. Captopril treatment in adult rats

Captopril (Sigma Chemicals, Poole, Dorset, UK.) was mixed into drinking water at the concentration of 1g/L and provided to adult rats for a period of two weeks. The dose of captopril was chosen on the basis of previous work by Langley-Evans & Jackson (1995) and Lee *et al* (1991). The amount of captopril consumed was calculated on a daily basis from recorded fluid intake. The animals had free access to dry feed (standard laboratory chow) and no other fluids were provided.

2.5.2. Early captopril treatment

Captopril treatment of 2-week-old rats began at a time when the rats were still housed in litters of up to 8 with their mothers. For this reason calculations of actual drug intake for individual animals were approximate. Each litter was given captopril in their drinking water at a concentration of 0.5g/L for two weeks. Approximate intake per pup was calculated by subtracting the estimated consumption of the mother from the total consumed by the mother and litter, and then dividing by the number of offspring in the cage.

Table 2.1. Day to day and within day variation of systolic blood pressure measurements in rats, using an inflatable tail cuff.

	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean±SEM
Day 1	120	119	146	140	155	136±7
Day 2 am	126	128	144	143	156	139±6
Day 2 pm	128	128	146	146	145	139±4
Day 3	137	118	149	141	153	140±6
Day 4	134	122	144	153	149	140±6
CoV	5.2%	3.9%	1.4%	3.6%	3.0%	

Day to day and within day variation of systolic blood pressure was assessed by making repeated blood pressure measurements on 5 12-week-old male rats over a 4-day period. Blood pressure was measured using the tail cuff method and is expressed as systolic blood pressure in mmHg. CoV = Coefficient of variation.

Table 2.2. Effect of time spent in the restraint tube on the systolic blood pressures of rats using the tail cuff method.

	Rat 1	Rat 2	Rat 3	Mean±SEM
2 minutes	136	153	148	146±5
5 minutes	141	146	158	148±5
10 minutes	141	154	147	147±4
CoV	2.4%	3.1%	4.2%	

12-week-old male rats were placed in the restraint tube and left to settle for different lengths of time before making blood pressure measurements. Systolic blood pressure (mmHg) was measured using the tail cuff method. CoV = Coefficient of variance.

2.5.3. Early losartan or nifedipine treatment.

At two weeks of age, entire litters (still housed with their mothers) were treated with either losartan or nifedipine in their drinking water. Drug concentration in both cases was 100mg/L of drinking water, chosen on the basis of previous work by other groups (Goodfriend *et al*, 1996). Treatment continued for two weeks, until the offspring were 4 weeks of age.

2.6. Urine collections

24-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 easily calculated. The urine drains off separately and is collected in a small container at the bottom of the cage. The volume of urine collected over 24 hours was measured gravimetrically and samples were acidified using 6M HCl, to preserve them, and stored at -70^{0}C, for a period of up to 6 months.

2.7. Culling and tissue collection

All animals were culled by either decapitation, or CO_2 asphyxia and cervical dislocation and the major organs (heart, liver, lung, kidneys and spleen) rapidly dissected. All organs were frozen immediately in liquid nitrogen for preservation and later stored at -70^{0}C for further analysis, for a period of up to 6 months. Blood was collected into heparinised tubes containing a cocktail of peptidase inhibitors (to prevent further production of angiotensin II, appendix 3) and immediately placed on ice. It was then centrifuged at 2000rpm and 0^{0}C for 10 minutes and the plasma layer removed and stored in aliquots at -70^{0}C for later analysis. Analyses took place within 6 months of sample collection.

2.8. Recruitment of human subjects

96 healthy 9-10 year old children (50 male and 46 female) took part in the study. All the children were recruited from the Princess Anne Hospital Growth Study (58 subjects) (T.Wheeler) and the MRC Salisbury study (38 subjects) (Clark *et al*, 1996, Law *et al*, 1991, 1995). Maternal details and birth characteristics such as birth weight, length, head circumference, ponderal index and placental weights were available for each of the

children from previous studies, as well as recent measurements of size and blood pressure (Drummond, unpublished, Law *et al*, 1991, 1995, Clark *et al*, 1996).

Children from the Salisbury study were selected at random from different birth weight groups, from a study population of 190 children who had participated in previous studies (Clark *et al*, 1996, Law *et al*, 1991, Law *et al*, 1995), whereas all of the children who were contactable from the Princess Anne Hospital Growth study were recruited.

Children from the Salisbury study provided 24-hour urine samples which were stored in aliquots at -80^{0}C within 72 hours of collection. These samples had been in storage for approximately 2 years (Clark *et al*, 1996). Parents of the children on the Princess Anne Hospital Growth Study were contacted to arrange urine collections. Each of the children collected a single early morning sample in their own homes on specific dates arranged. Sterile containers were provided and sent by post. The samples were collected from the family homes on the day that the children had made the urine collection. The samples were acidified using 6M HCl and then stored at -70^{0}C.

Urinary Prostaglandin E_2 concentrations were measured in each of the samples (as described in section 2.9.5) and expressed per mg creatinine (method described in section 2.9.7).

2.9. Biochemical Measurements

2.9.1. Determination of pulmonary angiotensin converting enzyme (ACE) activity

ACE is a peptidase and its activity was determined in lung tissue by the method of Hayakiri *et al* (1987). This is a spectrophotometric method which measures hippuric acid generated by the enzymatic hydrolysis of hippuryl-L-histidyl-L-leucine (HHL). Lung tissue was homogenised in buffer, pH 8.3, (see appendix 4 for all reagents) and 100µl placed in a glass test tube. All samples were assayed in duplicate. 250µl of 20mM potassium phosphate (K_2HPO_4, pH 8.3) was added and the tubes incubated at 37^{0}C for 5 minutes. 100µl of HHL (3mM made up in distilled water) was added and the tubes were

mixed and incubated again at 37^{0}C for 30 minutes. The tubes were then placed in a boiling water bath for 10 minutes to halt the enzyme activity and then 3 ml 0.2M K_2HPO_4 (pH 8.3) was added. 1.5mls of 3% cyanuric chloride in 1,4-dioxane was added and the tubes mixed and centrifuged at 3000rpm for 10 minutes. Absorbance was read at 382nm. Hippurate production was determined against a standard curve of 0 - 500µg hippuric acid. Standards of hippuric acid were not incubated prior to the cyanuric chloride addition. Activities were expressed as units per mg of protein where a unit of enzyme activity is the amount of enzyme that hydrolyses 1µmol of HHL to hippuric acid in 1 minute at 37^{0}C. The within assay variation was less than 5% and the variation between assays was less than 15%. Storage of the lung homogenates did not affect ACE activity, as demonstrated by determination immediately after culling (without freezing) and repeating after 6 weeks storage at -70^{0}C. Variation was found to be less than 5% over this time.

2.9.2. Determination of protein content of tissue

The protein content of the lung homogenates was determined using the Bicinchoninic acid (B.C.A.) method of Smith *et al* (1985) adapted for use on a microplate reader. 200µl of B.C.A. solution was added to 10µl of standard (0-1mg/ml bovine serum albumin (BSA)) or sample and incubated at 37^{0}C for 30 minutes. The absorbance was measured at 550nm (Appendix 5). Intra-assay and inter-assay variation were found to be 6.3% and 5.9% respectively. All samples were measured in duplicate.

2.9.3. Determination of plasma renin activity

Blood was collected at the time of culling into heparinised tubes (containing peptidase inhibitors to prevent further production of angiotensin II, appendix 3) on ice and centrifuged within 20 minutes of collection. The plasma layer was removed and stored in aliquots at -70^{0}C. Plasma renin activity was measured by Dr. J. Morton at the MRC Blood Pressure Unit, Glasgow using the method of Morton & Wallace (1983). The enzyme assay is based upon radioimmunoassay of the product and is carried out in the cold (all details of chemicals and reagents are shown in appendix 6). Two sets of tubes were prepared for each sample, one of which was for incubation and the other

represented zero time. 20µl of each sample (measured in duplicate) was pipetted into the two sets of tubes and 20µl of antibody trapping mixture was added. All tubes were centrifuged at 2000rpm for 2 minutes and then the zero time tubes placed immediately into ice. All other tubes (including the standards) were incubated at 37^0C for 30 minutes and then placed into the ice bath with the zero time tubes to halt the reaction. 250µl of cold 50mM Tris/HCl buffer (pH 7.4) was added to all of the tubes followed by 50µl [^{125}I]-angiotensin I. 1ml of cold dextran/charcoal was added to each tube, which were then vortex-mixed and centrifuged at 3000rpm for 15 minutes. The supernatants were removed by batch decantation and drainage using absorbent paper and the radioactivity present in the charcoal pellet counted using an automatic gamma-counter. A standard curve of 0 - 200pg angiotensin I and total counts were also run (in duplicate). Renin activity was calculated using the following method:

1) pg Angiotensin I (blank) - pg Angiotensin I (30 minutes) = pg Angiotensin I/20µl plasma/30 minutes

2) Plasma renin activity = pg Angiotensin I * 0.1 = ng/ml/hr

2.9.4. Determination of plasma angiotensin II concentrations

Peptidase inhibitors (see appendix 3) were added to blood samples immediately after collection to prevent further hydrolysis of angiotensin I. Samples were then centrifuged and the plasma layer stored at -70^0C in aliquots. Plasma angiotensin II concentrations were measured by Dr. J. Morton at the MRC Blood Pressure Unit, Glasgow, using the method of Morton & Wallace (1983). The method used is a radioimmunoassay, which is carried out at room temperature, following the extraction of angiotensin II from the plasma samples (appendix 7). See appendix 7 for all chemicals and reagents. 400 µl of Tris/HCl buffer (pH 7.4) was added to all plasma extracts (in duplicate), mixed thoroughly and placed in an ultrasonic bath for 10 minutes. Tubes were mixed again and centrifuged at 3000rpm for 10 minutes. 100µl of unknown extract was placed into tubes and 100µl angiotensin II antiserum added. [^{125}I]-Angiotensin II was added to all tubes, which were then mixed and kept at 4^0C for 18 hours. Standards (0-100pg) and total counts were also run. After 18 hours 1ml of cold dextran/charcoal was added to all tubes

which were then mixed and centrifuged at 3000rpm for 10 minutes. The supernatant was removed using a Pasteur pipette and a suction water pump. The radioactivity present in the charcoal pellet was counted using an automatic gamma-counter. Adjustments were made for sample dilution, recovery and plasma volume to obtain a final plasma concentration in pg/ml.

2.9.5. Determination of plasma angiotensinogen concentrations

Angiotensinogen concentrations were assessed by measuring the amount of angiotensin I produced in the presence of excess renin, as described by Tewksbury & Dart (1982). The production of angiotensin I was induced by incubating 50µl diluted plasma (1:1000 dilution) with 445µl of buffer (0.1M Tris/HCl, pH 7.4, 0.025M EDTA, 1g/l BSA), 50ng renin (porcine) and 5µl phenylmethylsulphonylfluoride (PMSF, 50g/l in ethanol) for 1 hour at 37^0C. Angiotensin I was then determined using an angiotensin I [^{125}I] radioimmunoassay kit (NEN Life Science Products). 100µl of standard (range 0.1-10 ng/ml) or sample (plasma incubation solution) was pipetted into test tubes in duplicate followed by 100µl of angiotensin I [^{125}I] tracer. 100µl blank antiserum was added to the tubes containing the blanks and the same quantity of angiotensin I antiserum (rabbit) added to all other tubes. Tubes were vortex mixed and incubated for 2 hours at room temperature. 0.5ml of the second antibody was then added to each tube and mixed, followed by a further incubation at room temperature for 30 minutes. All tubes were centrifuged at a speed of 1000g for 10 minutes at room temperature and the supernatants decanted off and discarded. All tubes were then counted using a gamma counter. Angiotensin I concentrations in each tube were calculated automatically by the counter program. Net counts were calculated for each sample and standard by subtracting the average blank count from the average total count for each duplicate pair. Average net counts for each standard and sample were expressed as a percentage of the average net counts for the zero standard (termed percent bound or $\%B/B_0$).

$$\%B/B_0 = \frac{\text{Average Net Counts of Standard or Sample}}{\text{Average Net Counts of Zero Standard}} \times 100$$

$\%B/B_0$ was plotted against concentration of angiotensin I on a semi-logarithmic Figure and sample concentrations calculated from the standard curve. Results were multiplied by the appropriate dilution factor and expressed in units of angiotensinogen/ml of plasma. Units are equal to ng of angiotensin I produced in the presence of excess renin activity. Within assay variation was shown to be 5%, by measuring controls within the assay (n = 48). Between assay variation was 8%, assessed by repeated measurement of the same controls in successive assays (n = 14).

2.9.6. Determination of urinary prostaglandin E_2 concentrations

Prostaglandin E_2 was determined using a Biotrak enzymeimmunoassay system (Amersham). All reagents are described in appendix 8. The assay was carried out at room temperature, which increases the range over which PGE_2 can be measured (0-300pg/well). A microtitre plate was set up with sufficient wells to run all blanks, standards and samples in duplicate. 100µl of assay buffer was added to the non-specific binding (NSB) wells and 50µl into the zero standard wells. 50µl of each standard (0-300pg/well) and sample were pipetted into the appropriate wells followed by 50µl of antibody. 50µl of conjugate was added to all wells except the blank and the plate was incubated at room temperature for 1 hour on a microtitre plate shaker. The wells were then washed with wash buffer and emptied and blotted dry. 150µl of enzyme substrate was immediately pipetted into all of the wells and the plate was incubated at room temperature on a microtitre plate shaker for exactly 30 minutes. The optical density (OD) was then read at 630nm. The results were calculated by working out the percentage bound for each standard and sample using the following relationship:

$$\%B/B_0 = \frac{\text{(standard or sample OD - NSB OD)}}{\text{Zero standard OD - NSB OD}} * 100$$

A standard curve (0-320pg/well) was then generated by plotting the percent B/B_0 as a function of the log prostaglandin E_2 concentration and the pg/well value of the samples was read directly from the Figure. Results were expressed as either ng/24 hours or as

pg/mg of creatinine. Within assay variation was shown to be 9%, by measuring controls within the assay (n = 34). Between assay variation was 13%, assessed by repeated measurement of the same controls in successive assays (n = 29).

2.9.7. Determination of urinary 6-keto-$PGF_{1\alpha}$ concentration

6-keto-$PGF_{1\alpha}$ was determined using a Biotrak enzymeimmunoassay system (Amersham). The assay was carried out at room temperature following the same procedure as described above for the prostaglandin E_2 assay. All reagents are described in appendix 8. The range of standards was 0-50pg/well. Results were expressed as either ng/24 hours or as pg/mg of creatinine. The within assay variation for this kit was 5% and the between assay variation is given as 15%(Amersham), which corresponds closely to the values determined for the PGE_2 kit (2.9.6).

2.9.8. Determination of urinary creatinine

Urinary creatinine was determined by the alkaline picrate method using the Jaffe reaction. All reagents are described in appendix 9. All standards and samples were assayed in duplicate. 25mg of Fuller's earth was placed in centrifuge tubes followed by either 100µl of standard or 25µl of urine sample. 3mls of distilled water and 0.2 mls of saturated oxalic acid were added and the tubes vortex mixed and allowed to stand for 10 minutes. The tubes were then centrifuged for 10 minutes at 5000g and the supernatant decanted. Tubes were then overturned to drain for approximately 20 minutes. The precipitate was resuspended in 3mls of distilled water by vortex mixing and 1ml of freshly made alkaline picrate added. The tubes were mixed and allowed to stand at room temperature for 15 minutes while orange colour developed. The absorbance was read at 520nm. The creatinine concentrations of the urine samples were determined from a standard curve (0 - 400µg/ml). Intra-assay variation was 5.9% while inter-assay variation was 15.5%.

2.9.9. Determination of 15-hydroxy prostaglandin dehydrogenase (PGDH).

PGDH was assayed in placenta, kidney, lung, liver and spleen using the method of Nagai *et al* (1991). Tissue samples were homogenised in 10mM potassium phosphate buffer (pH 7.4) at a dilution of 1g tissue/5mls buffer. 0.1ml of each sample was then incubated with 0.9ml assay reagent (1M Tris/HCl, pH 10, 1mM NAD, 30μM PGE_2) for 90 minutes at 40^0C. The reaction was then terminated by the addition of 2ml 0.5M NaOH. Absorbance was measured at 500nm against a blank of Tris/HCl/NAD and NaOH. Units of activity are equivalent to nmol product formed/min/mg protein, determined against a standard curve of 15-keto PGE_2 (0-20nmoles) made up in Tris/HCl/NAD/NaOH solution. Variability was less than 5% within and between assays.

2.10. Statistical analysis

All results are expressed as the mean ± SEM or as the mean ± st.dev as stated in the text. Results were analysed using ANOVA and Students' t-tests or regression analysis. Standard curves were calculated using Stanley (computer package) and results were analysed using Minitab or SPSS computer packages. Specific methods are stated in the appropriate chapters and sections.

In early studies, results were analysed twice using the litter and the offspring as the statistical unit. This was to ensure that statistically significant results were not merely due to litter bias. Results that were found to be significantly different when analysed by offspring also reached statistical significance when analysed by litter. Later results were, therefore, only analysed by offspring number. The number of litters used is also given for each study.

Chapter 3: part 1

The effect of feeding a low protein diet upon maternal weight gain and growth of the offspring.

3.1 Introduction

A large body of evidence indicates that human hypertension and therefore cardiovascular disease is determined in part, by maternal nutrition. Epidemiological studies have shown that there is a relationship between intrauterine growth retardation and an increased risk of hypertension in later life (Barker *et al*, 1993). Recent studies have demonstrated relationships between maternal diet during pregnancy and placental and fetal growth (Godfrey *et al*, 1996) and blood pressure in the offspring (Campbell *et al*, 1996). Experiments in animals unequivocally support the hypothesis that the nutritional profile of the mother permanently programmes the metabolic and physiological functions of the fetus, as reviewed by Langley-Evans (1998). Rats exposed to a maternal low protein diet *in utero* have significantly elevated systolic blood pressures (Langley & Jackson, 1994). This rat model of maternal-diet-induced hypertension is being used to study the possible mechanisms of programming.

As in the human population (Barker, 1994), the high blood pressure arising from intrauterine protein restriction in rats is associated with patterns of disproportionate fetal and placental growth (Langley-Evans *et al*, 1996b) and is of early onset (Langley-Evans *et al*, 1994). Rats exposed to a maternal low protein diet *in utero* have a low body mass at birth and an increased placental weight (Langley-Evans, 1994). Restriction of protein during pregnancy has been shown to accelerate fetal growth between days 14 and 20 of gestation, however, this accelerated growth does not continue until the end of gestation and the resulting offspring are of low to normal birth weight (Langley-Evans *et al*, 1996b). Placental growth is also accelerated by the restriction of protein in the maternal diet (Langley-Evans *et al*, 1996b).

In human populations the pattern of growth of the fetus is dependant on the timing of the nutritional restriction (Barker, 1994). If the fetus is undernourished in early gestation it

will be proportionately small at birth (Barker, 1994). Disproportionate growth occurs when the fetus is undernourished in either mid or late gestation. Each of these periods are associated with a different pattern of fetal growth and an increased risk of different pathologies in adult life. Nutritional restriction in mid-gestation is associated with babies who are of low birth weight and are thin in proportion to body mass (Divon *et al*, 1996) and have an increased risk of developing non-insulin-dependant diabetes, hypertension and coronary heart disease in adult life (Barker, 1994). Individuals who are undernourished in late gestation may be of normal birth weight but are short in relation to head circumference and have an increased risk of cardiovascular disease (Barker *et al*, 1995).

The aim of the first experiment described in the present chapter was to examine the effects of maternal protein restriction on maternal growth during rat gestation. Fetal and placental growth were assessed in the second experiment and the postnatal growth in experiments 3 and 4. Blood pressures of the offspring were determined in the final experiment of this section. These studies also considered the effects of targeting protein restriction to specific periods of gestation.

3.2 Methods

The methods of animal production, dietary administration and blood pressure measurement are as described in chapter 2. Eleven pregnant female rats were fed a 9% casein diet throughout pregnancy and eleven were fed an 18% casein diet. Five female rats were fed a 9% casein diet during week one of gestation and four were fed a 9% casein diet during week two only of gestation. Maternal weight and food intake, were measured every 2-3 days during gestation. Rats were fed either an 18% casein diet (n=5) or a 9% casein diet (n=5) throughout pregnancy (d0-14), or a 9% casein diet during week one only (d0-7, n=5) or week two (d8-14, n=4) only of gestation and culled at d14 pregnancy using CO_2 as previously described in chapter 2, and immediately dissected. The fetuses and placentae were removed and weighed. All other animals were culled by decapitation, and blood and tissues collected as described in chapter 2.

3.3 Results

3.3.1 Effect of feeding a low protein diet during pregnancy on maternal dietary intake and weight gain.

Six virgin female Wistar rats were mated and given free access to a low protein (9% casein) diet throughout gestation. A further six were fed a control diet (18% casein) throughout pregnancy. Two of the rats from the low protein group were later found not to be pregnant and were excluded from the analysis. The low protein fed rats tended (p=0.06) to consume more diet than the controls (11% higher) (Table 3.1). This, however, did not significantly increase their protein intake. The weight gain in the low protein group was 11% less than the controls (Table 3.2). The difference in dietary intake was greater in the first week of pregnancy whilst the decreased weight gain in the low protein fed group was only significant in the final week of pregnancy (Tables 3.1 & 3.2). Feed efficiency (weight gain/food intake) increased progressively during pregnancy for both the control and low protein groups (Table 3.3), but was consistently lower for the low protein exposed group compared to the controls (Table 3.3, p<0.05). Efficiency ratios for the low protein diet compared with the control diet increased throughout pregnancy (Table 3.3). Average efficiency of the low protein diet was 81% of the control diet (Table 3.3).

3.3.2. Effect of feeding a maternal low protein diet on the fetal and placental weights at day 14 of pregnancy.

Nineteen pregnant female Wistar rats were fed either an 18% casein diet (n=5) or a 9% casein diet (n=5) throughout pregnancy (d0-14), or a 9% casein diet during week one only (d0-7, n=5) or week two (d8-14, n=4) only of gestation, in order to study the effects of protein restriction during different periods of pregnancy. The rats were culled at day 14 of pregnancy and the fetuses and placentae removed and weighed. Fetal weights of rats exposed to a low protein diet during any period of pregnancy were significantly higher than in the control group ($p \leq 0.02$). Those exposed to a low protein diet only during week one of gestation had the highest fetal weights in comparison to all other groups (p<0.001) (Table 3.4). Placental weights were also highest in the group exposed

to a low protein diet during week one only ($p<0.001$) (Table 3.4). There were no significant differences in fetal:placental ratio between the groups (not shown).

3.3.3 Effect of feeding a maternal low protein diet on the body weights of the offspring.

Six virgin female rats were mated and given free access to an 18% casein (control) diet and a further 6 were fed a 9% casein diet throughout gestation. Two rats were excluded from the low protein group (section 3.3.1). Birth weights of the pups were not significantly altered by the low protein feeding and litter sizes did not differ between the groups (Table 3.5). Female offspring only were used in the following studies. At weaning body weights of the offspring were higher in the low protein exposed group (n=18), compared to the controls (n=15, not significant) and at 9 and 12 weeks of age the low protein group still weighed significantly more than the control rats. Results were analysed by both litter and individual offspring number (Table 3.6).

3.3.4. The effect of feeding a maternal low protein diet on the organ weights of the offspring.

Female offspring of rats exposed to either an 18% (n=6) or a 9% (n=9) casein diet during pregnancy were culled at 12 weeks of age and blood and tissue samples collected. Organ weights, and organ weights expressed per Kg body weight, are shown in Table 3.7. No significant differences were found in the lung, liver, heart or kidney weights between the dietary groups. The spleen was however, found to be smaller in the rats exposed to a maternal 9% protein diet compared to the controls, both in absolute and relative terms (Table 3.7).

3.3.5 The effect of feeding a maternal low protein diet on the blood pressures of the offspring.

The offspring of rats fed a 9% protein diet throughout pregnancy had higher systolic blood pressures than control animals from 4 weeks of age (Table 3.8). The offspring of rats fed a low protein during only week 1 (d0-7), or week 2 (d8-14), or week 3 (d15-22) of gestation had significantly elevated blood pressures compared with control rats (Table

3.8). Results from male and female rats were combined as no sex differences in blood pressure were observed. Systolic blood pressure remained elevated until 12 weeks of age, in rats exposed to the low protein diet throughout gestation (27mmHg, Figure 3.1). Results were analysed by litter and by total number of offspring per dietary group.

Table 3.1. The effects of feeding a low protein diet on food intake during pregnancy compared to a control diet.

	Food Intake (g/day)			
	Week 1	week 2	week 3	Average
18% protein diet	24 ± 1	26 ± 2	28 ± 1	26 ± 1
9% protein diet	30 ± 2	30 ± 1	26 ± 1	29 ± 1
P	0.05	0.06	NS	0.06

Pregnant female rats were individually housed and given free access to either an 18% protein control diet (n=6) or a 9% protein diet (n=4). Food intake was calculated throughout gestation. Values given are expressed as mean ± SEM and the *p* value denotes the level of significance of the difference from the control. Results were analysed using t-tests.

Table 3.2. The effect of feeding a low protein diet on weight gain during pregnancy, compared to a control diet.

	Maternal weight gain (g)			
	Week 1	week 2	week 3	Full gestation
18% protein	27 ± 1	44 ± 2	51 ± 2	117 ± 1
9% protein	23 ± 3	40 ± 3	41 ± 1	104 ± 4
P	NS	NS	0.007	0.08

Pregnant female rats were individually housed and given free access to either an 18% protein control diet (n=6) or a 9% protein diet (n=4). Weight gain was calculated throughout gestation. Values given are expressed as the mean ± SEM and the *p* value denotes the level of significance of the difference from the control. Results were analysed using t-tests.

Table 3.3. The effect of feeding a low protein diet on feed efficiency during pregnancy, compared to a control diet.

Feed efficiency (weight gain g/ food intake g)				
	Week 1	week 2	week 3	Full gestation
18% protein	0.16±0.01	0.24±0.01	0.26±0.01	0.21±0.01
9% protein	0.11±0.01*	0.19±0.01*	0.23±0.01*	0.17±0.01*
Efficiency ratio	0.69	0.79	0.88	0.81

Pregnant female rats were individually housed and given free access to either an 18% protein control diet (n=6) or a 9% protein diet (n=4). Weight gain and food intakes were calculated throughout gestation. Values given are the weight gained in grams per gram of food intake, and represent feed efficiency. Efficiency ratios are the efficiency of the 9% casein diet compared to the 18% protein control diet. *$p<0.05$ where p denotes the difference from the control group.

Table 3.4. The effect of feeding a low protein diet during different discrete periods of pregnancy on fetal and placental weights at day 14 of gestation.

Dietary Group (n)	Fetal weight (g)	Placental weight (g)
18% casein (62)	$0.142 \pm 0.002^{\ddagger\dagger}$	0.179 ± 0.005
9% casein (46)	$0.150 \pm 0.002^{*\dagger}$	$0.175 \pm 0.006^{\dagger}$
9% d0-7 (49)	$0.194 \pm 0.006^{**\ddagger\ddagger\dagger}$	$0.224 \pm 0.007^{**\ddagger\ddagger\dagger}$
9% d8-14 (55)	$0.164 \pm 0.006^{**\ddagger}$	$0.187 \pm 0.008^{\ddagger}$

Nineteen pregnant Wistar rats were fed either an 18% casein diet (control) or a 9% casein diet throughout gestation or a 9% casein diet during week one only or week two only of gestation. Rats were culled on day 14 of pregnancy and the fetuses and placentae removed and weighed. Weights are expressed as mean ± SEM and $^{*}p<0.05$, $^{**}p<0.001$ denotes the difference from the control group. $^{\ddagger}p<0.05$, $^{\ddagger\ddagger}p<0.001$ denotes the difference from the 9% casein group, while $^{\dagger}p<0.05$, $^{\dagger}p<0.001$ shows the difference from the 9% casein day 8-14. Results were analysed using an ANOVA and post hoc t-tests.

Table 3.5. The effects of feeding a maternal low protein diet on birth weights of the offspring and litter size.

	Birth weight (g)	Litter size
18% protein control diet	5.0 ± 0.1 (n = 65)	11 ± 2
9% low protein diet	5.0 ± 0.1 (n = 56)	14 ± 1
P	NS	NS

Pregnant female rats were individually housed and given free access to either an 18% protein control diet (n = 6) or a 9% protein diet (n = 4). The offspring were weighed and counted shortly after birth and the results expressed as mean ± SEM. The results were analysed using a t-test and *p* denotes the statistical significance of the difference from the control. NS = not significant.

Table 3.6. The effects of feeding a maternal low protein diet on the body weights of the offspring at 4, 9 and 12 weeks of age.

	Weight at 4 weeks (g)(n)	Weight at 9 weeks (g) (n)	Weight at 12 weeks (g) (n)
18% protein control diet	58 ± 1 (15)	177 ± 3 (15)	203± 2 (6)
9% low protein diet	76 ± 2 (18)	199 ± 5 (18)	214± 1 (9)
P	<0.001, <0.01	<0.005, <0.05	<0.01, =0.08

Pregnant female rats were individually housed and given free access to either an 18% protein control diet (n = 6) or a 9% protein diet (n = 4). The female offspring were weighed after weaning at four weeks of age and again at 9 and 12 weeks of age and the results expressed as the mean weight in g ± SEM. At 10 weeks of age half of the rats were treated with captopril, as described in Chapter 5, and excluded from further analysis resulting in the lower number of rats (n) in each group at 12 weeks. At 12 weeks the rats came from a total of 6 litters, 3 litters from the low protein group and 3 from the controls. The difference in the body weights of the low protein group compared to the controls was analysed using a t-test. *p* denotes the statistical significance of the difference from the control. Results were analysed both by offspring and by litter and both *p* values are given respectively.

Table 3.7. The effect of feeding a maternal low protein diet on the organ weights of the offspring at 12 weeks of age.

	n	Weight (g)				
		Lung	Liver	Heart	Spleen	Kidney
18% protein	6	1.39±0.03	10.1±0.40	0.88±0.03	0.72±0.03	0.79±0.03
9% protein	9	1.43±0.05	9.9±0.25	0.94±0.04	0.63±0.02*	0.80±0.01
		g/kg body weight				
18% protein	6	6.85±0.28	49.8±2.23	4.33±0.17	3.57±0.19	3.88±0.12
9% protein	9	6.67±0.20	47.5±1.56	4.53±0.25	3.04±0.16*	3.82±0.11

Pregnant female rats were given free access to either an 18% protein control diet or a 9% protein diet throughout pregnancy. At 12 weeks of age all animals were culled and organ weights measured. Organ weights are expressed as mean ± SEM. Results were analysed using ANOVA and t-tests. *$p \leq 0.05$, where p denotes the significance of the difference from the control group.

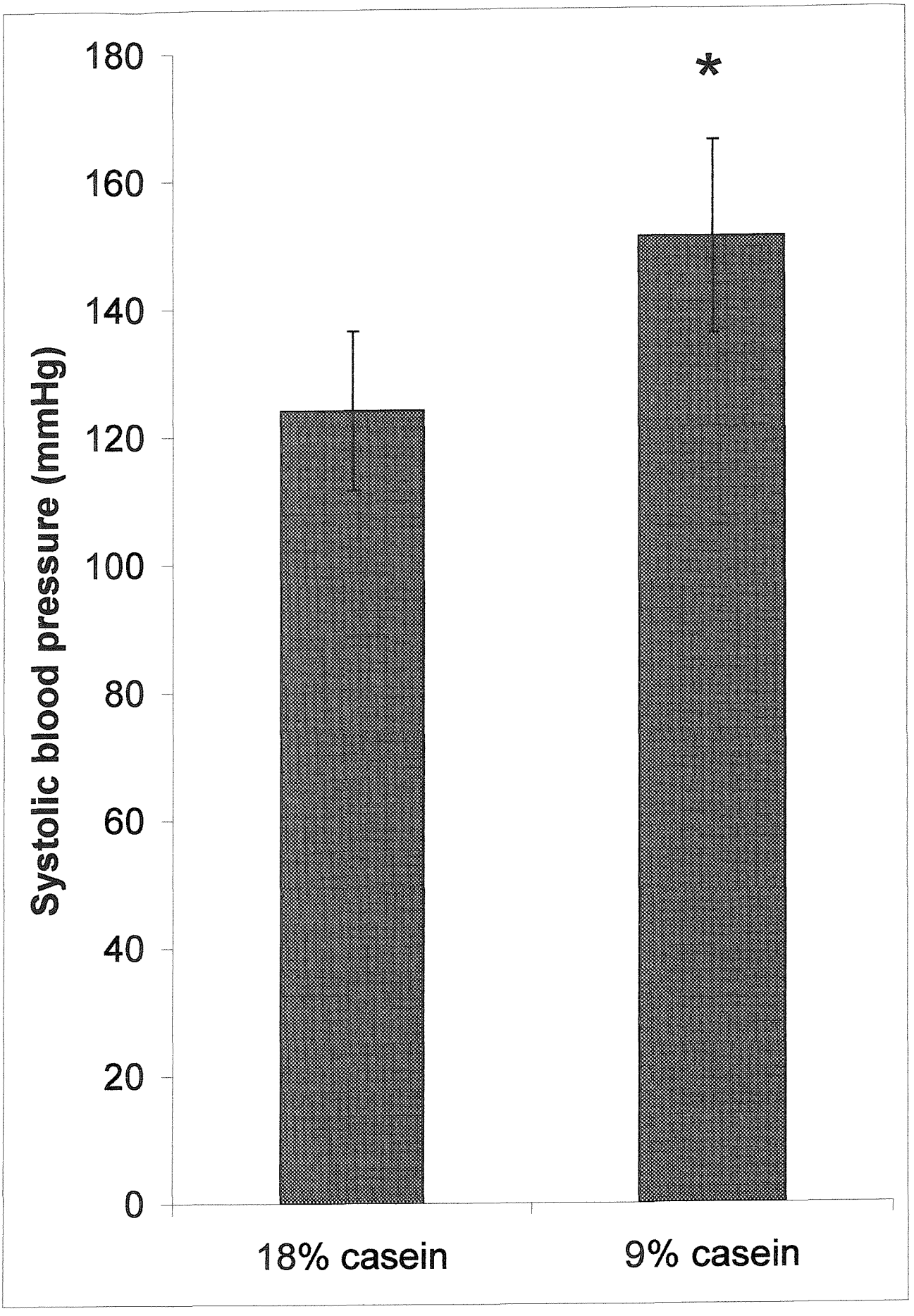
Table 3.8. Effect of feeding a maternal low protein diet on the blood pressures of the offspring at 4 weeks of age.

	Systolic blood pressure (mmHg)	N
18% protein control	96 ± 3	15
9% protein	122 ± 3*	18
9% protein d0-7	111 ± 4*‡	20
9% protein d8-14	105 ± 3*‡	20
9% protein d15-22	112 ± 5*	11

Pregnant female rats were housed individually and given free access to either an 18% or 9% protein diet throughout gestation or a 9% protein diet during discrete one week periods of pregnancy. The systolic blood pressures of the offspring were measured using a tail cuff at 4 weeks of age. Results are expressed as the mean ± SEM and were analysed using ANOVA and t-tests. *$p<0.05$ denotes the difference from the control group and ‡ $p<0.05$ denotes statistically significant difference relative to 9% protein.

Figure 3.1. The effect of feeding a maternal low protein diet on the systolic blood pressure of the offspring at 12 weeks of age.

Pregnant female rats were housed individually and given free access to either an 18% or 9% protein diet throughout gestation. The systolic blood pressures of the offspring were measured using a tail cuff at 12 weeks of age. Results are expressed as the mean ± SEM and were analysed using ANOVA and t-tests (n=6 and 9 for the control and low protein groups respectively). $^{*}p<0.0001$ denotes the difference from the control group when analysed by the number of offspring. $^{\ddagger}p<0.005$ denotes the difference from the control group when analysed by litter number (n=3 for both the low protein and control groups).


180
160
140
120
100
80
60
40
20
0
Systolic blood pressure (mmHg)
*
18% casein
9% casein

3.5 Discussion

Epidemiological evidence has suggested that intrauterine growth retardation is associated with an increased risk of hypertension in adult life (Barker *et al*, 1990). This has received support from studies with rat models where hypertension has been associated with the feeding of a low protein diet *in utero* (Langley & Jackson, 1994). Previous studies with this rat model of hypertension, have shown that the low protein diet fed during pregnancy effects the intrauterine and postnatal growth of the offspring (Langley-Evans *et al*, 1996a, 1996b, 1996c, 1996d, 1996e, McCarthy *et al*, 1994, Langley-Evans & Nwagwu, 1998).

The aim of the present study was to characterise the effects of feeding a maternal low protein diet on maternal weight gain, fetal and placental growth and the growth of the offspring. Maternal weight gain was lower in the rats fed a low protein diet whilst their dietary intake tended to be greater in comparison to the control rats. At day 14 of gestation the fetuses of all of the low protein fed mothers weighed significantly more than the controls, whilst placental weight only differed in the rats exposed to a low protein diet between day 0 and 7 of gestation. Birth weights and litter sizes did not differ between the two dietary groups. By weaning, body weights of the low protein exposed offspring were slightly higher than the control group (not significant) and by 9 weeks of age this difference was significant. The rats exposed to a low protein diet during the periods of day 0-7, day 8-14 or day 15-22 pregnancy had elevated systolic blood pressures compared to the controls. This relative hypertension was observed from 4 weeks of age. The feeding of a maternal low protein diet led to a smaller spleen in the offspring but had no other effects on organ sizes.

Pregnant female rats were fed either an 18% casein control diet or a 9% casein, low protein diet *ad libitum* throughout gestation or a 9% casein diet during different discrete periods of pregnancy. The rats fed a low protein diet had an increased food intake in early-mid gestation compared to the controls. It may be suggested that on a mild dietary restriction the rats may increase their intake to compensate for the decrease in protein. The rats did not increase their intake enough to make a statistically significant difference

to their protein intake, which remained at approximately 75% of their requirement (Clarke *et al*, 1978). Although dietary intake was increased and therefore energy intake was higher, the low protein group underwent a smaller pregnancy related weight gain than the controls. The difference in weight gain between the low protein group and the control group was most significant in the last trimester. This could be due to a fetal growth rather than maternal weight gain, as a large percentage of fetal growth occurs during the final period of gestation (Langley-Evans *et al*, 1996b), however, this is unlikely as birth weights did not differ between the two groups. Fetal body mass in the control animals has previously been shown to increase by 90% in the final 2 days of pregnancy, while there is only a 70% increase in body mass in rats exposed to a maternal low protein diet, during this period (Langley-Evans *et al*, 1996b). Birth weight *per se* is not significantly different in the low protein exposed group, however, fetal growth patterns are altered (Langley-Evans *et al*, 1996b, Harding & Johnston, 1995).

Feed efficiency data showed that the 9% protein diet was, on average, 80% efficient compared with the control diet. This corresponds well with the suggestion that the rats were receiving approximately 75% of their protein requirement (Clarke *et al*, 1978). However, it appears that the efficiency of the diet increased over the period of gestation, and this may be due to the 'programming' of the pattern of growth by early undernutrition (Harding & Johnston, 1995). It is possible that the mother stores protein in early gestation for use in later pregnancy when the demand is increased for rapid fetal growth (Langley-Evans *et al*, 1996b, Harding & Johnston, 1995, Naismith & Morgan, 1976). It has been suggested that, during rat pregnancy, protein is laid down and stored during the first two weeks (Naismith & Morgan, 1976), and it was during this period that the mothers fed low protein increased their food intake and there was little difference in weight gain from the controls. In the third week the protein stored is used for the growth and development of the fetus (Naismith & Morgan, 1976) and it was during this time that the low protein group gained less weight and their food intake returned to control levels. These results are supported by Desai *et al* (1996) who also found increased food intake in

the first two weeks, along with a smaller weight increase in the final week, in rats fed a low protein diet during gestation (80g protein /kg).

At day 14 of gestation the fetuses and placentae weighed significantly more in the rats exposed to a low protein diet than the controls. This is consistent with the study of Langley-Evans *et al* (1996b) which indicated that day 14 fetuses and placentae were significantly heavier in the rats fed 9% casein, but that on days 12 and 16 the fetal/placental weights were unaltered. At day 20 of pregnancy fetal and placental weights were greater in the low protein exposed animals but by day 22 birth weights are found to be low to normal (Langley-Evans *et al*, 1996b). This illustrates the complex nature of the processes involved with growth and development. Taken together these observations indicate patterns of disproportionate growth in mid to late gestation and suggest that adaptive mechanisms are active in the fetal tissues. It is suggested that the growth of the fetal brain is spared at the expense of the trunk (Barker, 1994) and, therefore, other organs such as the liver and kidney. It is thought that the increase in placental weight is an attempt to increase the blood and nutrient supply to the fetus when necessary. Owens *et al* (1989) suggest that the placenta maintains a priority of growth in mid-gestation and that the fetus may provide energy substrate for this process. The placenta has the ability to adapt in order to supply the nutrient demands of the fetus and therefore greater placental growth should lead to an increase in fetal growth, as shown at day 14 in the present study.

In the present experiments birth weights did not differ between the groups which is inconsistent with later studies in this thesis. It is difficult to compare birth weights between groups with differing litter sizes. Although the litter sizes between groups are not statistically different in this study, they do differ slightly. Birth weights of the offspring also depend on maternal size and the posistion of the fetus on the uterine horn (Levy & Jackson, 1993). Maternal size did not differ significantly between these rats, but obviously did vary slightly. These factors all influence birth weight so that in studies of

small numbers the effect of maternal nutrition may not be observed. Birth weights have usually been found to be low to normal in these animals (Langley-Evans *et al*, 1996b).

At weaning body weights were higher in the low protein group than in the controls. This difference was greater by 9 weeks of age suggesting that metabolism and/or dietary intake had been programmed *in utero* to give an altered growth pattern and possibly body composition. It has previously been shown that maternal food restriction in rats influences body composition of the offspring and leads to an increased risk of obesity in later life (Anguitta *et al*, 1994). Using the same rat model as described in the current studies, maternal protein restriction has been associated with increased adiposity in the offspring (McCarthy *et al*, 1994). Studies of men who were fetuses when their mothers were exposed to famine in the Dutch Hunger Winter, suggest that rates of obesity are increased in adulthood (Ravelli *et al*, 1976).

It is thought that disproportionate growth of the fetus will adversely affect the growth and development of certain organs, which will then impact upon health in later life. In previous studies the growth of the kidney has been shown to be impaired by fetal undernutrition (Langley-Evans *et al*, 1996g). Gross size of the kidney has been reported to be reduced in rats exposed to a maternal 9% casein diet during week 2 or 3 of gestation (Langley-Evans *et al*, 1996g). In this study, organ weights (other than the spleen) did not differ between the groups. This may be due to the low numbers used in the study. In contrast Zeman reported that the offspring of protein deprived rats had small kidneys that were histologically imature (1968), whilst undernourished sheep fetuses have been shown to have kidneys which are larger than normal (Harding & Johnston, 1995). Despite the lack of gross size changes it is still possible that the development of these organs may have been altered and that they may be structurally and/or functionally different. Previous studies have shown that maternal protein restriction results in lower glomerular number in the kidneys of the offspring (Zeman, 1968, Hinchcliffe *et al*, 1992, Langley-Evans *et al*, 1999) and impaired renal function (Hall & Zeman 1968). Rats exposed to a

maternal 9% protein diet exhibit significantly reduced renal blood flow but unchanged glomerular filtration rates (Langley-Evans *et al*, 1998).

Maternal protein restriction also affects the metabolic zonation of the liver (Desai *et al*, 1995) and liver growth impairment has been associated with coronary heart disease in humans (Barker *et al*, 1995). Similarly the structural development of the vasculature of the offspring may be altered by a maternal low protein diet. Rats exposed to a maternal 9% protein diet exhibit a reduced arterial elastin content and aortic compliance compared to control animals (Langley-Evans, unpublished observation). Maternal protein restriction is associated with altered glutathione metabolism in the offspring and increased susceptibility to sulfur dioxide induced lung injury (Langley-Evans *et al*, 1997). The reduction in size of the spleen observed in the low protein exposed animals, suggests a possible effect upon the immune system. Studies have shown that exposure of rats to a maternal low protein diet effects their response to endotoxin challenge in early adulthood (Langley *et al*, 1994). Studies in the Gambia have provided evidence that people undernourished *in utero* may be more susceptible to infection in later life (Moore *et al*, 1997).

At 4 and 12 weeks of age the low protein group had significantly elevated blood pressures as first shown previously by Langley & Jackson (1994). Rats exposed to a maternal low protein diet during any discrete period of pregnancy also exhibited significantly elevated systolic blood pressures compared to the control. Other animal models have also shown raised blood pressures in association with altered patterns of intrauterine growth. Woodall *et al* (1996) found that mothers subjected to a total food restriction to 30% of normal *ad libitum* intake produced offspring with systolic blood pressures 5-8 mmHg above the controls. Guinea pigs subjected to unilateral uterine artery ligation (which reduces placental blood flow to one of the uterine horns, thereby impeding fetal growth), produced offspring with a raised MAP of 7.5 mmHg above the controls (Persson & Jansson, 1992). Both of these procedures are very severe compared with the mild protein restriction (75% of requirement (Clarke *et al*, 1978)) used in this

study, yet the increase in blood pressure is not as great. This suggests that the composition of the diet is a very important factor in the programming of the fetus. Further evidence for the importance of the composition of the diet is that an 8% protein diet with different fat and carbohydrate sources does not lead to an elevated blood pressure in the offspring (Lucas *et al*, 1996, Langley-Evans, In Press 1999). In rats, blood pressure in early adulthood has been correlated with maternal protein intake (Langley & Jackson, 1994, Langley-Evans *et al*, 1994). In the human population maternal protein and carbohydrate intakes have been correlated with fetal growth (Godfrey *et al*, 1996) and blood pressure in the offspring at 40 years of age (Campbell *et al*, 1996).

In conclusion the results from the present studies have supported the hypothesis that maternal diet affects fetal growth and programmes the growth pattern and the blood pressure of the offspring. The following studies will investigate the possible mechanisms underlying the current findings. In particular the role of the renin-angiotensin system in the elevation of blood pressure in this model will be investigated as previous studies have suggested that it is altered in the low protein exposed rats compared with the control animals (Langley & Jackson, 1994, Langley-Evans & Jackson, 1995).

Chapter 3: part 2

The effect of feeding a maternal low protein diet on the renin-angiotensin system in the offspring.

3.6 Introduction

Experiments in animals support the hypothesis that undernutrition in the mother leads to persistent elevation of the blood pressure in later life (Langley-Evans *et al*, 1996g, chapter 3: part 1). Feeding an iron-free diet to female rats prior to mating and throughout pregnancy results in offspring, which are hypertensive in adult life (Crowe *et al*, 1993). Rats exposed to a maternal low protein diet *in utero* have significantly elevated systolic blood pressures in later life (Langley-Evans *et al*, 1996g, chapter 3: part 1). These hypertensive rats exposed to a maternal low protein diet, have been shown to have elevated plasma and pulmonary angiotensin converting enzyme (ACE) activities (Langley & Jackson, 1994). Captopril, which is an ACE inhibitor, was shown to reversibly normalise the blood pressures in these rats, suggesting a possible role for the renin-angiotensin system in the maintenance of high blood pressure in this model (Langley-Evans & Jackson, 1995). In studies of the human population, Konje and colleagues (1996) have shown that cord blood renin concentrations are inversely correlated with birth weight, whilst in later life (50-53 years) concentrations of active and inactive renin tend to be greater in the individuals who were heavier at birth (Martyn *et al*, 1996).

The data of Langley-Evans & Jackson (1995) and Konje *et al* (1996) appear to support a role for the renin-angiotensin system in the programming of hypertension, whilst Martyn *et al* (1996) suggest that the renin-angiotensin system may be suppressed in older men who were of low birth weight. This apparently contrasting evidence makes interpretation of the role of the renin-angiotensin system difficult. Hence, the aim of this study was to further characterise the effects of a maternal low protein diet upon the renin-angiotensin system in the offspring. This was intended as a preliminary approach to an investigation

of how renin-angiotensin system action may be involved in the prenatal programming of hypertension.

3.7 Methods

The methods of animal production, dietary administration, culling, and tissue collection are as described in chapter 2. Female rats were fed either a 9% or an 18% casein diet throughout pregnancy or a 9% casein diet during week one and/or week two and/or week three only of pregnancy. Pregnant rats were culled at either day 14 or day 20 of gestation using CO_2 as described in Chapter 2. Offspring were culled at birth, 4 weeks or 12 weeks of age by decapitation. Pulmonary ACE activity was measured using the method of Hayakiri *et al* (described in section 2.9.1). Plasma renin activity and angiotensin II measurements were performed by the MRC Blood Pressure Unit, Glasgow as described in sections 2.9.3 & 2.9.4. Plasma angiotensinogen concentrations were assessed by measuring the amount of angiotensin I produced in the presence of excess renin, as described by Tewksbury & Dart (1982). The angiotensin I produced was determined using a radioimmunoassay kit (NEN Life Sciences Products) as described in Section 2.9.5. Results were analysed using ANOVA (one or three-way) and t-tests.

3.8 Results

3.8.1 The effect of feeding a maternal low protein diet on the plasma renin activity, angiotensin II and angiotensinogen concentrations in the offspring.

Fifteen virgin female Wistar rats were mated and housed singly with free access to either an 18% casein (control) diet or a 9% casein diet throughout pregnancy (d0-22) or a 9%casein diet during week 1 only (d0-7), week 2 only (d8-14) or week 3 only (d15-22) of gestation. At 4 weeks of age the offspring were culled and blood and tissue collected. The offspring of the rats fed a 9% protein diet during week 3 only of gestation had significantly higher plasma renin activity than any other group (Table 3.9). Plasma angiotensin II concentrations tended to be lower, in all groups exposed to a maternal low protein diet during any period of gestation, in comparison to the control group and were

significantly lower in those animals exposed to a maternal low protein diet during week 1 of gestation only (Table 3.9).

At 12 weeks of age angiotensin II concentrations were greater in rats exposed to both a 9% casein and an 18% casein diet throughout gestation than at 4 weeks of age. Rats exposed to a 9% casein diet *in utero*, tended to have higher angiotensin II concentrations than controls at 12 weeks of age, although this was not statistically significant ($p<0.1$, Table 3.10). Plasma renin activity did not alter with age and did not differ between dietary groups (Table 3.10). At 12 weeks of age plasma angiotensinogen concentrations were similar in the two dietary groups (Table 3.10).

3.8.2 The effect of feeding a low protein diet during gestation on pulmonary ACE activity in the mother and the offspring.

Pregnant Wistar rats were fed either an 18% casein diet or a 9% casein diet throughout gestation or a 9% casein diet during different discrete periods of gestation (week 1, week 2, week 3). Numbers of litters and offspring used are given in the appropriate tables (Tables 3.11-3.13). Pulmonary ACE activity was measured as the lung contains the highest ACE activity of all the tissues (Allen, 1991). For the measurement of maternal pulmonary ACE activity the pregnant females were culled on day 14 of pregnancy and fetal ACE activity was measured in day 20 fetuses. Pulmonary ACE activity was not measured at day 14 of pregnancy as the lungs are too small at this stage. Pulmonary ACE activity was also measured at the neonatal stage and at 4 and 12 weeks of age. There was no significant effect of diet upon pulmonary ACE activity in the pregnant rat at day 14 of gestation (Table 3.11) or fetal pulmonary ACE activity at day 20 of gestation (Table 3.12). At the neonatal stage the rats exposed to a maternal low protein diet throughout gestation had pulmonary ACE activities that were similar to those of control pups. Those animals exposed to a maternal low protein diet during any discrete period of gestation had significantly elevated pulmonary ACE activity compared to the controls (Table 3.13). Rats exposed to a maternal 9% protein diet between days 8 and 14 of pregnancy had

elevated pulmonary ACE activities in comparison to both the 18% and 9% protein exposed groups ($p<0.06$).

By 4 weeks of age pulmonary ACE activity was lower in all groups, relative to activity at the neonatal stage, but followed a similar pattern of results to the neonatal rats. The animals exposed to a maternal 9% protein diet during discrete periods of pregnancy showed the highest levels of ACE activity (Table 3.14). Rats exposed to a maternal 9% protein diet between days 15 and 22 of gestation had significantly elevated pulmonary ACE activities in comparison to all other groups ($P<0.05$). By 12 weeks of age the offspring of rats exposed to a low protein diet throughout pregnancy had significantly higher pulmonary ACE activity than the controls (Figure 3.2).

Table 3.9. The effect of intrauterine exposure to a low protein diet on plasma renin and angiotensin II concentrations at 4 weeks of age.

Dietary Group	(n)	Renin (ng/ml/hr)	Angiotensin II (pg/ml)
18% protein (control)	(7)	7 ± 1	22 ± 5
9% protein	(6)	5 ± 1	13 ± 2
9% protein d0-7	(8)	6 ± 1	8 ± 1*
9% protein d8-14	(6)	6 ± 1	13 ± 3
9% protein d15-22	(8)	10 ± 2*†	15 ± 3

Pregnant Wistar rats were fed either an 18% protein (control) diet or a 9% protein diet throughout gestation or a 9% protein diet during discrete one week periods of pregnancy. At 4 weeks of age the offspring were culled and blood and tissue collected. Results are expressed as mean ± SEM and were analysed using an ANOVA and t-tests. * $p<0.05$ where p denotes the difference from the control group. $^{\dagger}p<0.05$ where p denotes the difference from all other groups. Litter n=3 for each dietary group.

Table 3.10. The effect of intrauterine exposure to a low protein diet on plasma renin, angiotensin II and angiotensinogen concentrations at 12 weeks of age.

Dietary Group	(n)	Angiotensinogen (μg/ml)	Renin (ng/ml/hr)	Angiotensin II (pg/ml)
18% protein (control)	(19)	2.7 ± 0.1	7 ± 1	38 ± 8
9% protein	(29)	2.6 ± 0.1	7 ± 1	57 ± 11

Pregnant Wistar rats were fed either an 18% protein (control) diet or a 9% protein diet throughout gestation. At 12 weeks of age the offspring were culled and blood and tissue collected. Results are expressed as mean ± SEM and were analysed using an ANOVA and t-tests. No significant differences were observed. Litter n=3 and 4 for the 18% protein group and the 9% protein group respectively.

Table 3.11. The effect of feeding a low protein diet during pregnancy on pulmonary ACE activity in the mother at day 14 of pregnancy.

Dietary Group	(n)	ACE activity (units/mg protein)	*p*
18% protein (control)	(5)	27 ± 2	
9% protein	(4)	31 ± 8	NS
9% protein d0-7	(5)	37 ± 8	NS
9% protein d8-14	(5)	31 ± 5	NS

Pregnant Wistar rats were fed either an 18% protein (control) diet or a 9% protein diet throughout gestation or a 9% protein diet during discrete one week periods of pregnancy. At day 14 of pregnancy the rats were culled and blood and tissue collected. Results are expressed as mean ± SEM and were analysed using an ANOVA and t-tests. *p* denotes the difference from the control group. One unit of ACE activity is the amount of enzyme which hydrolyses 1nmol of HHL to hippuric acid in 1 minute at 37^0C.

Table 3.12. The effect of feeding a low protein diet during pregnancy on the pulmonary ACE activity of the fetus at day 20 gestation.

Dietary Group	(n)	ACE activity (units/mg protein)	*p*
18% protein (control)	(5)	6.3 ± 0.3	
9% protein	(4)	6.8 ± 0.4	NS

Pregnant Wistar rats were fed either an 18% protein (control) diet or a 9% protein diet throughout gestation. At day 20 of pregnancy the rats were culled and fetal tissue collected. Results are expressed as mean ± SEM and were analysed using an ANOVA and t-tests. *p* denotes the difference from the control group. One unit of ACE activity is the amount of enzyme which hydrolyses 1nmol of HHL to hippuric acid in 1 minute at 37^0C. All of the rats were taken from different litters.

Table 3.13. The effect of feeding a low protein diet during pregnancy on neonatal pulmonary ACE activity in the offspring at birth.

Dietary Group	(n^1,n^2)	ACE activity (units/mg protein)	*p*
18% protein (control)	(29,9)	47 ± 2	
9% protein	(15,5)	53 ± 6	NS
9% protein d0-7	(23,6)	61 ± 4	0.003
9% protein d0-14	(15,5)	62 ± 3	0.0003
9% protein d8-14	(14,6)	68 ± 5	0.0007
9% protein d8-22	(7,5)	59 ± 4	0.04
9% protein d15-22	(28,8)	61 ± 4	0.003

Pregnant Wistar rats were fed either an 18% protein (control) diet or a 9% protein diet throughout gestation or a 9% protein diet during discrete one or two week periods of pregnancy. Shortly after birth the rats were culled and blood and tissue collected. Results are expressed as mean ± SEM and were analysed using an ANOVA and t-tests. *p* denotes the difference from the control group. One unit of ACE activity is the amount of enzyme which hydrolyses 1nmol of HHL to hippuric acid in 1 minute at 37^0C. n^1 = number of offspring used, n^2 = number of litters offspring were taken from.

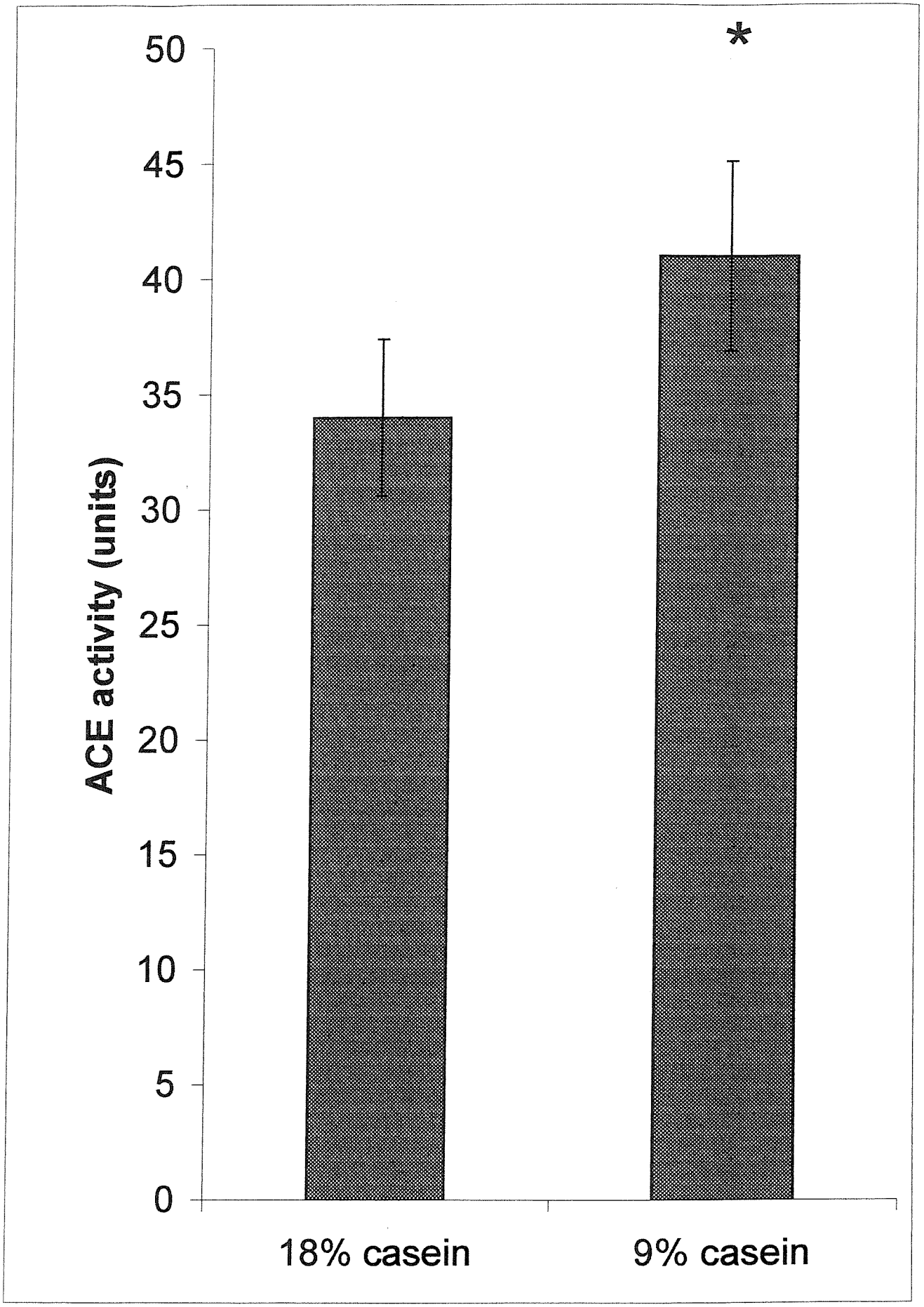
Table 3.14. The effect of feeding a maternal low protein diet during pregnancy on the pulmonary ACE activity of the offspring at 4 weeks of age.

Dietary Group	(n^1,n^2)	ACE activity (units/mg protein)	*p*
18% protein (control)	(12,3)	24 ± 1	
9% protein	(5,3)	27 ± 4	NS
9% protein d0-7	(19,5)	29 ± 2	0.03
9% protein d8-14	(25,6)	34 ± 2	0.000
9% protein d15-22	(18,5)	44 ± 4*	0.000

Pregnant Wistar rats were fed either an 18% protein (control) diet or a 9% protein diet throughout gestation or a 9% protein diet during discrete one week periods of pregnancy. At 4 weeks of age the rats were culled and blood and tissue collected. Results are expressed as mean ± SEM and were analysed using an ANOVA and t-tests. *p* denotes the difference from the control group, $*p<0.05$ where *p* denotes the difference from all other groups. One unit of ACE activity is the amount of enzyme which hydrolyses 1nmol of HHL to hippuric acid in 1 minute at 37^0C. n^1 = number of offspring used, n^2 = number of litters offspring are taken from.

Figure 3.2. The effect of intrauterine exposure to a maternal low protein diet on pulmonary ACE activity in later life.

Pregnant Wistar rats were fed either an 18% protein (control) diet or a 9% protein diet throughout gestation. At 12 weeks of age the offspring of these rats were culled and blood and tissue collected. Results are expressed as mean ± SEM and were analysed using an ANOVA and t-tests. *p<0.05 where p denotes the difference from all other groups. One unit of ACE activity is the amount of enzyme which hydrolyses 1nmol of HHL to hippuric acid in 1 minute at 37^0C. n = 6 and 9 for the control and low protein group respectively.


50
45
40
35
30
25
20
15
10
5
0
ACE activity (units)
*
18% casein
9% casein

3.9 Discussion

A rat model has been developed whereby the feeding of a low protein diet *in utero* has been associated with hypertension in later life (Langley & Jackson, 1994). This model is currently being used to elucidate the mechanisms of cardiovascular programming. Previous studies with this rat model of hypertension, induced by fetal exposure to maternal low protein diets have demonstrated elevated pulmonary ACE activity (Langley & Jackson, 1994) and a reduction in blood pressure with captopril treatment (Langley-Evans & Jackson, 1995). The aim of this study was to further investigate the effects of feeding a maternal low protein diet on the renin-angiotensin system in the offspring.

The results of the present study indicated that plasma renin activity was unaltered in rats exposed to a maternal 9% protein diet, except for those that were exposed to the maternal 9% protein diet during week 3 (d15-22) only, which had significantly greater plasma renin activities than all other groups. Angiotensin II concentrations tended to be lower in rats exposed to a maternal 9% protein diet during any discrete period of pregnancy, compared to the controls at 4 weeks of age. This was significant in the group exposed to the low protein diet between days 0 and 7 of gestation only. By 12 weeks of age angiotensin II concentrations appeared higher in the low protein exposed rats compared to the controls (not significant). Pulmonary ACE activity was elevated in all rats exposed to a maternal low protein diet during any discrete period of pregnancy compared to the controls from 4 weeks of age. At the neonatal stage the group exposed to a maternal low protein diet during week 2 of gestation only had pulmonary ACE activities significantly greater than both the 18% and 9% protein groups. At 4 weeks of age the group exposed to a maternal low protein diet during week 3 of gestation only had pulmonary ACE activities significantly greater than all other groups.

It has previously been suggested that angiotensinogen availability is the rate-limiting step in the renin-angiotensin pathway (Gould & Green, 1971). However, in the present study angiotensinogen was found to be available in excess in both the low protein exposed rats and the controls. Angiotensinogen concentrations compared well with published rat data (Bohlender *et al*, 1996).

Plasma renin activity was generally, although not significantly, lower in rats exposed to a maternal low protein diet except for those exposed in late gestation only, in which significantly elevated plasma renin activity was observed. This suggests that the timing of the nutritional insult is particularly important in the programming of renin activity. This may be related to the fact that the development of the kidney takes place mainly in late pregnancy as renin is produced in the kidney (Hinchliffe *et al*, 1992). Evidence from both human and animal studies suggests that the kidney is structurally and functionally altered in offspring subjected to poor maternal nutrition or intrauterine growth. Human fetuses, which are small for gestational age, have smaller, kidneys, which are thinner in shape (Konje *et al*, 1996) which also contain fewer nephrons than appropriately grown fetuses Hinchcliffe *et al*, 1992). Studies in rats have indicated that exposure to a maternal low protein diet *in utero* impairs renal development and function (Merlet-Bernichou *et al*, 1968, Zeman, 1968, Hall & Zeman, 1968). Experiments using the rat model described in this thesis, have shown that the offspring of mothers fed a moderately low protein diet in mid or late pregnancy, show different developmental patterns in the kidney during late gestation (Langley-Evans *et al*, 1999). Rats exposed to a maternal low protein diet throughout gestation have fewer nephrons in later life, relative to controls (Langley-Evans *et al*, 1999).

In studies of the human population Konje *et al* (1996) have shown that active renin concentrations in cord blood are higher in low birth weight babies suggesting that the renin-angiotensin system is programmed from early life. However active and inactive renin concentrations appear to be higher in adults who were heavier at birth (Martyn *et al*, 1996). Martyn and colleagues (1996) also showed that inactive renin was inversely associated with both systolic and diastolic blood pressure, whilst no relationship was found between active renin concentrations and blood pressure. This is supportive of the results from the present study where plasma renin activity does not appear to be related to blood pressure. Elevated blood pressure causes a decrease in renin secretion, so the relationship between high blood pressure and decreased renin activity is perhaps not surprising. The apparent inconsistency between the findings of Martyn *et al* (1996) and Konje *et al* (1996) may be explained by the possibility that the high renin concentrations

found in cord blood are not of fetal origin. It is also possible that they are high due to the different role of the renin-angiotensin system in the fetus. Plasma renin levels in chronically catheterised fetal sheep are higher than in the pregnant ewe (Lumbers 1995) and renal renin levels in human fetuses are 20-fold greater than the levels in the adult (Broughton Pipkin, 1993).

Angiotensin II is a potent vasoconstrictor and it is therefore surprising that low levels of this hormone are found in these animals with elevated blood pressures, however, a rise in blood pressure leads to a reduction in renin secretion and therefore a decrease in the angiotensin I produced. This does not appear to be consistent with the observation that levels of ACE activity were increased and blood pressure has been shown to be normalised by treatment with an ACE inhibitor (Langley-Evans & Jackson, 1995). Plasma angiotensin II levels may not be important in the regulation of blood pressure, for example circulating levels rise in association with angiotensin II antagonist treatment (due to the blockade of the negative feedback mechanism) which leads to a decrease in blood pressure (Morton *et al*, 1992). It should not be assumed that circulating angiotensin II concentrations are the same as the effective levels of angiotensin II at the receptor sites. It is possible that elevated blood pressure is leading to reduced renin secretion and therefore angiotensin II concentrations in the low protein exposed rats, but that these animals have an increased sensitivity to angiotensin II. This may be why ACE inhibition leads to a decrease in blood pressure despite an apparent excess of renin or angiotensin II. In support of this argument rats exposed to a maternal low protein diet *in utero* demonstrate an increased pressor response to angiotensin II compared to controls (Gardner *et* al, 1998).

ACE activity was found to be elevated in the lungs of the offspring of rats exposed to a maternal low protein diet from early life, whilst maternal pulmonary ACE activity was not affected by exposure to the low protein diet. This provides evidence that the ACE activity in the offspring is independent of maternal ACE and that the low protein diet does not directly modulate ACE activity. This suggests that other regulators must be working in the tissues of the offspring and that ACE activity may be programmed by

intrauterine exposure to a low protein diet. Elevated plasma ACE activity has also been observed in rats exposed to a maternal 9% protein diet (Langley-Evans & Jackson, 1995). Glucocorticoids have been well characterised as having a programming effect upon the developing fetus and are known to induce ACE activity (Langley-Evans, 1997, Mendelsohn *et al*, 1982). They also induce the expression of the angiotensin II type 1 (AT_1) receptor in vascular smooth muscle tissue (Sato *et al*, 1994). 11-β hydroxysteroid dehydrogenase (11β-HSD) activity in the placenta protects the fetus from exposure to maternal glucocorticoids by metabolising them to inactive forms (Edwards *et al*, 1993). The placental activity of this enzyme is correlated with fetal weight (Benediktsson *et al*, 1993, Edwards *et al*, 1993). Treatment with dexamethasone (a glucocorticoid which is poorly metabolised by 11β-HSD) during pregnancy in the rat causes hypertension in the offspring (Benediktsson *et al*, 1993), supporting the hypothesis that increased fetal glucocorticoid exposure, programmes elevated blood pressure in later life (Benediktsson *et al*, 1993, Edwards *et al*, 1993).

Placental 11β-HSD activity is significantly reduced in pregnant rats fed low protein diets (Phillips *et al*, 1994). Metyrapone treatment during pregnancy (which inhibits glucocorticoid synthesis) prevents the onset of hypertension in the offspring of rats fed a low protein diet (Langley-Evans *et al*, 1996f). The replacement of maternal corticosterone reverses this effect, suggesting that exposure to glucocorticoids during fetal life is necessary for the programming of hypertension (Langley-Evans, unpublished data). Maternal adrenalectomy prior to pregnancy also prevents the effects of feeding a maternal low protein diet on the blood pressures of the offspring (Langley-Evans *et al*, unpublished data). This evidence together suggests that fetal exposure to an excess of glucocorticoids is an important first step in the programming of hypertension. Fetal ACE activity is not altered by diet and it is therefore suggested that elevated pulmonary ACE activity in the low protein exposed rats after birth is due to increased glucocorticoid action in late pregnancy. Increased glucocorticoid action is evident in the low protein exposed fetuses at this time as shown by the massive increase in glycerol 3-phosphate dehydrogenase (GPDH) activity between day 20 and day 22 of gestation (Langley-Evans

et al, 1996c). GPDH is a glucocorticoid-inducible enzyme and such an increase in activity is not observed in the control animals (Langley-Evans *et al*, 1996c).

Pulmonary ACE activity is significantly increased in those animals whose mothers were exposed to a low protein diet during discrete periods of gestation (particularly weeks 2 and/or 3) at the neonatal stage and at 4 weeks of age. These results follow a similar pattern to those of the glucocorticoid-inducible enzymes (tyrosine aminotransferase (TAT), glutamine synthase (GS) and GPDH), which also exhibit increased activity in rats exposed to a maternal low protein diet during discrete periods of pregnancy, particularly the last two weeks (Langley-Evans *et al*, 1996g, Langley-Evans & Nwagwu, 1998). This evidence supports the hypothesis that the elevated ACE activity observed in the offspring of low protein fed rats in the current study is induced by fetal exposure to excess glucocorticoids.

From these results it is clear that the renin-angiotensin system is affected by exposure to a low protein diet *in utero* and that further studies are necessary to elucidate the role. Pulmonary ACE activity is elevated by exposure to a maternal low protein diet, an effect which is observed from an early age and is attributable to fetal exposure to excess glucocorticoids. Raised ACE activity does not result in an increase in circulating angiotensin II concentrations. It is therefore important to determine whether the increased ACE activity observed, is involved in either the programming or maintenance of hypertension in this model. Further studies will therefore determine the effects of ACE inhibition on the blood pressure of these animals.

Chapter 4: part 1

The effect of feeding a maternal low protein diet on prostaglandin excretion in the offspring.

4.1. Introduction

Intrauterine growth retardation is associated with an increased risk of hypertension in later life (Barker *et al*, 1993, Langley-Evans *et al*, 1996g). The rat model of maternal diet-induced hypertension has been used to study the possible mechanisms involved in the programming of hypertension (Langley-Evans *et* al, 1996g). As discussed in the previous chapter, experiments have indicated that the renin-angiotensin system may have a role in the development and maintenance of hypertension in this model (Langley-Evans *et al*, 1996g, Chapter 3). Prostaglandins are locally acting hormones, which are derived from polyunsaturated fatty acids and affect a wide variety of physiological processes (Currie & Needleman, 1984, Wagner & Kurtz, 1998). They are synthesised from arachidonic acid, which is found in mammalian tissue mainly as structural lipids in the cellular and sub-cellular membranes (Wagner & Kurtz, 1998). Tissue concentrations of arachidonic acid are dependent on both dietary linoleic acid intake (Wagner & Kurtz, 1998) and protein intake (De Tomas *et al*, 1980, De Tomas *et al*, 1983, Narce *et al*, 1991). Renal prostaglandins are known to mediate blood flow and their interactions with the renin-angiotensin system are of particular interest in hypertension (Wagner & Kurtz, 1998).

Prostaglandins PGE_2 and PGI_2 are potent renal vasodilators and help to maintain renal blood flow, specifically in the presence of vasoconstrictor hormones such as angiotensin II (Wagner & Kurtz, 1998). Angiotensin II stimulates the release of PGE_2 and PGI_2 by the kidney, which subsequently blunts the peptide-induced vasoconstriction (Wagner & Kurtz, 1998). PGE_2, PGI_2 and 6-keto-PGE_1 have a stimulatory effect on the release of renin, which is thought to be mediated by cyclic AMP (Jackson *et al*, 1982, Wagner & Kurtz, 1998). PGE_2 has been shown to act as a vasoconstrictor in rat kidney, at high concentrations, where it potentiates the action of angiotensin II and noradrenaline (Baer & McGiff, 1979, Wagner & Kurtz, 1998).

The conversion of polyunsaturated fatty acids to prostaglandins is dependent upon the activity of desaturases (Hassam *et al*, 1975). The activities of desaturases involved in the conversion of linoleic acid to arachidonic acid, the precursor of PGE_2, have been shown to be reduced in animals subjected to a maternal low protein diet during pregnancy and lactation (De Tomas *et al*, 1983). Protein deprivation during pregnancy and lactation exerts a marked effect on the fatty acid composition of liver phospholipids, including arachidonic acid (De Tomas *et al*, 1983). This fatty acid pattern was reversed when protein deficient animals were placed on a control diet at birth (De Tomas *et al*, 1980 & 1983, Narce *et al* 1991). Spontaneously hypertensive rats (SHRs) also show abnormalities of desaturase activity and apparent changes in overall fatty acid profiles. Six month old SHRs have particularly high desaturase activities and the hepatocyte membrane fatty acid composition indicates an impairment of n-6 polyunsaturated fatty acid metabolism (Narce *et al*, 1994). Pathways of prostaglandin synthesis may thus be subject to programming effects of maternal diet, resulting in defects of prostaglandin metabolism associated with hypertension. The pathway of prostaglandin degradation is also of interest. 15-Hydroxyprostaglandin dehydrogenase (PGDH) is considered to be the key enzyme responsible for the biological inactivation of prostaglandins (Tai *et al*, 1979, Nagai *et al*, 1991). Decreased activity of PGDH has been found in the kidneys of SHR's (Tai *et al*, 1979). These animals also exhibit increased urinary PGE_2 excretion (Dunn, 1978).

The renin-angiotensin system and prostaglandins interact to a large extent, therefore the aim of the present study was to determine whether prostaglandin metabolism is altered in low protein exposed rats, with known alterations to renin-angiotensin system activities.

4.2. Methods

The methods of animal production, dietary administration, culling, urine and tissue collection were as described in chapter 2. Pregnant Wistar rats (n=30) were fed on either an 18% casein (control) diet (n=15) or a 9% casein diet (n=15) throughout gestation. Some of the mothers were culled on days 14 or 20 (n=3 for each group) of gestation in order to collect fetal and placental tissue. Others were allowed to continue to full term

and the offspring were culled at the neonatal stage, 4 weeks, 7 weeks or 12 weeks of age (n=8 offspring from 3 litters for each group). Prostaglandins PGE_2 and 6-keto-$PGF_{1\alpha}$ were measured in urine samples using an enzymeimmunoassay (Amersham) (Sections 2.9.6. and 2.9.7). 24-hour urine collections were made in the offspring at 4 and 12 weeks of age using metabolic cages as described in section 2.6. PGDH activity was determined in tissue samples using the method of Nagai *et al* (1991) (section 2.9.9) and expressed per mg protein (section 2.9.2). Results were analysed using ANOVA and t-tests.

4.3. Results

4.3.1. The effect of feeding a maternal low protein diet upon urinary prostaglandin excretion in the offspring.

24-hour urine samples were collected from 4-week-old or 12-week-old female offspring of rats fed either an 18% protein diet or a 9% protein diet throughout pregnancy. The volume of urine excretion did not differ between the dietary groups (data not shown). Urinary PGE_2 concentrations were found to be significantly higher in the rats exposed to a maternal low protein diet compared to the controls at both ages (Figure 4.1). At 12 weeks of age urinary PGE_2 excretion was lower than had been observed at 4 weeks of age ($p<0.001$). Urinary 6-keto-$PGF_{1\alpha}$ concentrations at 12 weeks (Figure 4.2) tended to be higher in the low protein exposed rats, however this was not significant.

4.3.2. The effect of feeding a maternal low-protein diet on tissue PGDH activity in the offspring.

Pregnant Wistar rats (n=30) were fed on either an 18% casein (control) diet (n=15) or a 9% casein diet (n=15) throughout gestation. Some of the mothers were culled on days 14 or 20 (n=3 for each group) of gestation in order to collect fetal and placental tissue. Others were allowed to continue to full term and the offspring were culled at the neonatal stage, 4 weeks, 7 weeks or 12 weeks of age (n=8 offspring from 3 litters for each group). PGDH activity was assessed in placenta, kidney, liver, lung, and spleen. At day 14 of gestation PGDH activity was elevated in the placental tissue of the low protein exposed mothers ($p=0.05$, Table 4.1). This difference was no longer statistically significant by

day 20 of gestation. Renal PGDH activity followed an altered developmental pattern in rats exposed to a maternal low protein diet (Figure 4.3). In both the control and low protein fed animals PGDH activity was markedly increased over the last two days of gestation, such that at birth it was 70% of mature activity. At both day 20 of gestation and the neonatal stage, renal PGDH activity did not differ between the two dietary groups. However, by 4 weeks of age, activity in the kidney was significantly lower in the offspring of the rats exposed to a low protein diet during pregnancy compared to the controls ($p<0.001$, Figure 4.3). By 12 weeks of age this difference in renal activity had disappeared. There were no significant differences between dietary groups in PGDH activity in the liver, lung or spleen at the ages studied (Table 4.2, Figures 4.4 - 4.5). The developmental pattern of PGDH activity was similar in both the lung and liver in the control animals, with a peak in activity between 4 and 7 weeks of age (Figures 4.4 and 4.5). However the low protein exposed rats showed a linear increase in pulmonary PGDH activity between birth and 12 weeks of age (Figure 4.5).

Figure 4.1. The effect of feeding a low protein diet during pregnancy on urinary prostaglandin E_2 excretion in the offspring.

Pregnant Wistar rats were fed either an 18% protein (control) diet (n=6) or a 9% protein diet (n=6) throughout gestation. At 4 and 12 weeks of age 24-hour urine samples were collected from offspring from each litter (n=12). Results are expressed as mean ± SEM and were analysed using an ANOVA and t-tests. *$p<0.05$ where p denotes the difference from the control group.

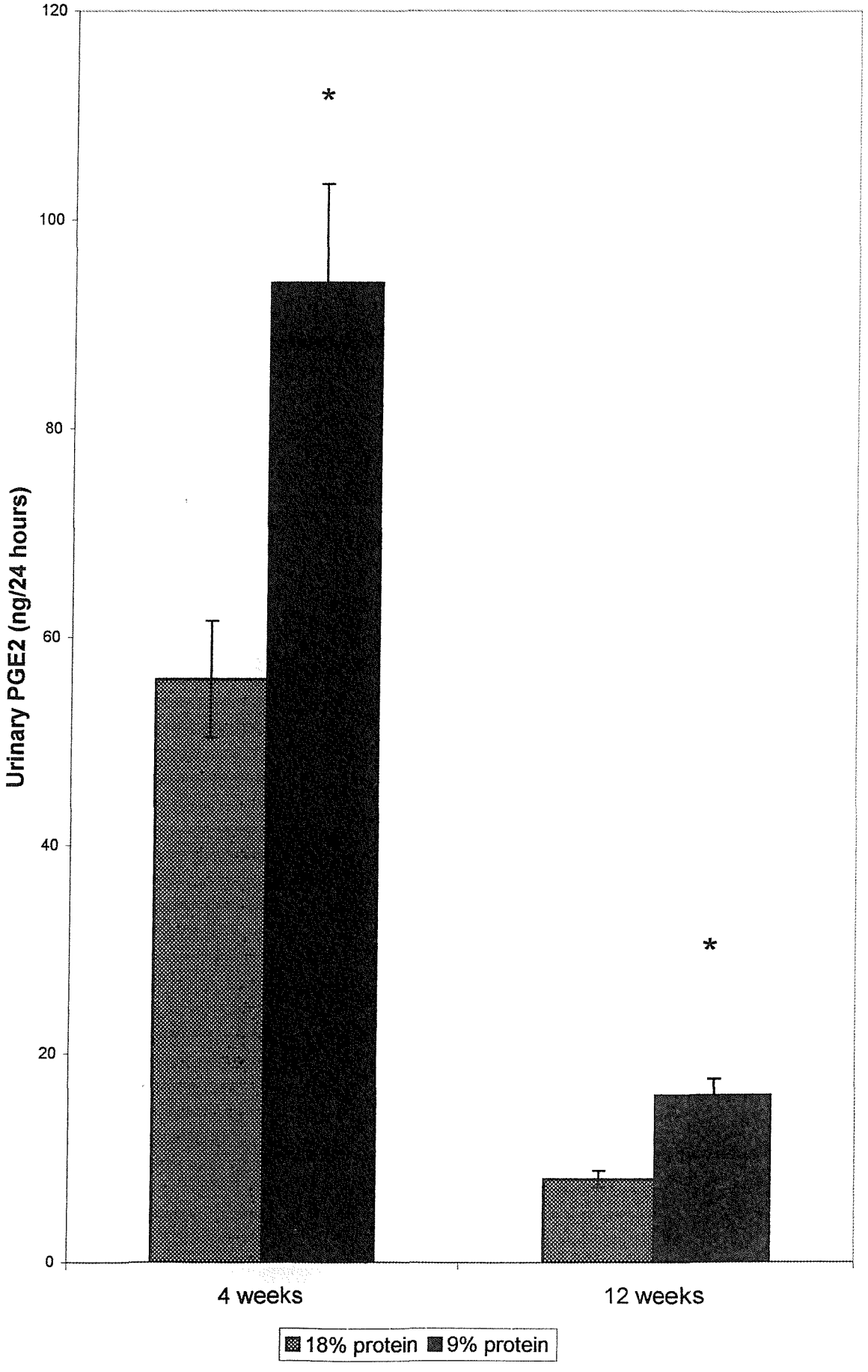

120
100
80
60
40
20
0
Urinary PGE2 (ng/24 hours)
*
*
4 weeks
12 weeks
18% protein
9% protein

Figure 4.2. The effect of feeding a low protein diet during pregnancy on urinary 6-ketoPGF$_{1\alpha}$ excretion in the offspring.

Pregnant Wistar rats were fed either an 18% protein (control) diet (n=6) or a 9% protein diet (n=6) throughout gestation. At 12 weeks of age 24-hour urine samples were collected from offspring from each litter (n=12). Results are expressed as mean ± SEM and were analysed using an ANOVA and t-tests. No significant differences were observed.

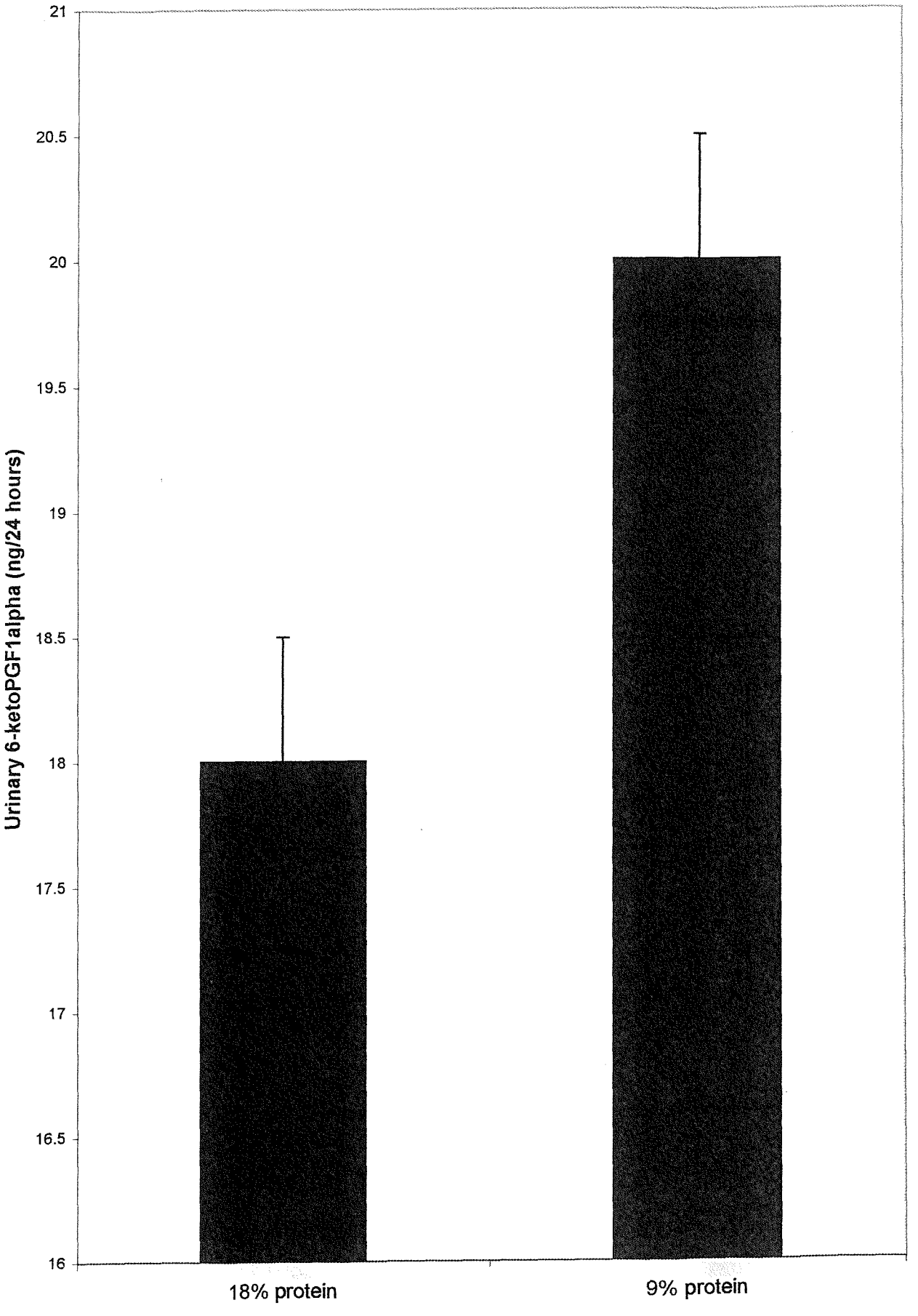

21
20.5
20
19.5
19
18.5
18
17.5
17
16.5
16
Urinary 6-ketoPGF1alpha (ng/24 hours)
18% protein
9% protein

Table 4.1. The effect of feeding a maternal low protein diet on the PGDH activity in the placentae of the offspring.

Age	Units activity/mg protein		*p*
	18% casein	9% casein	
Day 14	39 ± 0.2	59 ± 0.5	0.05
Day 20	57 ± 0.2	79 ± 0.1	NS

Pregnant Wistar rats were fed either an 18% casein (control) diet or a 9% casein diet throughout gestation. Mothers were culled at either day 14 or day 20 of pregnancy to allow for the collection of fetal and placental tissue. Results are expressed as mean ± SEM in units of activity per mg protein where 1 unit is equal to 1 pmol product formed/min. Results were analysed using t-tests. *p* denotes the difference in activity observed in the low protein exposed rats compared to the controls. NS denotes no significant difference from the controls.

Table 4.2. The effect of feeding a maternal low protein diet on the PGDH activity in the spleen of the offspring.

Age	Units activity/mg protein		*p*
	18% casein	9% casein	
Day 14	39 ± 0.2	59 ± 0.5	0.05
Day 20	57 ± 0.2	79 ± 0.1	NS
4 weeks	31 ± 0.0	37 ± 0.1	NS
12 weeks	31 ± 0.3	34 ± 0.4	NS

Pregnant Wistar rats were fed either an 18% casein (control) diet or a 9% casein diet throughout gestation. Offspring were culled at 4 weeks of age and 12 weeks and tissue collected. Results are expressed as mean ± SEM in units of activity per mg protein where 1 unit is equal to 1 pmol product formed/min. Results were analysed using t-tests. *p* denotes the difference in activity observed in the low protein exposed rats compared to the controls. NS denotes no significant difference from the controls.

Figure 4.3. The effect of feeding a low protein diet during pregnancy on renal PGDH activity in the offspring.

Pregnant Wistar rats were fed either an 18% protein (control) diet (n=6) or a 9% protein diet (n=6) throughout gestation. Tissue samples were collected from offspring from each litter at the time points shown (n=12). Results are expressed as mean ± SEM and were analysed using an ANOVA and t-tests. *$p<0.001$, where p denotes a significant difference from the control group. Units are equivalent to nmol product formed/min/mg protein.

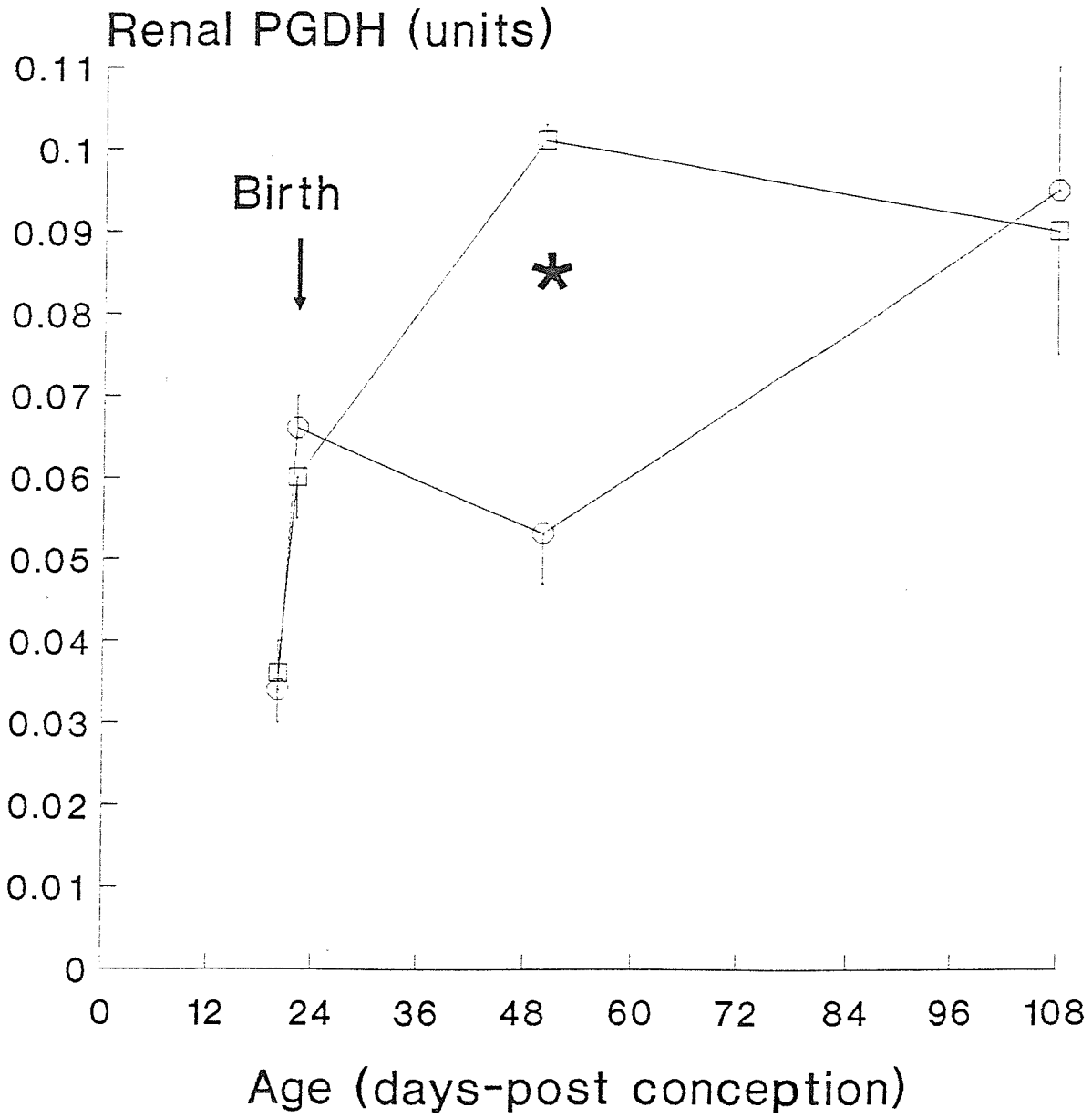

Renal PGDH (units)
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Birth
*
0
12
24
36
48
60
72
84
96
108
Age (days-post conception)

Figure 4.4. The effect of feeding a low protein diet during pregnancy on hepatic PGDH activity in the offspring.

Pregnant Wistar rats were fed either an 18% protein (control) diet (n=6) or a 9% protein diet (n=6) throughout gestation. Tissue samples were collected from offspring from each litter at the time points shown (n=12). Results are expressed as mean ± SEM and were analysed using an ANOVA and t-tests. Results did not differ significantly.

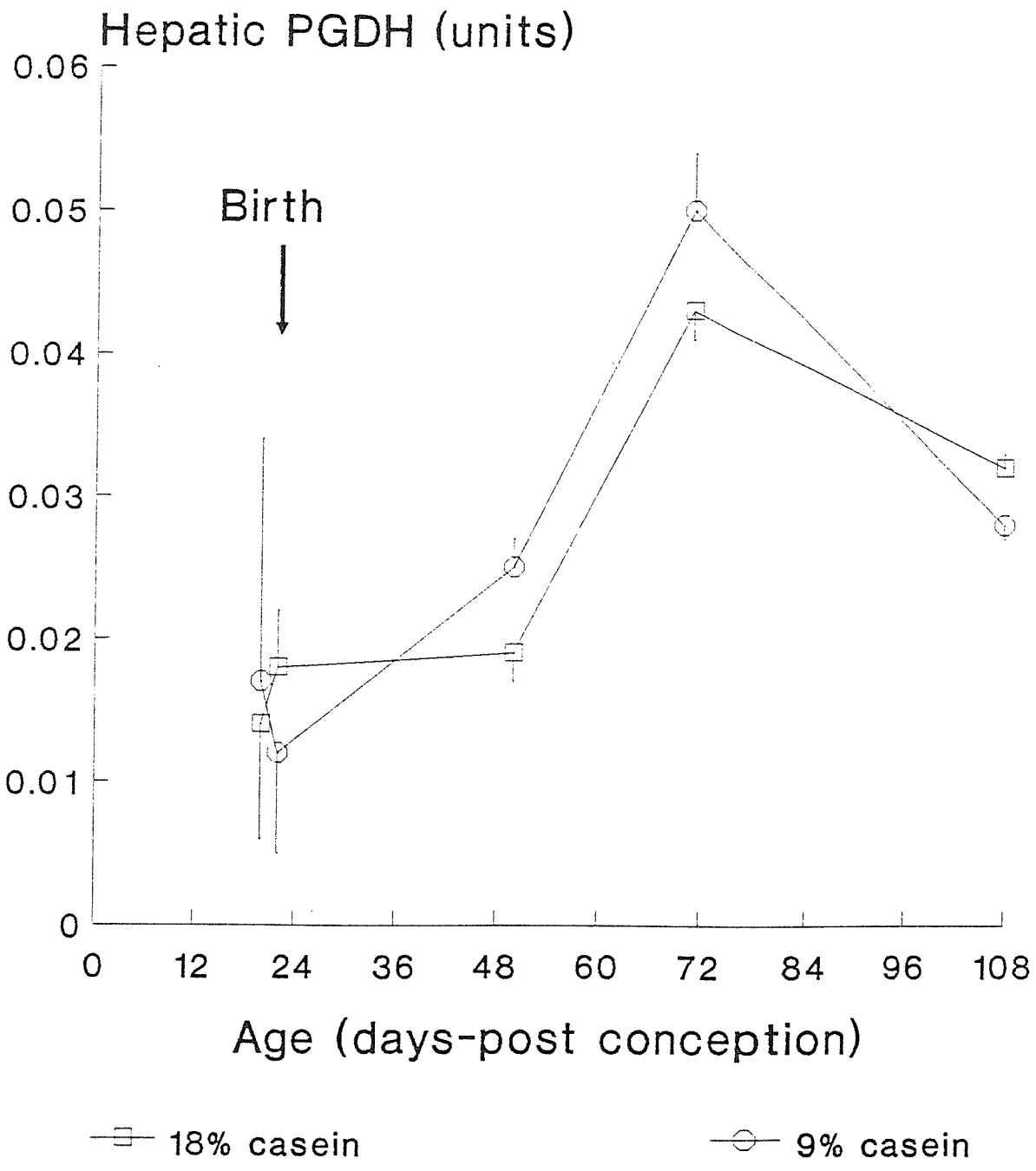
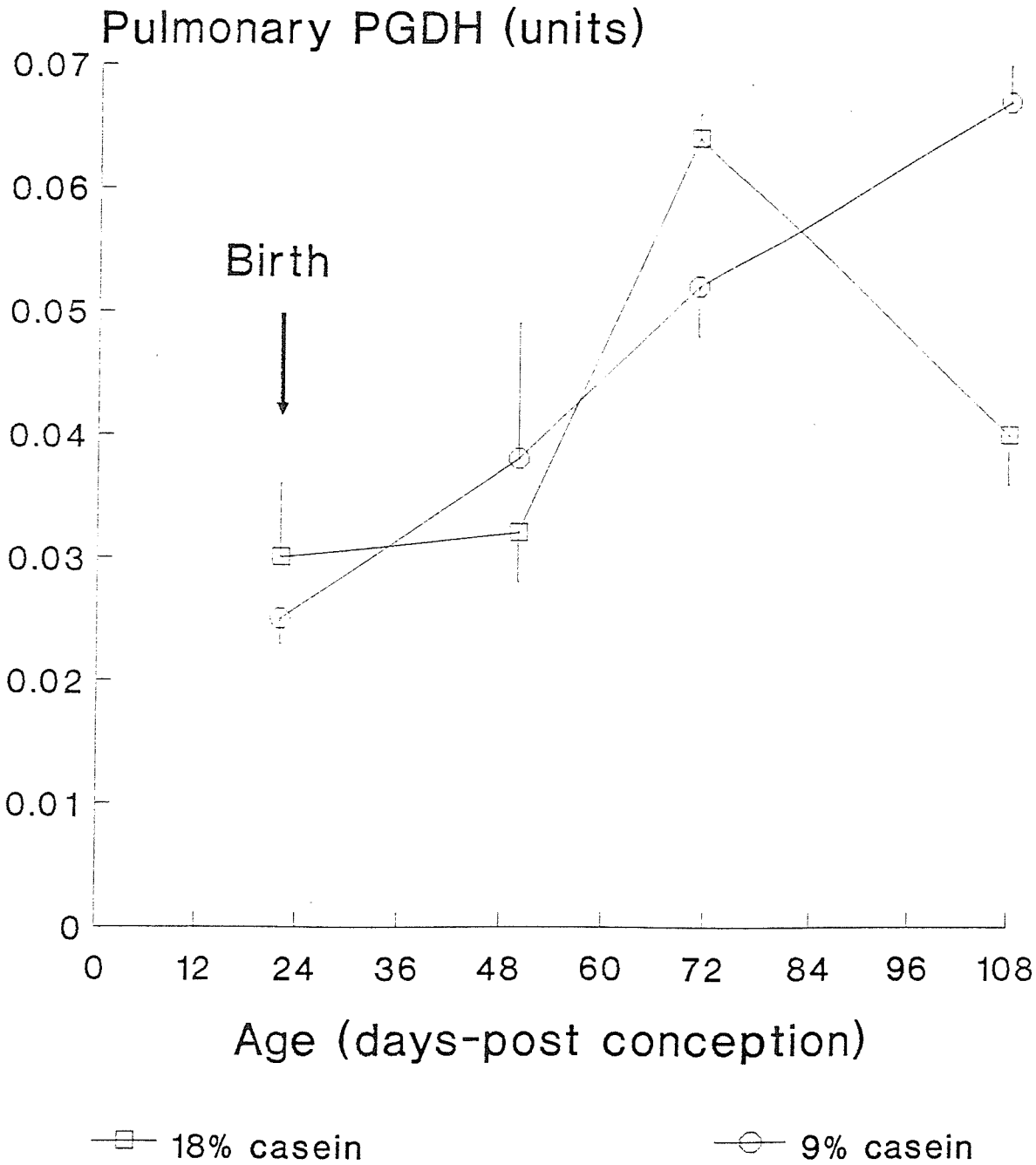

Hepatic PGDH (units)
0.06
0.05
0.04
0.03
0.02
0.01
0
Birth
0
12
24
36
48
60
72
84
96
108
Age (days-post conception)
18% casein
9% casein

Figure 4.5. The effect of feeding a low protein diet during pregnancy on pulmonary PGDH activity in the offspring.

Pregnant Wistar rats were fed either an 18% protein (control) diet (n=6) or a 9% protein diet (n=6) throughout gestation. Tissue samples were collected from offspring from each litter at the time points shown (n=12). Results are expressed as mean ± SEM and were analysed using an ANOVA and t-tests. No significant differences were observed.


Pulmonary PGDH (units)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Birth
0
12
24
36
48
60
72
84
96
108
Age (days-post conception)
18% casein
9% casein

4.4. Discussion

The aim of the current study was to assess prostaglandin metabolism in the rat model of maternal-diet-induced hypertension. This was considered important due to the way in which the prostaglandins interact with the renin-angiotensin system, which shows evidence of programming in this model. The spontaneously hypertensive rat (SHR) model, which exhibits similar disturbances of the renin-angiotensin system to the low protein rat model, also exhibits patterns of altered prostaglandin metabolism (Tai *et al*, 1979, Konieczkowski *et al*, 1983, Dunn, 1978).

Urinary PGE_2 excretion was found to be significantly elevated in rats exposed to a maternal low protein diet, both at 4 and 12 weeks of age (Figure 4.1). This finding is consistent with observations of increased urinary PGE_2 excretion in the SHR strain (Dunn *et al*, 1978). This has also been independently verified in our laboratory (data not shown). However PGE_2 excretion was significantly lower at 12 weeks in both the low protein exposed and the control animals compared to excretion at 4 weeks of age. In the SHR elevated prostaglandin excretion appears to be due to both an increase in synthesis (Konieczkowski *et al*, 1983) and a reduction in degradation by PGDH (Tai *et al*, 1979). In the current study degradative activity tended to increase between birth and 12 weeks of age, possibly explaining the reduction in the concentration of urinary PGE_2 at 12 weeks of age compared to 4 weeks of age.

Urinary 6-keto-$PGF_{1\alpha}$ excretion appeared to be slightly elevated in the low protein exposed animals at 12 weeks of age but this was not statistically significant. 6-keto-$PGF_{1\alpha}$ is the stable metabolite of PGI_2 (prostacyclin), which acts in a similar way to PGE_2 to affect blood pressure. Urinary concentrations of the PGI_2 metabolite were similar to the levels of PGE_2 excretion observed at 12 weeks of age. Concentrations at 4 weeks were not compared.

At day 14 of pregnancy, PGDH activity was elevated in the placenta of rats fed a low protein diet. The same trend was observed at day 20 but the difference was no longer found to be statistically significant. The developmental profile of PGDH activity was

altered in the kidney by fetal protein restriction. It was also disturbed, to a lesser extent, in the lung, but fetal exposure to the low protein diet had no effect upon either hepatic or spleen activity. PGDH activity was significantly reduced in the kidney of rats exposed to a maternal 9% casein diet (by approximately 50%) at 4 weeks of age, but by 12 weeks this difference had disappeared. In the lungs, the enzyme activity tended to be higher (not significant) at 12 weeks of age. Similar observations were noted in the SHR strain. Markedly lower PGDH activity in the kidney and greater pulmonary activity were reported (Tai *et al*, 1979). These effects, however, were apparently not transient and were observed in both 6 and 12 week old animals (Tai *et al*, 1979). There were no other significant differences in PGDH activity, between dietary groups, in other organs at any given age.

Very little has been published on the action, regulation or role of PGDH activity. It occurs in many tissues and may have tissue specific roles, for example in the placenta it may be necessary to reduce PGE_2 concentrations to prevent the early onset of labour. Placental PGDH activity increased during pregnancy, which may relate to increasing PGE_2 levels around the time of birth.

PGE_2 is a potent vasodilator, so elevated levels in urine would appear to be inconsistent with elevated blood pressure. However, PGE_2 has been shown to act as a vasoconstrictor in rat kidney, at high concentrations, where it potentiates the action of angiotensin II and noradrenaline (Baer & McGiff, 1979). PGE_2 release is stimulated by angiotensin II and also stimulates the release of renin (Currie & Needleman 1984, Jackson *et al*, 1982). Neither renin activity nor angiotensin II concentrations appear to differ in the low protein exposed rats compared to the controls. PGE_2 excretion does not appear to be related to angiotensin II concentrations in the different age groups. Angiotensin II concentrations appear to increase with age whilst urinary PGE_2 amounts decrease, suggesting that the PGE_2 excretion is not directly related to the circulating angiotensin II concentrations.

Elevated PGE_2 excretion in this model is likely to be related to either an increase in production (for example through increased desaturase activity) or a decrease in the rate of

breakdown. PGDH is the key enzyme responsible for the biological inactivation of PGE_2 (Tai *et al*, 1979, Nagai *et al*, 1991). Elevated activity in the placenta may lead to a decrease in prostaglandins and therefore a decrease in perfusion of the placenta and a decrease in the supply of oxygen and nutrients to the fetus, which would further contribute to mid-late gestational growth retardation (Langley-Evans *et al*, 1996b). PGDH activity is regulated by glucocorticoids (Tsai & Brown, 1987), which may be of particular significance in this model (Langley-Evans, 1998). 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 is an enzyme in the placenta which protects the fetus from maternal glucocorticoids (Seckl, 1997). It has been demonstrated that placental 11β-HSD type 2 activity is decreased in the low protein rat model (Phillips *et al*, 1994), in association with an increase in glucocorticoid-inducible enzyme activities (Langley-Evans & Nwagwu, 1998). This suggests an increase in glucocorticoid stimulation of the tissue, which may promote increased placental PGDH expression as is suggested by the activity data shown in the present study.

Rats exposed to a low protein diet in fetal life have increased glucocorticoid receptor numbers (Langley-Evans *et al*, 1996c) and are therefore more sensitive to the actions of glucocorticoids in adult life. The hypertension observed in the low protein rat model is glucocorticoid-dependent and is prevented by the pharmacological blockade of glucocorticoid synthesis (Langley-Evans, 1997). Dexamethasone, which is a synthetic glucocorticoid, has been shown to upregulate PGDH expression (Tsai & Brown, 1987). However, the results from the current study show that these animals, which are hypersensitive to glucocorticoids, exhibit decreased PGDH activity in the kidney in association with elevated urinary PGE_2 and elevated blood pressure. It is possible that the reduced renal PGDH activity observed in the low protein exposed rats is due to impaired renal development. The kidneys of such animals have been shown in some studies to be small in proportion to body size and to contain fewer nephrons than the kidneys of control animals (Langley-Evans *et al*, 1997). It is, therefore, possible that the cells expressing PGDH may be present in fewer numbers in the kidneys of the low protein exposed rats.

In general PGE_2 is a vasodilator, so the elevated levels observed in this model, in association with hypertension, are difficult to explain. Elevated urinary PGE_2 has however, been associated with elevated blood pressure in other rat models (Konieczkowski *et al*, 1983). Spontaneously hypertensive rats show both elevated desaturase activity and decreased PGDH activity (Dunn, 1978, Tai *et al*, 1979). PGE_2 has been shown to act as a vasoconstrictor, in high concentrations, in the rat kidney (Baer & McGiff, 1979). Thus it may be the case that, in the low protein exposed rats, the PGE_2 synthesised in the kidney acts locally to increase renal vascular resistance, due to ineffective clearance by PGDH.

At 4 weeks of age the decrease in renal PGDH activity in the low protein exposed rats appears consistent with the observed increase in PGE_2 excretion (both approximately 50%). Although the period of decreased renal PGDH activity is transient, other studies have also identified the period between 2 and 4 weeks of age as a critical period for the programming of the cardiovascular system (Sherman & Langley-Evans, 1998). The studies described in Chapters 5 and 6 have shown that the onset of hypertension can be prevented in the low protein exposed rats by treatment with ACE inhibitors or angiotensin II receptor antagonists, between 2 and 4 weeks of age. This suggests that this is an important period in the development of the hypertension observed in these animals.

In conclusion, rats exposed to a maternal low protein diet in fetal life exhibit disturbances of prostaglandin metabolism. The observations are similar to those made in the spontaneously hypertensive rat strain. It is possible that the differences noted in the low protein exposed rats compared to the control animals, may have a role in the disturbance of the renal control of blood pressure in this model. Due to the unusual association between elevated urinary prostaglandin excretion and raised blood pressure, and the relative ease of the non-invasive measurement, urinary PGE_2 concentration would be a useful measurement to make in the human population.

Chapter 4: part 2

Urinary prostaglandin excretion in 10 year old children and its relationship with the risk of hypertension in later life.

4.5. Introduction

The offspring of rats fed a low protein diet during pregnancy become hypertensive in later life (Chapter 3, Langley-Evans *et al*, 1997). They also show disturbances of the renin-angiotensin system and prostaglandin metabolism, which may contribute to the onset and/or maintenance of the elevated blood pressure observed in this model (Chapter 3 and Chapter 4: part 1, Langley-Evans *et al*, 1997, 1998).

Rats exposed to a maternal low protein diet *in utero* have significantly elevated urinary PGE_2 excretion rates (Chapter 4: part 1). This is also associated with altered pattern of activity of renal PGDH (Chapter 4: part 1). PGDH, which is a key enzyme responsible for the breakdown of prostaglandins, had a significantly reduced level of activity in the kidneys of 4-week old rats exposed to a maternal low protein diet compared to control animals (Chapter 4: part 1). The spontaneously hypertensive rat (SHR) model, which shows some similarities to the low protein exposed rat model of hypertension, such as elevated PGE_2 excretion (Dunn, 1978), also exhibits a decreased activity of renal PGDH (Tai *et al*, 1979), as well as high desaturase activities which are involved in the production of precursors of the prostaglandins (Narce *et al*, 1994).

Prostaglandin concentrations are useful measurements to make in this model as they are vasoactive agents, which regulate blood pressure and the actions of the renin-angiotensin system. Prostaglandin excretion can also be measured relatively easily without using invasive techniques. It is therefore possible to use these measurements to compare the rat model with cohorts of human subjects studied with respect to the 'fetal origins hypothesis'.

Although there are many similarities between the rat model and the human disease, there is very little biochemical evidence that the mechanisms linking fetal nutrition and later

hypertension identified in the rat model, are the same as those involved in the progression of human disease. The aim of this study was to determine whether similar disturbances of prostaglandin excretion to those noted in Chapter 4:part 1, are found in the human population in relation to the risk of hypertension. Such a comparison of rat and human observations is a unique step in the study of the fetal origins of hypertension.

4.6. Methods

The subjects were recruited from the Princess Anne Hospital Growth Study and the MRC Salisbury cohort with the help of Mr T. Wheeler (Department of Obstetrics and Gynaecology, Princess Anne Hospital, Southampton) and Dr P. Clark (MRC Environmental Epidemiology unit, Southampton General Hospital, Southampton) respectively. The methods of recruitment and urine collection are as described in chapter 2, as are the methods of PGE_2 determination. Results were analysed using linear regression analysis by Professor C. Osmond (MRC Environmental Epidemiology unit, Southampton General Hospital, Southampton).

A preliminary study was carried out to assess the effects of different methods of sample collection on the stability and measurement of urinary PGE_2 and creatinine. Five volunteers collected 4 urine samples at different times of the day. Each sample was divided into 4 aliquots, the first of which was frozen at -70^0C immediately. The second aliquot was acidified (6M HCl) and frozen immediately, whilst the third and fourth aliquots were acidified and frozen after 6 and 72 hours respectively. Urinary PGE_2 was determined in each sample and expressed per mg creatinine. The addition of acid and the length of time before freezing had no significant effect on the PGE_2 concentration (variation was $< 10\%$). The variation between samples collected at different times of the day was $\leq 15\%$

4.7. Results

4.7.1. Birth Characteristics

All of the subjects were born at full term in either the Princess Anne Hospital, Southampton or in Salisbury where their birth measurements were made by hospital staff for their own clinical records. 96 children were included in the study (50 boys and 46 girls). The birth weight, ponderal index and head circumference measurements for each group are shown in Table 4.3. The children born in Salisbury were, on average, smaller at birth than those born in Southampton and were thinner with smaller head circumference i.e. disproportionately smaller (Table 4.3).

4.7.2. Current characteristics

All of the children on the study were considered to be normal and in good health. The age of the children at the time of urine collection was 9.66 ± 0.14 years. The current height, weight and blood pressure of the children is shown in Table 4.4. Multiple regression analysis showed that current systolic blood pressure was correlated with birth weight (R=-0.314, p=0.05), ponderal index (R=-0.332, p=0.046) and head circumference (R=0.331, p=0.047) after data was corrected for child's current weight and height. Diastolic blood pressure did not show the same level of correlation with birth characteristics. Urinary PGE_2 was measured using an enzymeimmunoassay kit (Amersham) and was expressed per creatinine. A summary of the results is shown in Table 4.4. Children born in Salisbury were, on average, slightly older than those born in Southampton and tended to have higher diastolic blood pressures (p<0.05), as well as being shorter (not significant) and weighing less (not significant, Table 4.4).

4.7.3. The relationship between birth characteristics, current characteristics and urinary PGE_2 concentrations

Regression analyses were performed to determine the relationship between urinary PGE_2 concentrations and birth and current characteristics, which may relate to the risk of hypertension in later life. PGE_2 concentrations did not correlate with any of the current characteristics such as age, height, weight, systolic blood pressure or diastolic blood

pressure (Shown in Figures 4.6 – 4.10). No correlation was noted between PGE_2 and birth weight (Figure 4.11). Urinary PGE_2 was found to be negatively correlated with both ponderal index (Figure 4.12, $p<0.05$) and head circumference (Figure 4.13, $p<0.04$). Data was corrected for sex, which strengthened both relationships ($p<0.02$ and $p<0.03$ for ponderal index and head circumference respectively). PGE_2 by tertiles of ponderal index and head circumference are shown in Table 4.5. The above effects were not observed when the two groups of children were analysed separately.

Table 4.3. Birth characteristics of study subjects

	All	Salisbury	Southampton
Birth weight (g)	3430 ± 562	3266 ± 499	3539 ± 579*
Head circumference (cm)	35.20 ± 1.50	34.33 ± 1.13	35.62 ±1.41**
Ponderal index	27.08 ± 3.02	25.48 ± 2.33	27.95 ±3.01**

Subjects were recruited from the MRC Salisbury study or the Princess Anne Hospital Growth Study. Birth characteristics were taken from hospital records. Results were expressed as the mean ± standard deviation. *p<0.05, **p<0.001 where p shows the difference between the Southampton group and the Salisbury group.

Table 4.4. Current characteristics of study subjects

	All	Salisbury	Southampton
Age (years)	9.66 ± 0.14	9.80 ± 0.13	9.62 ± 0.11*
Height (cm)	136.57 ± 6.26	134.30 ± 6.40	137.30 ± 6.10
Weight (kg)	33.14 ± 6.06	31.42 ± 5.58	33.71 ± 6.15
Diastolic pressure (mmHg)	54.78 ± 6.05	57.10 ± 5.77	54.01 ± 5.98*
Systolic pressure (mmHg)	97.72 ± 8.54	100.83 ± 11.4	96.68 ± 7.18
PGE_2 (pg/mg creatinine)	3.77 ± 1.72	4.02 ± 1.85	3.61 ± 1.61

Subjects were recruited from the MRC Salisbury study or the Princess Anne Hospital Growth Study. Birth characteristics were taken from hospital records and anthropometric measurements were made on the respective cohort studies. Urine samples were collected and urinary PGE_2 concentrations were measured using an enzymeimmunoassay (Amersham) and were expressed per creatinine. Results were expressed as the mean ± standard deviation. *$p \leq 0.05$ where p shows the difference between the Southampton and Salisbury studies.

Figure 4.6. Urinary PGE_2 concentration plotted against age

Subjects were recruited from the MRC Salisbury study or the Princess Anne Hospital Growth Study. Birth characteristics were taken from hospital records and anthropometric measurements were made on the respective cohort studies. Urine samples were collected and urinary PGE_2 concentrations were measured using an enzymeimmunoassay (Amersham) and were expressed per creatinine. R = -0.058, p=0.66. Units = pg/mg creatinine.

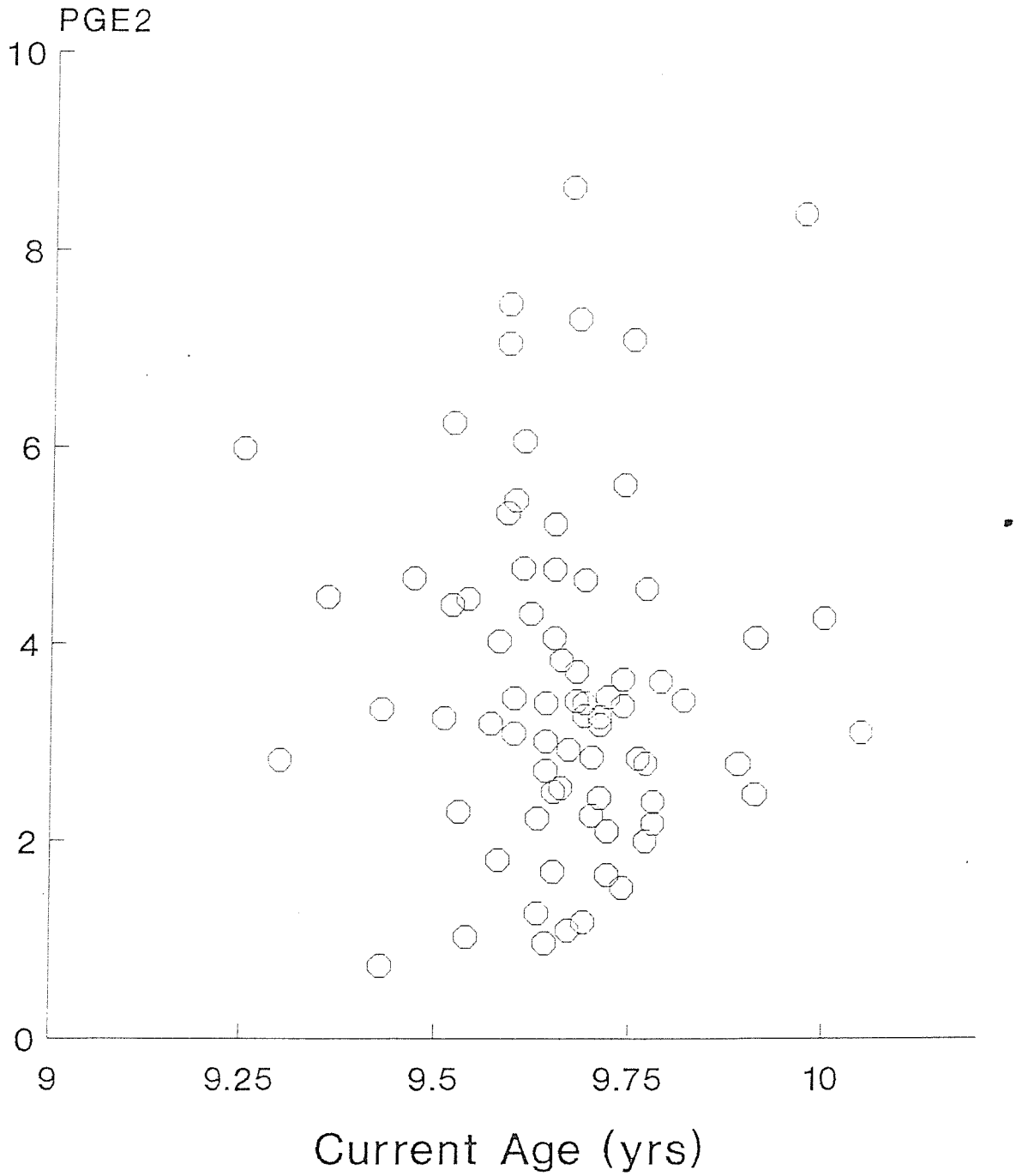

PGE2
10
8
6
4
2
0
9
9.25
9.5
9.75
10
Current Age (yrs)

Figure 4.7. Urinary PGE_2 concentration plotted against current height in cms

Subjects were recruited from the MRC Salisbury study or the Princess Anne Hospital Growth Study. Birth characteristics were taken from hospital records and anthropometric measurements were made on the respective cohort studies. Urine samples were collected and urinary PGE_2 concentrations were measured using an enzymeimmunoassay (Amersham) and were expressed per creatinine. R = 0.096, p =0.41. Units = pg/mg creatinine.

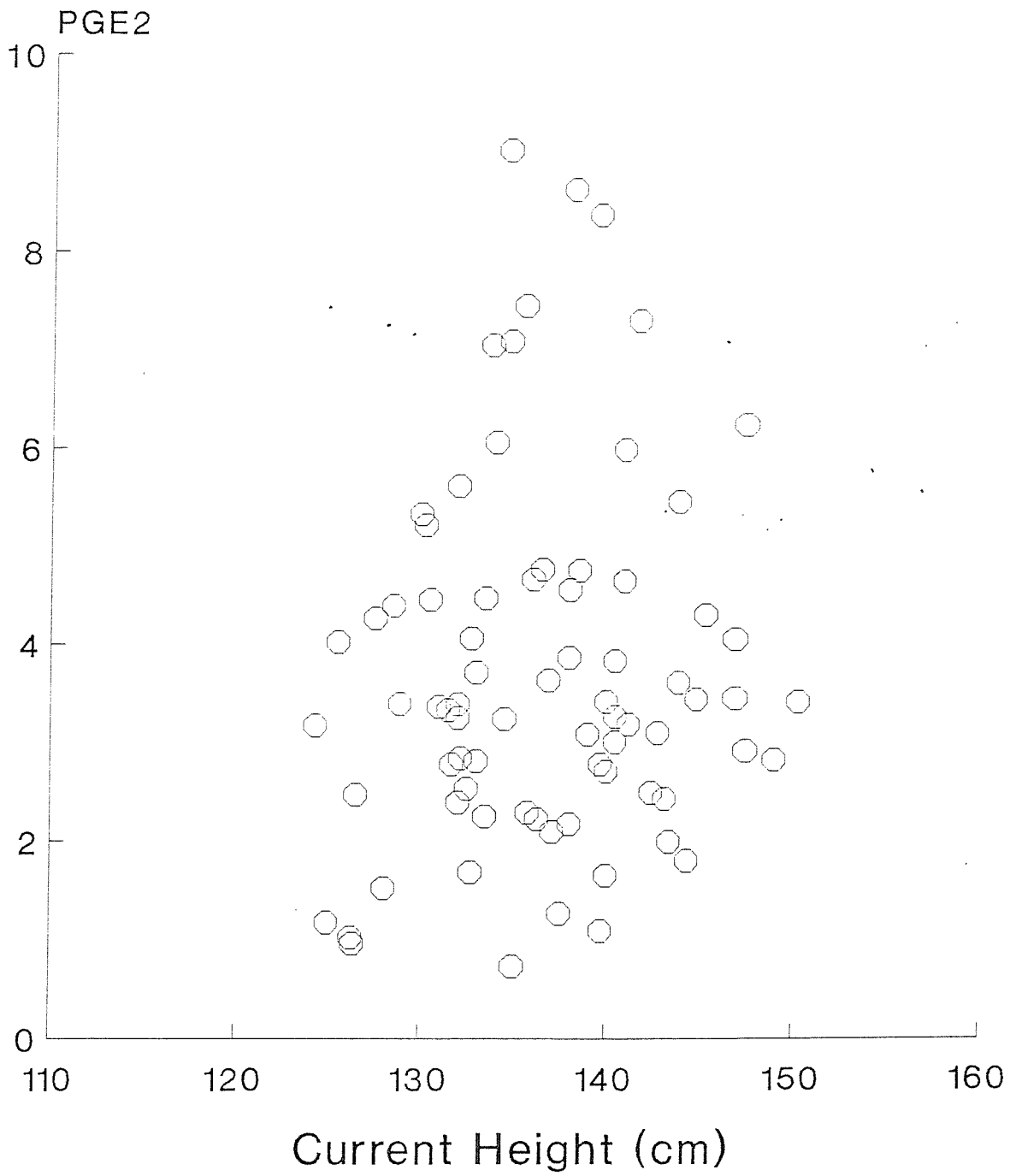

PGE2
10
8
6
4
2
0
110
120
130
140
150
160
Current Height (cm)

Figure 4.8. Urinary PGE_2 concentration plotted against current weight in kgs

Subjects were recruited from the MRC Salisbury study or the Princess Anne Hospital Growth Study. Birth characteristics were taken from hospital records and anthropometric measurements were made on the respective cohort studies. Urine samples were collected and urinary PGE_2 concentrations were measured using an enzymeimmunoassay (Amersham) and were expressed per creatinine. R = -0.043, p =0.71. Units = pg/mg creatinine.

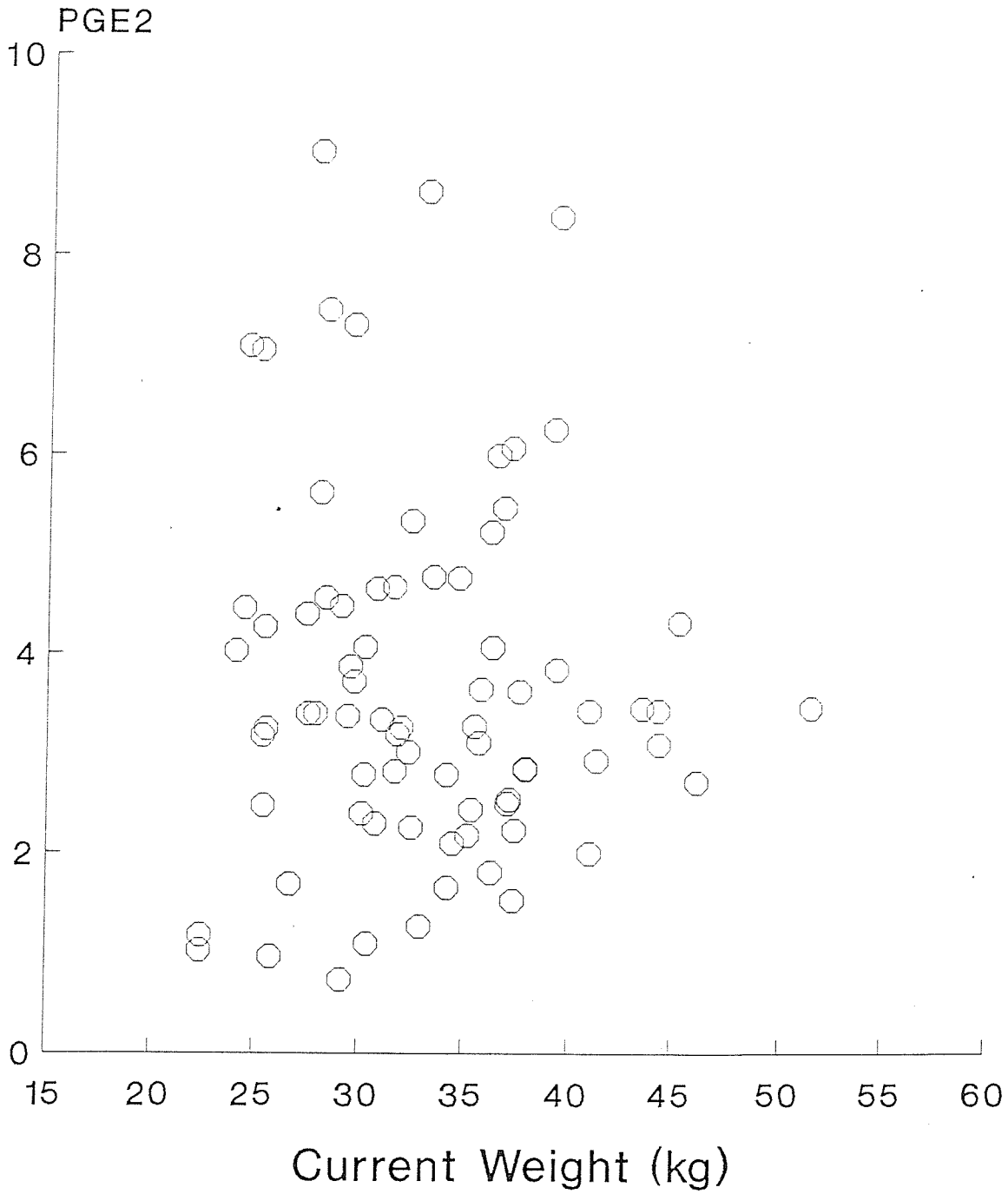

PGE2
10
8
6
4
2
0
15
20
25
30
35
40
45
50
55
60
Current Weight (kg)

Figure 4.9. Urinary PGE_2 concentration plotted against current systolic blood pressure

Subjects were recruited from the MRC Salisbury study or the Princess Anne Hospital Growth Study. Birth characteristics were taken from hospital records and anthropometric and blood pressure measurements were made on the respective cohort studies. Urine samples were collected and urinary PGE_2 concentrations were measured using an enzymeimmunoassay (Amersham) and were expressed per creatinine. R = -0.104, p =0.37. Units = pg/mg creatinine.

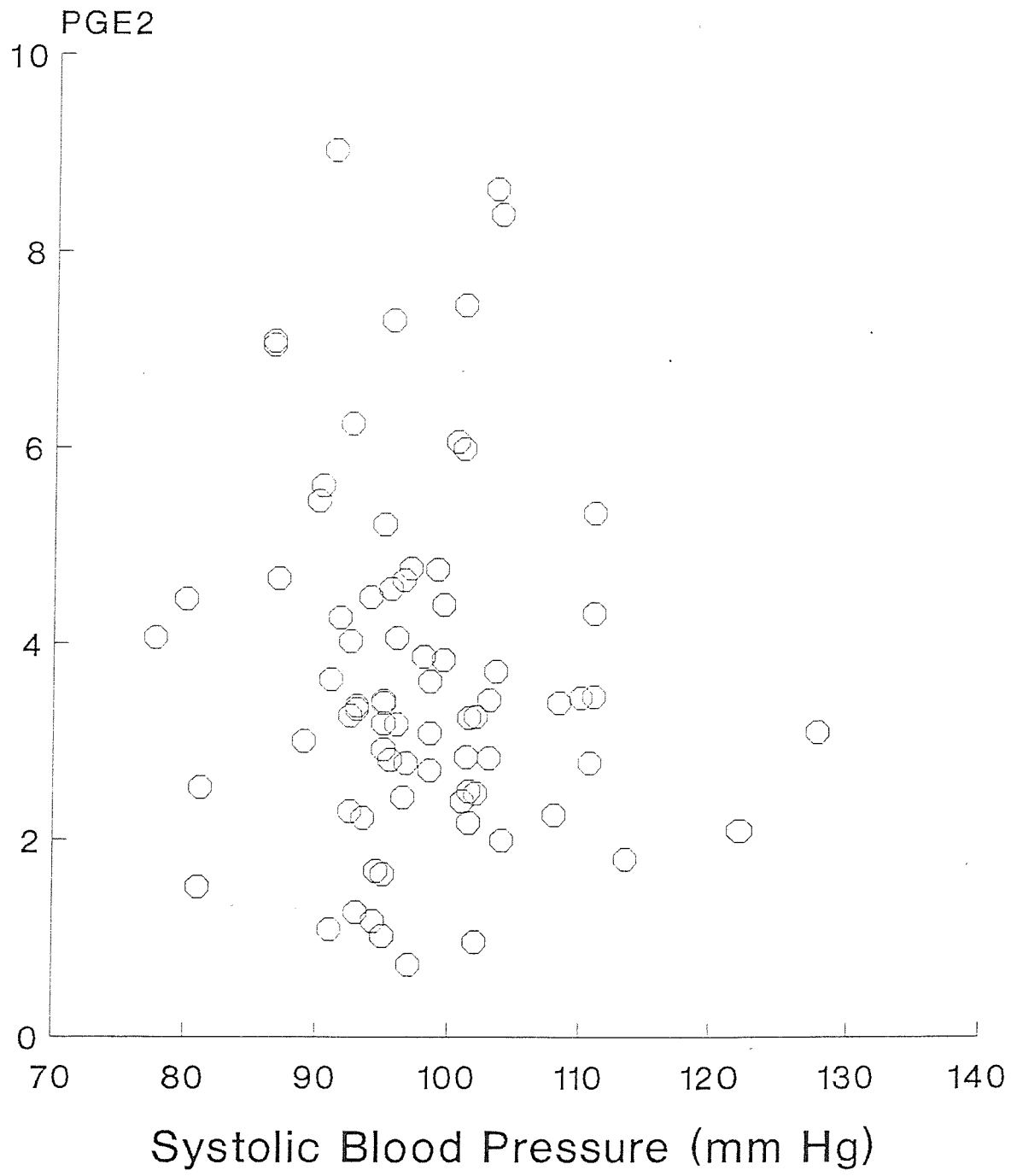

PGE2
10
8
6
4
2
0
70
80
90
100
110
120
130
140
Systolic Blood Pressure (mm Hg)

Figure 4.10. Urinary PGE_2 concentration plotted against current diastolic blood pressure

Subjects were recruited from the MRC Salisbury study or the Princess Anne Hospital Growth Study. Birth characteristics were taken from hospital records and anthropometric and blood pressure measurements were made on the respective cohort studies. Urine samples were collected and urinary PGE_2 concentrations were measured using an enzymeimmunoassay (Amersham) and were expressed per creatinine. R = -0.098, p =0.40.

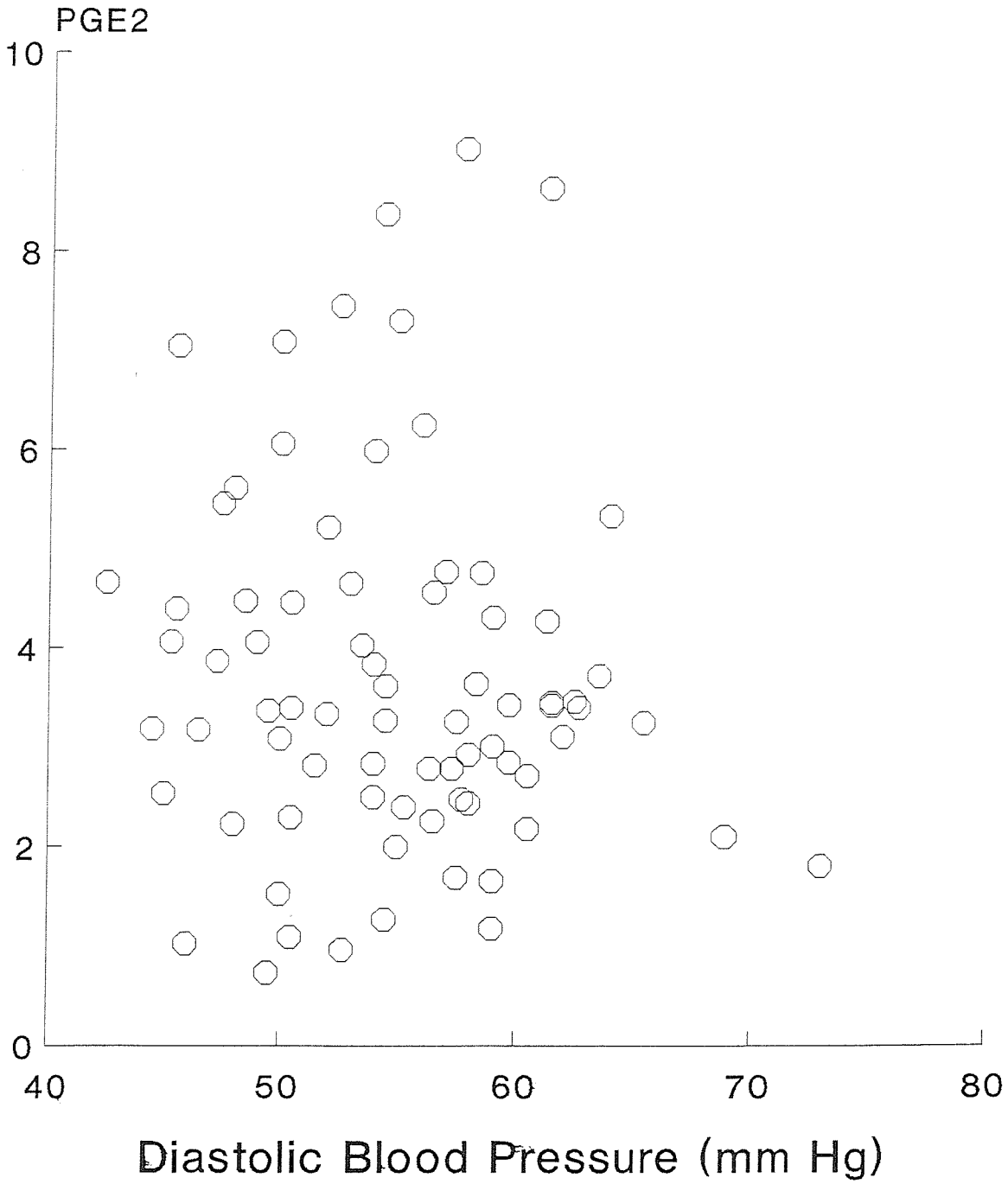

PGE2
10
8
6
4
2
0
40
50
60
70
80
Diastolic Blood Pressure (mm Hg)

Figure 4.11. Urinary PGE_2 concentration plotted against birth weight in grams

Subjects were recruited from the MRC Salisbury study or the Princess Anne Hospital Growth Study. Birth characteristics were taken from hospital records and anthropometric and blood pressure measurements were made on the respective cohort studies. Urine samples were collected and urinary PGE_2 concentrations were measured using an enzymeimmunoassay (Amersham) and were expressed per creatinine. R = -0.100, p =0.33.

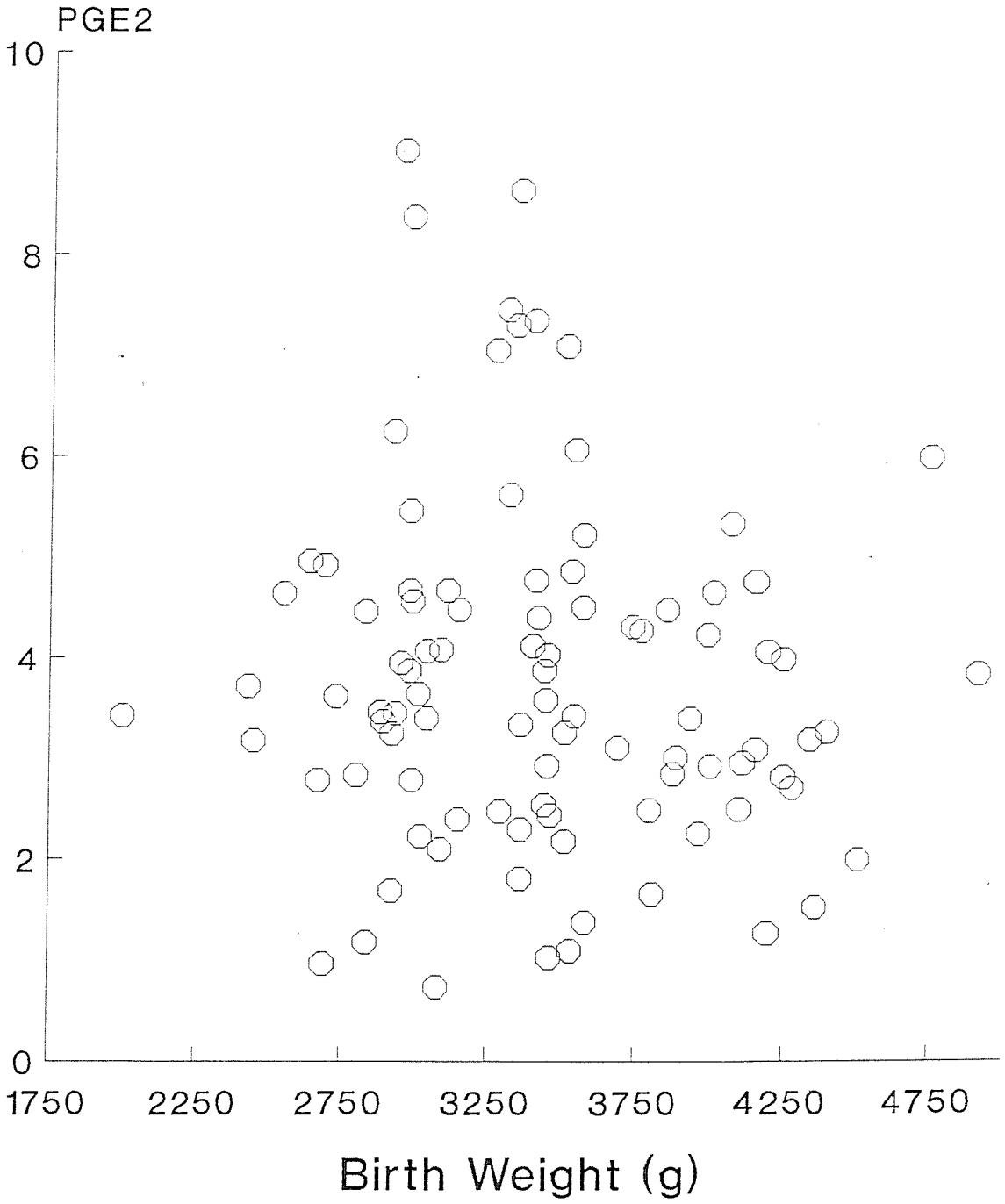

PGE2
10
8
6
4
2
0
1750
2250
2750
3250
3750
4250
4750
Birth Weight (g)

Figure 4.12. Urinary PGE_2 concentration plotted against ponderal index

Subjects were recruited from the MRC Salisbury study or the Princess Anne Hospital Growth Study. Birth characteristics were taken from hospital records and anthropometric and blood pressure measurements were made on the respective cohort studies. Urine samples were collected and urinary PGE_2 concentrations were measured using an enzymeimmunoassay (Amersham) and were expressed per creatinine. R = -0.211, p =0.047.

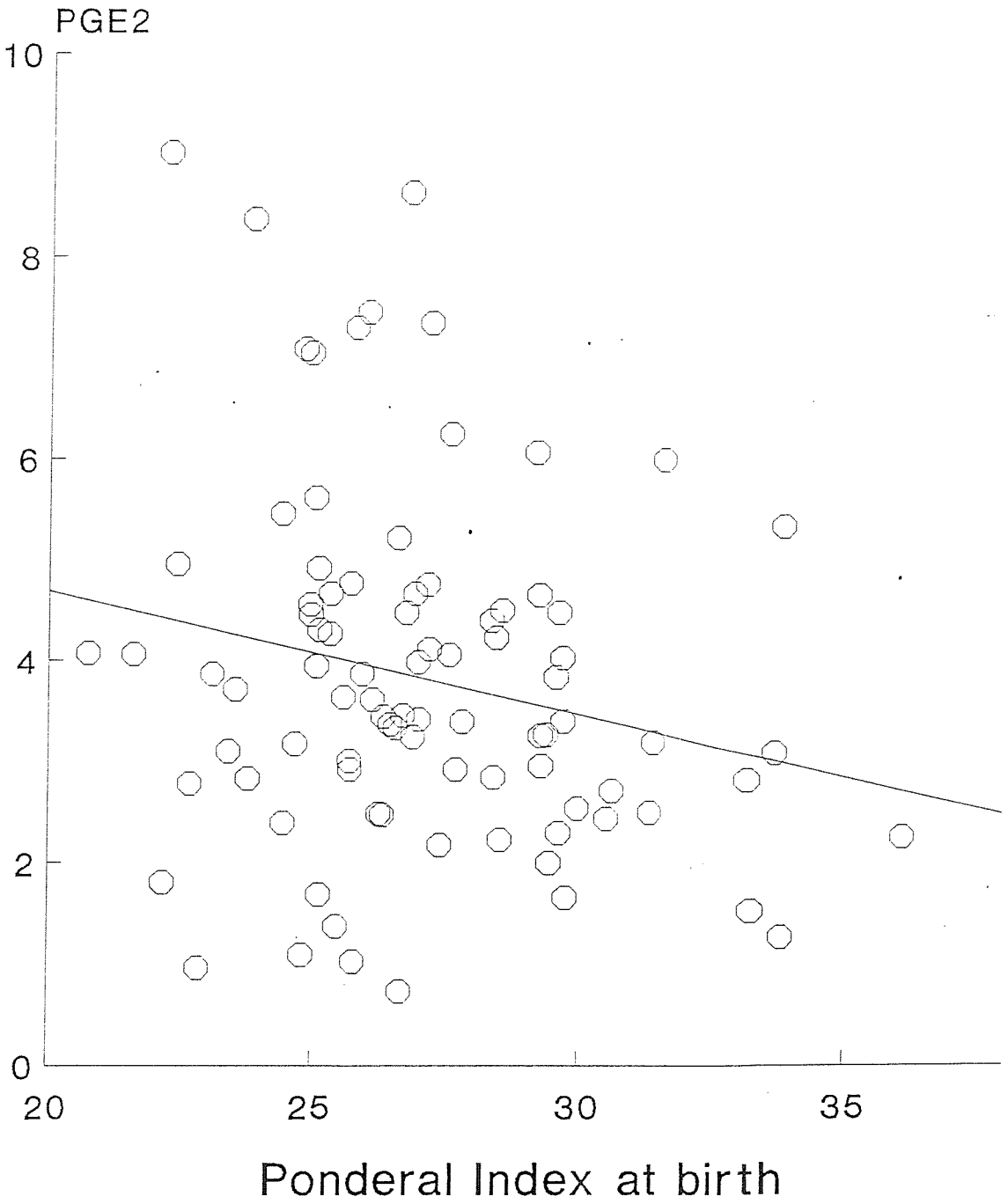

PGE2
10
8
6
4
2
0
20
25
30
35
Ponderal Index at birth

Figure 4.13. Urinary PGE_2 concentration plotted against head circumference at birth

Subjects were recruited from the MRC Salisbury study or the Princess Anne Hospital Growth Study. Birth characteristics were taken from hospital records and anthropometric and blood pressure measurements were made on the respective cohort studies. Urine samples were collected and urinary PGE_2 concentrations were measured using an enzymeimmunoassay (Amersham) and were expressed per creatinine. $R = -0.222$, $p = 0.037$.

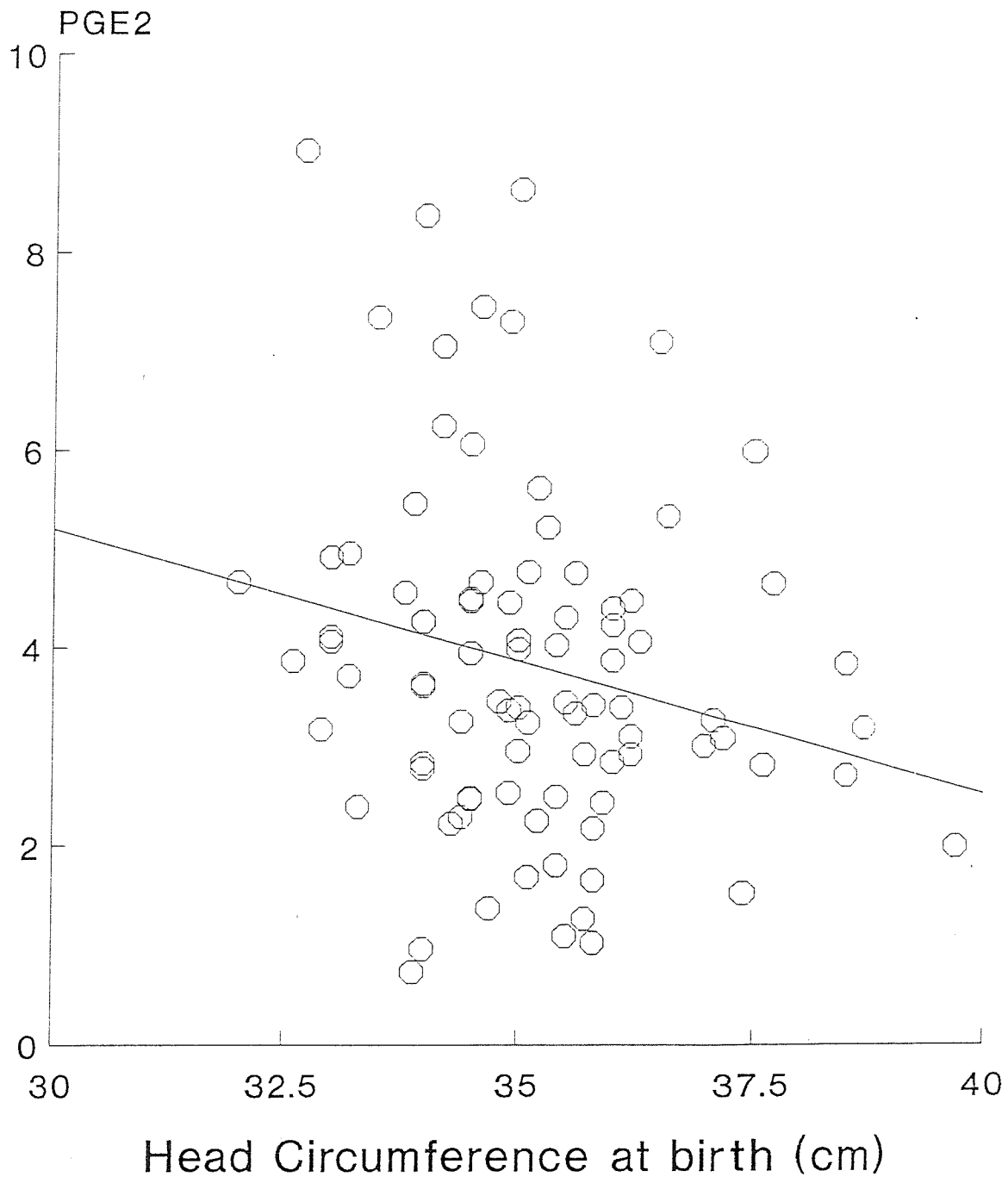

PGE2
10
8
6
4
2
0
30
32.5
35
37.5
40
Head Circumference at birth (cm)

Table 4.5. PGE_2 by tertiles of ponderal index and head circumference.

Ponderal index		n	Head circumference		n
<25.64	4.11 ± 2.0	29	<34.49	4.25 ± 2.06	26
25.65-28.01	4.06 ± 1.8	30	34.50-35.62	3.91 ± 1.82	27
>28.01	3.29 ± 1.3	29	>35.62	3.32 ± 1.37	29
P for trend	<0.02			<0.03	

Subjects were recruited from the MRC Salisbury study or the Princess Anne Hospital Growth Study. Birth characteristics were taken from hospital records and anthropometric and blood pressure measurements were made on the respective cohort studies. Urine samples were collected and urinary PGE_2 concentrations were measured using an enzymeimmunoassay (Amersham) and were expressed per creatinine. Results shown are corrected for sex.

4.8 Discussion

Epidemiological studies and animal studies have provided evidence that intrauterine growth retardation is associated with an increased risk of hypertension in later life (Barker *et al*, 1993, Langley-Evans *et al*, 1996b, chapters 3 & 4). A rat model of maternal diet induced hypertension has been used to investigate the mechanisms of cardiovascular programming (Langley-Evans *et al*, 1996g, chapters 3 & 4). Rats exposed to a low protein diet *in utero* have elevated urinary PGE_2 excretion rates associated with raised systolic blood pressures. The aim of this study was to determine urinary PGE_2 concentrations in a healthy human population to see whether similar disturbances of prostaglandin excretion accompany an increased risk of developing hypertension, associated with birth characteristics. The study assessed whether, in this respect, the low protein exposed rats are an appropriate model of the mechanism leading to human hypertension.

All of the subjects were chosen from two established cohort studies in Salisbury and Southampton. Data on birth weights and measurements and current anthropometric measurements and blood pressures were already available on each of the subjects in databases, held by the MRC Environmental Epidemiology Unit, Southampton. In the case of the Salisbury subjects 24-hour urine samples were already available (frozen at –80°C) and single urine samples were collected from each of the Southampton subjects. PGE_2 was measured in all urine samples and expressed per creatinine. PGE_2 was plotted against all birth and current characteristics and regression analyses performed.

The birth characteristics of the two study groups were significantly different. The values for birth weight, ponderal index and head circumference, were lower in the children born in Salisbury. This is possibly due to the different methods of selection of subjects used for each study. Children on the Salisbury study were randomly selected from the bottom, middle and upper quintiles, whilst the children from Southampton were a completely random group. Age at the time of urine collection was significantly different between the two studies. The difference between the mean ages of each study was less than 3 months

and the variation within each study was greater than this. There were no significant differences in the current characteristics of the children in the two cohorts.

The differences between the study groups, not only in the birth characteristics of the subjects, but also in the methods of urine collection, led to careful consideration about whether the results should be analysed together. Caution in interpretation of the results was necessary because one set of urine samples had been stored for 2 years without being acidified whilst the other set were recently collected and had been acidified before storage. The following points were considered to justify the joining of the two data sets for analysis. Primarily this was a preliminary study and although the birth weights differed between the two groups, the mean for both groups fell between the 25th and 75th percentile on standard birth weight charts (Tanner & Thomson, 1970). Secondly, urinary PGE_2 concentrations did not differ significantly between the two studies and followed the same pattern of normal distribution in both groups.

It was recognised that there is a large variation in creatinine excretion both within and between individuals which may have affected the ability to identify differences in urinary PGE_2 excretion. Creatinine excretion may vary within individuals due to a requirement for ATP. Urinary creatinine is a measure of muscle mass, which varies between individuals, and is a statement about their body composition and growth. Dietary consumption of meat also influences urinary creatinine excretion. It is recognised that these factors themselves may also influence blood pressure, and may have been influenced by fetal growth.

Markers of fetal growth i.e. size and shape at birth were correlated with current systolic blood pressure in this study, thus supporting the 'fetal origins hypothesis' (Barker, 1994). It was therefore assumed that birth characteristics were associated with the risk of becoming hypertensive in later life, for the purpose of interpreting the results of the study. Interestingly the smaller Salisbury babies had higher diastolic blood pressures as children than the larger Southampton babies.

Urinary PGE_2 concentrations were analysed against current characteristics as it was expected that characteristics such as current height and weight and blood pressure would be related to levels of a hormone, which is involved in the control of blood pressure. However, no correlation was found between PGE_2 and any of the current characteristics. This suggests that urinary PGE_2 excretion is independent of current growth and size and does not influence current systemic blood pressure. However, it is still possible that PGE_2 could have effects on local blood flow, for example in the kidney.

Birth measurements were used as indices of intrauterine growth and therefore as markers for risk of hypertension in later life (Barker *et al*, 1993). Since low protein exposed rats had higher urinary concentrations of PGE_2 compared to the controls, if this is a good model of programming in humans, it was expected that urinary prostaglandin excretion would be higher in the children with an increased risk of hypertension. The rats that have higher urinary excretion rates for PGE_2 also have elevated blood pressures at the time of measurement. It was therefore considered that the children chosen may not show increased PGE_2 levels, as they do not yet show signs of elevated blood pressures. Urinary PGE_2 concentrations were not correlated with birth weight *per se*. This may not be surprising as the low protein exposed rats are often not of low birth weight but show disproportionate growth (Langley-Evans *et al*, 1996b, Chapters 3 & 4). Urinary PGE_2 concentrations were, however, negatively correlated with both head circumference and ponderal index. Children who were born with smaller heads and proportionally thin bodies were excreting greater amounts of PGE_2 at age 9-10 years, which supports the results from the rat model. Head circumference has its peak velocity of growth at about 15-17 weeks of gestation (Tanner, 1989) which suggests that a low head circumference may be due to growth retardation in mid-gestation. Nutritional restriction during mid-gestation is also related to a low ponderal index and an increased risk of diabetes and hypertension in later life (Barker, 1994). The results from this study were remarkable considering that the subjects were young healthy children showing no signs of increased blood pressure and that relatively small numbers were available.

These data suggests that intrauterine growth retardation may lead to a disturbance in either the production or degradation of prostaglandins. Spontaneously hypertensive rats exhibit particularly high desaturase activities (Narce *et al*, 1994) and decreased activity of 15-hydroxyprostaglandin dehydrogenase (Tai *et al*, 1979). It is possible that the increased urinary PGE_2 excretion observed in the children who exhibit signs of disproportionate fetal growth, is related to impaired development of the kidney. Fetal kidney growth is disproportionate and slower in small fetuses than in those appropriate for gestational age (Konje *et al*, 1996) and nephron number is decreased in growth retarded babies (Hinchcliffe *et al*, 1992).

These results are particularly important as it is the first biochemical evidence that the rat model shares characteristics with human cohorts, which have already been investigated with respect to cardiovascular programming. The manipulation of the diet used in the rat model, which is a mild protein restriction with an increase the carbohydrate content, has been shown to be similar to deficits in the human diet, which have been associated with intrauterine growth retardation. The protein and carbohydrate content of the diet in the human population has been related to both fetal growth (Godfrey *et al*, 1996) and blood pressure in later life (Campbell *et al*, 1996). The rat model shows similar patterns of disproportionate fetal growth to those that occur in the human population (Langley-Evans *et al*, 1996b, Barker, 1994). The associations of poor maternal nutrition, fetal growth retardation and hypertension in later life observed in the human population are also clearly shown in the rat model. However, the association between increased urinary prostaglandin E_2 excretion and poor fetal growth is the first clear biochemical indicator that the mechanism underlying the fetal origins of hypertension may be the same in the human population, as it is in the rat model.

In conclusion, urinary PGE_2 excretion has been shown to be associated with markers of fetal growth and therefore the risk of hypertension in later life, in healthy 9-10 year old children. These results provide further evidence for the fetal programming of hypertension and show that the rat model is an appropriate and important tool in the study of the mechanisms involved in the fetal origins of human disease.

Chapter 5: Part 1

The effect of captopril treatment on 10-week-old rats exposed to a maternal low protein diet *in utero*.

5.1. Introduction

Studies by Konje *et al* (1996) and Martyn *et al* (1996) have indicated that disturbances of the renin-angiotensin system in humans may be associated with fetal growth retardation. Rat studies presented in Chapter 3 of this thesis indicate that fetal exposure to a maternal low protein diet increases pulmonary ACE activity, tends to increase plasma angiotensin II concentrations and may elevate plasma renin activity in a timing specific manner. These findings are complemented by evidence of disturbed prostaglandin metabolism in low protein exposed rats, that appear also to be evident in children who exhibited signs of disproportionate growth at birth (Chapter 4).

Rats exposed to a maternal low protein diet have been shown to have elevated ACE activity associated with raised blood pressures (Langley & Jackson, 1994, Chapter 3). Captopril, which is an ACE inhibitor, was shown to reversibly normalise the blood pressures in these rats (Langley-Evans & Jackson, 1995). These results suggest that the renin-angiotensin system has a role for the maintenance of the elevated blood pressure in this model.

The aim of the study reported in the present chapter, was to further investigate the effect of captopril, in particular looking at the effect of the drug on blood pressure, the renin-angiotensin system and the growth and development of the low protein exposed offspring.

5.2. Methods

The methods of animal production and dietary administration were as described in chapter 2. The rats used in the current study were taken from the group first described in chapter 3. Thirty-three adult female offspring were used (from 8 mothers), 18 of which were offspring of mothers fed a low protein diet (n = 4). Captopril was administered at

1g/L in the drinking water of individually housed rats, at 10 weeks of age for a two-week period, and daily intake calculated. The administered dose of captopril was approximately 182 mgs/kg body weight/day for both 18 and 9% casein exposed animals. Blood pressure was determined at days 0, 7 and 14 of the administration protocol. Rats were culled by decapitation at 12 weeks of age. Methods of blood pressure measurement, culling and blood and tissue collection were as previously described in chapter 2 (parts 2.4 and 2.7). ACE activity, plasma renin activity, plasma angiotensin II concentrations and urinary 6-keto-$PGF_{1\alpha}$ were analysed using assays described in section 2.9.

5.3. Results

The rats used in the current study were taken from the group first described in chapter 3. Thirty-three adult female offspring were used (from 8 mothers), 18 of which were offspring of mothers fed a low protein diet (n = 4). The male offspring were used in separate studies within our laboratory. Two of the rats from the 18% protein control group were culled due to illness and were therefore not included in later analyses. Birth weights for the two groups were not significantly different and litter sizes did not differ between the groups (Table 3.5). At weaning, body weights were higher in the low protein exposed group compared to the controls (Table 3.6). At 9 weeks of age the low protein group still weighed significantly more than the controls (Table 3.6).

5.3.1. The effect of captopril treatment on the blood pressures of rats exposed to a maternal low protein diet.

At 10 weeks of age captopril was administered in drinking water to rats from both the low protein group (n = 9) and control group (n = 7) at a concentration of 1g/L for a period of two weeks. The remainder of each dietary group were provided with water only, as controls. Fluid intake was similar in all groups of animals and intake of captopril solution did not differ from normal water intake (Table 5.1). The administered dose of captopril was approximately 182 mgs/kg body weight/day for both 18 and 9% casein exposed animals. Blood pressure was determined at days 0, 7 and 14 of the administration protocol. Baseline (day 0) blood pressure measurements indicated that animals exposed to a 9% casein diet *in utero* had significantly elevated blood pressures

relative to 18% casein exposed controls (15-21mmHg higher, $p < 0.05$, Table 5.2). Captopril had no significant effect upon the blood pressures of rats exposed to 18% casein *in utero* (Figure 5.1, Table 5.2), however treatment with captopril significantly reduced systolic blood pressure in the 9% protein exposed animals over a 14 day period back to a level not different to control rats ($p < 0.001$) (Figure 5.1, Table 5.2).

5.3.2. Effect of captopril treatment on 6-keto $PGF_{1\alpha}$ excretion.

24-hour urine collections were made from randomly selected rats (n=6 per group) after the period of captopril treatment. Equipment and time constraints limited the number of urine samples collected. Urinary 6-keto-$PGF_{1\alpha}$ concentrations were elevated, almost 2-fold, in association with captopril treatment in the low protein exposed animals (Table 5.3).

5.3.3. The effect of feeding a maternal low protein diet on the organ weights of the offspring.

At 12 weeks of age after 2 weeks of captopril treatment all rats were culled and blood and tissue samples collected. Organ weights expressed per kg body weight are shown in Table 5.4. Organ weights were expressed per kg body weight in order to assess whether specific organs had been affected by disproportionate growth. No significant differences between the dietary groups were found in the lung, liver, heart or kidney weights. The spleen, however, was found to be smaller in the rats exposed to a maternal 9% protein diet compared to the controls (Table 5.4) irrespective of drug treatment.

5.3.4. Effect of captopril treatment on pulmonary ACE activity.

ACE activity was analysed in lung tissue from 12 week old rats following 2 weeks of captopril treatment. Activity was found to be higher in the 9% protein exposed group in comparison to control and captopril treated groups. In both groups of animals treated with captopril, ACE activities were similar to activities in lungs of 18% protein control animals. Treatment with captopril significantly reduced ACE activity in the low protein exposed rats (Table 5.5).

Table 5.1. The effect of giving captopril in drinking water on fluid intake in rats exposed to either an 18% or a 9% protein diet *in utero*.

	Fluid Intake g/day	n
18% protein control diet	33 ± 2	6
9% protein diet	29 ± 4	9
18% protein diet & Captopril treatment	30 ± 7	7
9% protein diet & Captopril treatment	31 ± 5	9

Pregnant female rats were individually housed and given free access to either an 18% protein control diet (n = 4) or a 9% protein diet (n = 4). Thirty-three female offspring were successfully bred, 18 of which were from mothers fed a 9% protein diet. At 10 weeks of age 15 of the offspring (9 from the 9% protein exposed group) were treated with captopril which was administered in their drinking water for a two-week period. Captopril intake was measured gravimetrically every other day. Fluid intake was calculated over this two-week period and expressed as the mean intake in g/day ± SEM for each group. Offspring treated with captopril originated from 4 litters (2 litters from each dietary group).

Figure 5.1. Change in blood pressure with captopril treatment over two weeks in rats exposed to either a 9% or an 18% protein diet *in utero*

The offspring of mothers fed 18% and 9% protein diets throughout pregnancy were treated with captopril in drinking water at 1g/l for a period of 14 days. Captopril intake was 182mgs/kg/day for both the 18% (n=7) and 9% (n=9) protein groups. Systolic blood pressure was measured on days 0, 7 and 14 of treatment. Systolic blood pressure was also measured in non-captopril treated controls for both the 18% (n=6) and 9% (n=9) protein groups over the same period. $*p<0.05$ where p denotes the change in blood pressure over 14 days.

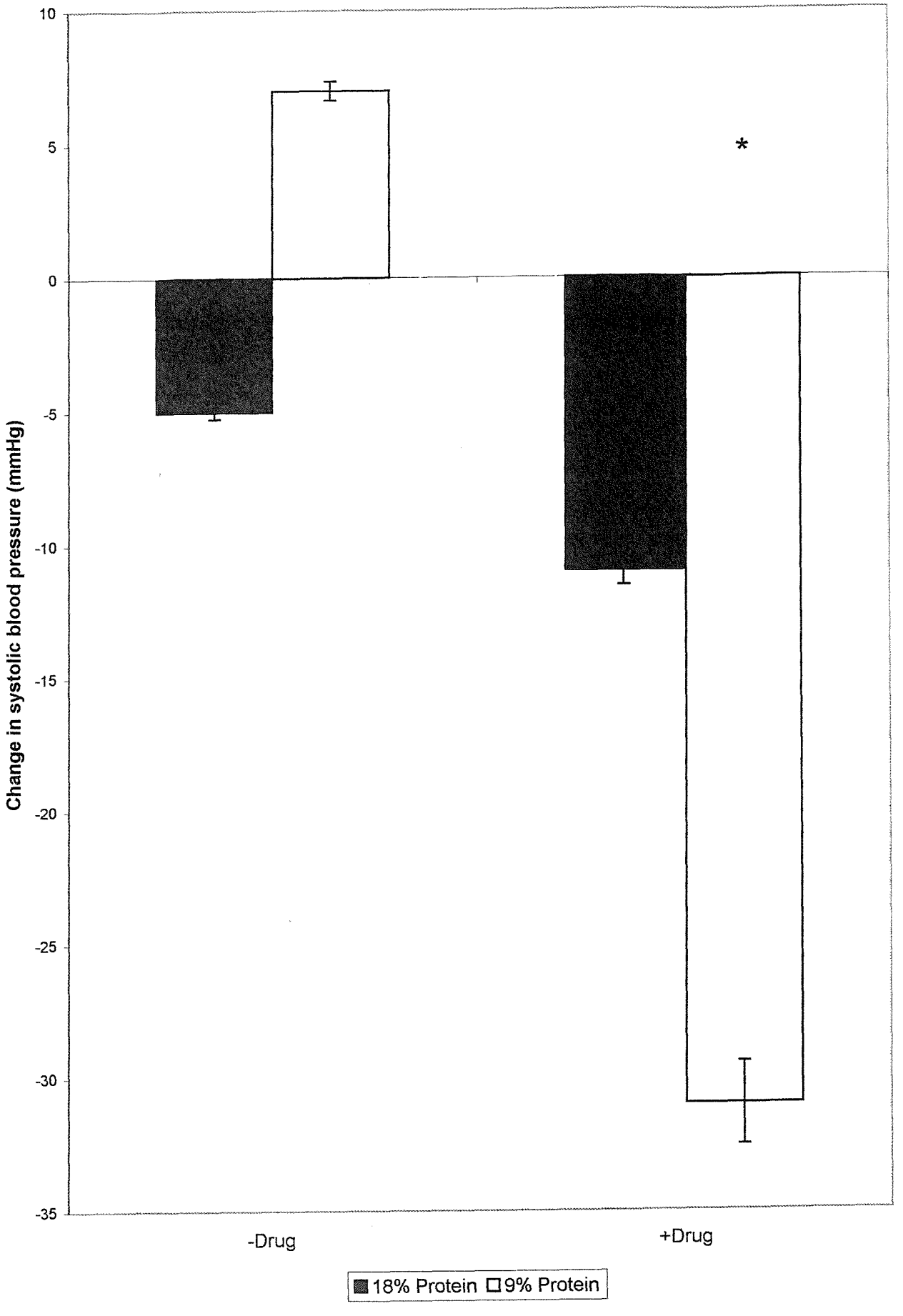

10
5
0
-5
-10
-15
-20
-25
-30
-35
Change in systolic blood pressure (mmHg)
*
-Drug
+Drug
18% Protein
9% Protein

Table 5.2. The effect of captopril on systolic blood pressure in rats exposed to either an 18% protein or a 9% protein diet *in utero*, over 14 day of treatment.

		Systolic Blood Pressure (mmHg)		
	n	Day 0	Day 7	Day 14
18% protein diet	6	127 ± 5	124 ± 3	122 ± 3
9% protein diet	9	142 ± 2*†	145 ± 3*†	149 ± 2*†
18% & Captopril	7	122 ± 6	117 ± 3	111 ± 3
9% & Captopril	9	148 ± 3*†	127 ± 4	117 ± 3‡

The offspring of mothers fed 18% and 9% protein diets throughout pregnancy were treated with captopril in drinking water at 1g/l for a period of 14 days. Captopril intake was 182mgs/kg/day for both the 18% and 9% protein groups. Systolic blood pressure was measured on days 0, 7 and 14 of treatment. Systolic blood pressure was also measure in non-captopril treated controls from both the 18% and 9% protein groups over the same period. Values are expressed as the mean systolic blood pressure for each group ± SEM and results were analysed using a 3-way ANOVA and t-tests. $^{*}p$=0.01 where p denotes the significance of the difference of the blood pressure of each group from the 18% protein group. $^{\dagger}p$=0.01 where p indicates a significant difference to the 18% protein group treated with captopril. $^{\ddagger}p$=0.01 where p denotes the difference of the blood pressures for each group on day 14 compared to day 1.

Table 5.3. The effect of maternal diet and captopril treatment on urinary 6-keto-PGF1α concentrations at 12 weeks of age.

Dietary Group	n	Ng/24 hours
18% protein (control)	6	17.92 ± 2.9
9% protein	6	20.14 ± 1.4
9% protein & Captopril	6	38.90 ± 4.6*†

The offspring of mothers fed 18% and 9% protein diets throughout pregnancy were treated with Captopril in drinking water at 1g/l for a period of 14 days. Captopril intake was 182mgs/kg/day for both the 18% and 9% protein groups. 24 hour urine collections were made from randomly selected rats and prostaglandin measurements made using an enzymeimmunoassay. Results are expressed as mean ± SEM and were analysed using ANOVA and t-tests. $^{*}p<0.05$ where p denotes the difference from the control. $^{\dagger}p<0.05$ where p denotes the difference from the 9% protein group.

Table 5.4. The effect of feeding a maternal low protein diet on the organ weights of the offspring at 12 weeks of age.

	n	g/kg body weight					
		Lung	Liver	Heart	Spleen	Kidney	Body weight
18% protein	6	6.9±0.3	49.8±2.2	4.3±0.2	3.6±0.2	3.9±0.1	203±2
9% protein	9	6.7±0.2	47.5±1.5	4.5±0.3	3.0±0.2*†	3.8±0.1	214±1
18% & drug	7	6.7±0.2	48.2±1.4	4.4±0.2	3.5±0.2	3.9±0.0	200±4
9% & drug	9	6.7±0.2	46.9±1.1	4.2±0.2	3.1±0.1*†	3.9±0.1	213±5

Pregnant female rats were given free access to either an 18% protein control diet or a 9% protein diet throughout pregnancy. Some of the offspring were treated with captopril at 10 weeks of age for a two-week period. At 12 weeks of age all animals were culled and organ weights measured. Organ weights are expressed as mean ± SEM in g/kg body weight. Results were analysed using ANOVA and t-tests. $^{*}p \leq 0.05$, where p denotes the significance of the difference of the organ weights of each group compared to the 18% protein group. $^{\dagger}p \leq 0.05$, where p denotes the difference from the 18% protein group treated with captopril.

Table 5.5. The effect of captopril treatment on pulmonary ACE activity in 12 week old rats exposed to a maternal low protein diet *in utero*.

Dietary Group	n	ACE (units)
18% protein	6	$33 \pm 2^{\dagger}$
9% protein	9	$40 \pm 2^{*\ddagger\S}$
18% & Captopril	7	$29 \pm^{\dagger}$
9% & Captopril	9	$31 \pm 3^{\dagger}$

The offspring of mothers fed 18% and 9% protein diets throughout pregnancy were treated with Captopril in drinking water at 1g/l for a period of 14 days. Captopril intake was 182mgs/kg/day for both the 18% and 9% protein groups. All rats were culled at 12 weeks of age and organs and plasma removed for analysis. Results are expressed as mean ± SEM and were analysed using ANOVA and t-tests. $^{*}p<0.01$ where p denotes the difference from the control group, $^{\dagger}p<0.01$ where p denotes the difference from the 9% protein group, $^{\ddagger}p<0.01$ where p denotes the difference from the 9% protein and captopril treated animals, $^{\S}p<0.01$, where p is the difference from the 18% protein group which were treated with captopril.

5.4. Discussion

Epidemiological evidence has suggested that there is a link between intrauterine growth retardation and an increased risk of hypertension in later life (Barker *et al*, 1990). This has received support from rat models where hypertension has been associated with the feeding of a low protein diet *in utero* (Langley & Jackson, 1994, chapter 3). Previous studies with this rat model of hypertension, induced by fetal exposure to maternal low protein diets, have demonstrated elevated pulmonary ACE activity (Langley & Jackson, 1994, chapter 3) and a reduction in blood pressure with captopril treatment (Langley-Evans & Jackson, 1995). The aim of this study was to show the effects of captopril treatment on ten week old rats exposed to a maternal low protein diet. ACE activity is elevated without an apparent effect on circulating angiotensin II concentrations in this model and it is therefore important to establish whether this is related in a causal manner to the raised blood pressure observed, or just coincidental. The present study differs from the original study of Langley-Evans & Jackson (1995), in that younger animals were used and that a separate group of untreated controls were used rather than using the period before treatment as an internal control.

Pregnant female rats were fed either an 18% casein control diet or a 9% casein, low protein diet throughout gestation *ad libitum*. Birth weights did not differ between the groups. The offspring of the mothers fed a 9% protein diet gained more weight in later life than the control animals. By 10 weeks of age blood pressure was significantly elevated in the rats exposed to a maternal low protein diet. The elevated blood pressure noted did not correlate with the increased body weight observed. At 10 weeks of age captopril was administered to a group of 18% protein exposed rats and a group of 9% protein exposed rats in their drinking water. Captopril treatment reduced blood pressure in the offspring of the low protein fed mothers over a period of two weeks. Organ weights were not significantly effected by maternal diet or captopril treatment, except the spleen which was significantly lighter in the low protein exposed rats. Pulmonary ACE activity was elevated in the 9% protein exposed rats and this was reduced to control

levels with captopril treatment. Captopril treatment had no significant effect on control animals.

Birth weights did not differ between the groups as shown in the previous chapter. Large numbers of offspring need to be included in the analysis to observe the effect of maternal diet on birth weight, as other factors such as slight variations in litter size or maternal size and the position of each fetus on the uterine horn will also affect fetal growth. The birth weights of the low protein exposed animals are generally found to be low to normal in this model indicating the moderate nature of the dietary restriction (Langley-Evans *et al*, 1996b, Chapter 3). As described in Chapter 3 organ weights were not significantly affected by maternal exposure to a low protein diet, except for the spleen, which was significantly smaller in the 9% protein treated animals compared to the controls. Captopril treatment had no effect upon organ weights. The reduction in size of the spleen observed in the low protein exposed rats suggests a possible effect upon the immune system. Studies have shown that exposure of rats to a low protein diet *in utero* affects their response to endotoxin challenge in early adulthood (Langley *et al*, 1994). Studies in the Gambia have shown that people who have suffered fetal undernutriton are more susceptible to infection in later life (Moore *et al*, 1997). Spleen weight, proliferation of spleen and thymus lymphocytes in response to B-cell and T-cell mitogens, and spleen natural killer cell activity, are all significantly reduced in the offspring of rats exposed to a maternal 9% protein diet in comparison to those exposed to an 18% protein control diet (Calder & Yacoob, 1999). This suggests that aspects of immune function are programmed *in utero* by maternal diet.

At ten weeks of age the low protein group had significantly elevated blood pressures relative to control rats, consistent with the results of previous studies (reviewed in Langley-Evans, 1996). Captopril was administered in the drinking water and intake was similar in both the low protein and the control animals. Although captopril has a strong smell in the drinking water, this did not appear to affect the intakes. The dose of 1g/L was chosen to allow a comparison with a previous study by Langley-Evans & Jackson

(1995), and studies with the Spontaneously Hypertensive Rat (Lee *et al*, 1991). The rats used in the Langley-Evans & Jackson study were 23 weeks of age and therefore had greater body weights than the rats in the present study, but had similar fluid intakes. This meant that the dose of captopril in mg/kg body weight per day in the present study was approximately double the dose used in the Langley-Evans & Jackson study (182mgs/kg/day and 98.2mgs/kg/day respectively). Administration of captopril significantly lowered systolic blood pressure in the low protein group only, and had no significant effect on the blood pressure of the control animals. ACE inhibition appeared to totally abolish hypertension in animals exposed to a maternal low protein diet. The effect of captopril on the low protein exposed rats was not due to an increased fluid intake in these animals and therefore an increased dose relative to that taken by control rats. In the previous study by Langley-Evans & Jackson (1995) the animals treated with captopril were much older (23 weeks) and blood pressure measurements were continued for a further 8 weeks after treatment. The blood pressures of the low protein exposed rats rose slowly and steadily and were significantly elevated compared to the control animals eight weeks after cessation of treatment. This suggests that captopril treatment in adult animals does not have a permanent effect and that blood pressure would eventually return to the pre-treatment level.

The main mechanism of action of captopril is through inhibition of ACE activity, which leads to a decrease in the production of angiotensin II. However plasma angiotensin II concentrations are not significantly different in low protein exposed animals compared to controls (Chapter 3). ACE activity was inhibited with captopril treatment but only in the low protein exposed animals. Studies of the efficacy of ACE inhibitors have shown that ACE activity is not totally abolished and that angiotensin I is still converted to angiotensin II (Vos *et al*, 1995). Angiotensin II is also generated by non-ACE pathways (Dzau, 1989). The enzymes tissue plasminogen activator, tonin, and cathepsin G are known to convert angiotensin I to angiotensin II *in vitro* (Tang *et al*, 1989, Klickstein *et al*, 1982), which may explain why angiotensin II is still produced in the presence of an ACE inhibitor. It is assumed that treatment with an ACE inhibitor causes a decrease in

angiotensin II concentrations and therefore an increase in renin activity. Discrepancies between blood pressure reduction induced by ACE inhibitors and angiotensin II concentrations have previously been explained by the difficulties in measuring angiotensin II in the presence of high angiotensin I concentrations, because of ex-vivo angiotensin II formation (Shalekamp *et al*, 1992).

Captopril may in fact influence blood pressure by methods other than ACE inhibition. Captopril treatment in the present study was shown to significantly increase urinary excretion of 6-keto-$PGF_{1\alpha}$ in the 9% protein exposed rats. This is supportive of other studies. Captopril treatment increases prostaglandin production, specifically PGE_2 and 6-keto-$PGF_{1\alpha}$ which are potent vasodilators (Swartz *et al*, 1979). Captopril is also a thiol-containing compound and a free radical scavenger (Sunman *et al*, 1993), which is reported to combine with nitric oxide to produce nitrosothiols which are potent relaxants of smooth muscle (Henry *et al*, 1989). ACE inhibitors also block the degradation of bradykinin, which is a vasodilator (Scholkens *et al*, 1987).

In conclusion the results from the present study have shown that the maternal diet induced hypertension is abolished by treatment with captopril, supporting the suggestion that the elevated ACE activity may have a role for the maintenance of hypertension in this model. It is important to recognise, however, that due to the other actions of the drug captopril, that the effect seen may not specifically due to an effect on the renin-angiotensin system. The main action of captopril is considered to be ACE inhibition, which leads to a decrease in the production of angiotensin II from angiotensin I. There is no evidence, however, that angiotensin II is elevated in the low protein exposed animals. It is therefore plausible that the relationship between elevated ACE activity and raised blood pressure may not be causal in this model. It is possible that angiotensin II may be elevated for a transient period during early life, and that measurements have not been made at an appropriate time to detect this. If this is the case then elevated ACE activity during this period, may be partially responsible for the initiation of hypertension. Spontaneously Hypertensive Rats do not develop the full hypertensive syndrome if

treated with an ACE inhibitor in early life, even after withdrawal of the drug. It is suggested that either an early increase in blood pressure or the actions of angiotensin II, may lead to structural vascular changes, such as vascular smooth muscle hypertrophy, which lead to both the maintenance and amplification of high blood pressure in these animals (Adams *et al*, 1990). The blood pressures of the low protein exposed rats rose slowly and steadily and were significantly elevated compared to the control animals eight weeks after cessation of treatment. This suggests that captopril treatment in adult animals does not have a permanent effect and that blood pressure would eventually return to the pre-treatment level. It is therefore considered important to assess whether captopril treatment also has such long-term effects in this nutritional model of programmed hypertension.

Chapter 5: Part 2.

Long Term Effects of Early Captopril Treatment on Rats Exposed to Maternal Low Protein Diets.

5.5. Introduction

Rats exposed to a maternal low protein diet *in utero* have significantly elevated systolic blood pressures in later life (Langley & Jackson, 1994, chapter 3). This raised blood pressure is observed from an early age and is persistent in to later life (Langley & Jackson, 1994). This rat model of maternal diet induced hypertension is being used to study the mechanism involved in the fetal programming of hypertension.

Captopril, which is an ACE inhibitor, was shown, in the previous study (Chapter 5: part 1), to normalise the blood pressures of adult low protein exposed rats, suggesting that the renin-angiotensin system may have a role for maintaining the elevated blood pressure observed in this model. In spontaneously hypertensive rats blood pressure can be reduced by treatment with an ACE inhibitor at an early age, and is maintained at lower levels even after treatment with the drug has ceased (Harrap *et al*, 1990). The aim of this study was to determine whether early captopril treatment could similarly prevent the development of elevated blood pressure in this rat model of maternal-diet-induced hypertension. Captopril administration is associated with birth defects if given during pregnancy. It was therefore given at the earliest age possible after birth when pups are able to drink from a water bottle, which was at 2 weeks of age. The effects on body weight, blood pressure and organ weights were assessed.

5.6. Methods

The methods of animal production and dietary administration were as described in chapter 2. Captopril was administered in the drinking water at 0.5g/L to litters for a two week period from two weeks of age. The daily intake of captopril was calculated for each litter, and individual doses estimated by subtracting the estimated maternal intake and dividing the remainder by the number of offspring in each litter. Methods of blood

pressure measurement, culling and blood and tissue collection were as described previously in chapter 2 (Sections 2.4 and 2.7). Lungs were dried over night at 60°C in order to assess dry lung weights.

5.7. Results

Eight virgin female Wistar rats were mated and given free access to a low protein (9% casein) diet throughout gestation. A further seven were fed a control diet (18% casein) throughout pregnancy. 158 offspring were born, 100 of which were from mothers fed a 9% protein diet during gestation. There was no significant difference in litter size between the 9% protein group (12.5±1 offspring) and the controls (9.5±1 offspring). All litters were reduced to a maximum of eight shortly after birth. Excess offspring from each litter were culled by decapitation. Birth weights were significantly lower in the low protein exposed animals compared to the controls ($p<0.005$, Table 5.6), and this dietary effect was stronger in the female offspring than in the male (Table 5.6).

5.7.1. The effects of captopril treatment on the body weights of rats exposed to a maternal low protein diet

At 2 weeks of age, eight of the litters (4 from 9% protein fed mothers and 4 from control mothers) were treated with captopril in their drinking water. The captopril was administered at 0.5g/l of drinking water and fluid intake did not differ between the two dietary groups. Captopril intake was 99.9 ± 5.4mgs/kg body weight/day and 98.9 ± 4.9mgs/kg body weight/day for the low protein exposed group and the 18% casein controls respectively. At 4 weeks of age the offspring which had been exposed to a maternal low protein diet were significantly heavier than the 18% casein controls (Tables 5.7 and 5.8). The male low protein exposed animals that had received captopril treatment weighed significantly less than the 18% casein controls ($p < 0.005$, Table 5.7), whereas the weights of the female low protein exposed captopril treated rats did not differ from those of the non-treated 18% casein fed controls (Table 5.8). Captopril treatment did not have a significant effect on the body weights of the 18% protein exposed male or female animals (Tables 5.7 and 5.8). By 12 weeks of age there were no significant differences in

body weight between the two dietary groups among either the males or females (Tables 5.7 and 5.8). The captopril treated 9% protein exposed males weighed significantly less than all other groups ($p < 0.05$, Table 5.7).

5.7.2. The effects of early captopril treatment on the blood pressures of rats exposed to a maternal low protein diet

Baseline blood pressure measurements were not made prior to treatment at two weeks of age as the rats are too small to make accurate measurements on at this age. Systolic blood pressures were measured at 4 and 12 weeks of age using the tail cuff method. No sex differences were noted. At 4 weeks of age systolic blood pressure was elevated in the low protein exposed animals compared to the 18% casein controls (26mmHg higher). However, the low protein exposed rats that had received captopril treatment had blood pressures that were similar to those of 18% protein exposed control rats (Table 5.9). Captopril treatment did not appear to alter the blood pressures of the 18% protein exposed animals (Table 5.9). By 12 weeks of age the blood pressures in all groups had increased compared to the pressures at 4 weeks of age ($p<0.001$). At 12 weeks of age the blood pressures of the low protein group remained elevated, compared to the 18% protein controls ($p<0.001$, Table 5.10). The rats exposed to a maternal low protein diet and subjected to early captopril treatment maintained their blood pressures at control levels and captopril treatment had no significant effect on the blood pressures of 18% protein exposed rats (Table 5.10).

5.7.3. The effect of maternal diet and captopril treatment on organ weights

All animals were culled at 12 weeks of age by decapitation. Lungs and kidneys were removed and weighed. Kidneys were studied as previous experiments have suggested that feeding a maternal low protein diet may affect the development of the kidney. Lungs were studied because they are the major site for the production of ACE, and captopril treatment may affect lung function. Organ weights were expressed as g/kg body weight. In the male offspring the kidney to body weight ratio was lower in the 9% protein exposed rats compared to the controls (7% smaller, Table 5.11), however kidney size in

the captopril treated low protein exposed animals did not differ from the controls (Table 5.11). Captopril treatment did not affect kidney size in the animals exposed to a maternal 18% protein diet (Table 5.11). In the female offspring kidney weight did not differ significantly between groups (Table 5.12). Lung weights in g/kg body weight did not differ between 9% and 18% protein exposed groups in either the males or females (Tables 5.11 and 5.12). Male and female animals exposed to a low protein diet *in utero* and subjected to early captopril treatment had significantly heavier lungs, than all other groups (Tables 5.11 and 5.12). Dry lung weights, however, did not differ between the groups (data not shown), suggesting an accretion of fluid in the lungs of captopril treated rats.

Table 5.6. The effect of feeding a maternal low protein diet on the birth weights of the offspring.

Dietary group	Birth weight (g)			
	Males	n	Females	n
18% protein (control) diet	5.77 ± 0.09	36	5.48 ± 0.07	22
9% protein diet	5.56 ± 0.07	59	5.14 ± 0.08	41
p (difference from control)	0.088		0.003	

Pregnant female Wistar rats were fed either an 18% protein (control) diet (n=7) or a 9% protein diet (n=8) throughout gestation. All offspring were weighed and sexed shortly after birth. Birth weights are expressed as the mean ± SEM. T-tests were used for statistical analysis. *p* denotes the statistical significance of the difference from the control group.

Table 5.7. The effects of early captopril treatment on body weights at 4 and 12 weeks of age, of male rats exposed to a maternal low protein diet

	n	4 weeks (g)	12 weeks (g)
18% protein (control) diet	11	$57 \pm 1^{\dagger}$	396 ± 6
9% protein diet	15	$66 \pm 2^{***}$	399 ± 5
18% protein diet & captopril	11	$52 \pm 3^{\dagger}$	402 ± 6
9% protein diet & captopril	12	$46 \pm 3^{**\dagger\ddagger}$	$377 \pm 6^{*\dagger\ddagger}$

Pregnant female Wistar rats were given free access to either an 18% protein control diet (n=7) or a 9% protein diet (n=8) throughout pregnancy. At two weeks of age 4 of the litters from each dietary group were treated with captopril in their drinking water for a two-week period. Body weights at 4 and 12 weeks are expressed as mean ± SEM. Data was analysed using 2-way-ANOVA and t-tests. $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.001$ where p denotes the statistical difference of the mean body weight in each group from the 18% protein control group. $^{\dagger}p<0.001$ where p denotes the difference from the 9% protein group. $^{\ddagger}p<0.05$ where p shows the effect of captopril relative to non-captopril treated animals in the same dietary group.

Table 5.8. The effects of early captopril treatment on body weights at 4 and 12 weeks of age, of female rats exposed to a maternal low protein diet

	n	4 weeks (g)	12 weeks (g)
18% protein (control) diet	9	52 ± 2†	225 ± 3
9% protein diet	16	60 ± 3*	231 ± 5
18% protein diet & captopril	9	57 ± 3†	229 ± 4
9% protein diet & captopril	14	54 ± 2†‡	231 ± 2

Pregnant female Wistar rats were given free access to either an 18% protein control diet (n=7) or a 9% protein diet (n=8) throughout pregnancy. At two weeks of age some of the offspring were treated with captopril in their drinking water for a two-week period. Body weights at 4 and 12 weeks are expressed as mean ± SEM. Data was analysed using ANOVA and t-tests. $^{*}p<0.05$ where p denotes the statistical difference of the mean body weight in each group from the control group. $^{\dagger}p<0.05$ where p denotes the difference from the 9% protein group. $^{\ddagger}p<0.05$ where p shows a significant effect of captopril relative to non-captopril treated animals in the same dietary group.

Table 5.9. The effects of early captopril treatment on systolic blood pressures at 4 weeks of age, of male and female rats exposed to a maternal low protein diet

	n	BP (mmHg)
18% protein (control) diet	20	103 ± 2†
9% protein diet	31	129 ± 2*
18% protein diet & captopril	20	105 ± 2†
9% protein diet & captopril	26	105 ± 1†‡

Pregnant female Wistar rats were given free access to either an 18% protein control diet (n=7) or a 9% protein diet (n=8) throughout pregnancy. At two weeks of age some of the offspring were treated with captopril in their drinking water for a two-week period. Systolic blood pressures were measured at 4 weeks using the tail cuff method. Blood pressures at 4 weeks are expressed as mean ± SEM. Data was analysed using 2-way-ANOVA and t-tests. $^{*}p<0.001$ where p denotes the statistical difference of the mean blood pressure in each group from the 18% protein control group. $^{\dagger}p<0.01$ where p denotes the statistical difference of the mean blood pressure in each group compared to the 9% protein exposed group. $^{\ddagger}p<0.001$ where p shows a significant effect of captopril treatment relative to non-captopril treated animals in the same dietary group.

Table 5.10. The effects of early captopril treatment on systolic blood pressures at 12 weeks of age, of rats exposed to a maternal low protein diet

	n	BP (mmHg)
18% protein (control) diet	19	$132 \pm 3^{\dagger\dagger}$
9% protein diet	29	$148 \pm 2^{*}$
18% protein diet & captopril	19	$135 \pm 1^{\dagger\dagger}$
9% protein diet & captopril	26	$139 \pm 3^{\dagger\ddagger}$

Pregnant female Wistar rats were given free access to either an 18% protein control diet (n=7) or a 9% protein diet (n=8) throughout pregnancy. At two weeks of age some of the offspring were treated with captopril in their drinking water for a two-week period. Systolic blood pressures were measured at 12 weeks using the tail cuff method. Blood pressures at 12 weeks are expressed as mean ± SEM. Data was analysed using 2-way-ANOVA and t-tests. $^{*}p<0.001$ where p denotes the statistical difference of the mean blood pressure in each group from the 18% protein control group. $^{\dagger}p<0.01$, $^{\dagger\dagger}p<0.001$ where p denotes the statistical difference of the mean blood pressure in each group compared to the 9% protein exposed group. $^{\ddagger}p<0.001$ where p shows a significant effect of captopril treatment relative to non-captopril treated animals in the same dietary group.

Table 5.11. The effects of early captopril treatment on kidney and lung weights at 12 weeks of age, of male rats exposed to a maternal low protein diet

	n	Kidney wt (g/kg body wt.)	Lung wt (g/kg body wt.)
18% protein (control) diet	11	$3.19 \pm 0.05^{\dagger\dagger}$	5.68 ± 0.22
9% protein diet	14	$2.98 \pm 0.05^{**}$	5.42 ± 0.22
18% protein diet & captopril	10	$3.10 \pm 0.08^{\dagger}$	6.21 ± 0.25
9% protein diet & captopril	13	$3.11 \pm 0.06^{\dagger}$	$6.84 \pm 0.30^{**\dagger\dagger}$

Pregnant female Wistar rats were given free access to either an 18% protein control diet (n=7) or a 9% protein diet (n=8) throughout pregnancy. At two weeks of age some of the offspring were treated with captopril in their drinking water for a two-week period. At 12 weeks of age all animals were culled by decapitation and organs were removed and weighed. Kidney weights and lung weights in g/kg body weight are expressed as mean ± SEM. Data was analysed using ANOVA and t-tests. $^{*}p<0.05$, $^{**}p<0.005$ where p denotes the statistical difference of the mean kidney or lung weight in each group from the 18% protein control group. $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.005$ where p denotes the statistical difference of the mean kidney or lung weight in each group compared to the 9% protein exposed group.

Table 5.12. The effects of early captopril treatment on kidney and lung weights at 12 weeks of age, of female rats exposed to a maternal low protein diet

	n	Kidney wt (g/kg body wt.)	Lung wt (g/kg body wt.)
18% protein (control) diet	8	3.27 ± 0.05	6.72 ± 0.22
9% protein diet	13	3.23 ± 0.11	6.73 ± 0.22
18% protein diet & captopril	9	3.38 ± 0.08	6.64 ± 0.25
9% protein diet & captopril	12	3.32 ± 0.06	7.84 ± 0.30*†

Pregnant female Wistar rats were given free access to either an 18% protein control diet (n=7) or a 9% protein diet (n=8) throughout pregnancy. At two weeks of age some of the offspring were treated with captopril in their drinking water for a two-week period. At 12 weeks of age all animals were culled by decapitation and organs were removed and weighed. Kidney and lung weights in g/kg body weight are expressed as mean ± SEM. Data was analysed using ANOVA and t-tests. $^{*}p<0.005$, where p denotes the statistical difference of the mean lung weight in each group from the 18% protein control group. $^{\dagger}p<0.001$, where p denotes the statistical difference of the mean lung weight in each group compared to the 9% protein exposed group.

5.8 Discussion

Epidemiological studies have provided evidence of a link between intrauterine growth retardation and hypertension in later life (Barker *et al*, 1993). Animal models have been developed which support the hypothesis that maternal nutrition affects the risk of hypertension in the offspring (Langley & Jackson, 1994). Rats fed a low protein diet during pregnancy produce offspring with elevated systolic blood pressures (Langley & Jackson, 1994).

In the previous study (Chapter 5: part 1), the elevated blood pressures observed in this rat model of maternal-diet-induced hypertension, were shown to be normalised with captopril treatment. From earlier work (Langley-Evans & Jackson, 1995) it is inferred that this effect is reversible. Studies with spontaneously hypertensive rats have shown that early treatment with an ACE inhibitor prevents the development of hypertension in these animals (Harrap *et al*, 1990, Morton, 1992). The aim of the present study was to assess the long term effects of early treatment with captopril on maternal low protein diet induced hypertension. Captopril was administered at the earliest possible date (when the rats began to drink from the water bottles) which was at the age of two weeks, and treatment lasted for a two week period.

The captopril dose for the previous study was chosen on the basis of work done by Langley-Evans & Jackson (1995) and Lee *et al* (1991). The dose was reduced by half for the present study to account for the lower body weights of the animals. Calculation of the actual dose received by individual rats is imprecise as the animals were still housed in litters with their mothers. It is also possible that the captopril is transferred in the mothers milk and that the offspring were also receiving the drug via this route. The pups were observed beginning to drink from the water bottle at two weeks of age but it is likely that by the end of the two weeks treatment they were consuming more water from the bottle and less milk from the mother, than at the beginning of treatment. This would lead to a progressive increase in the dose received during the treatment period, which would parallel their increase in weight over the time studied. As maternal blood pressure

is not raised by the low protein diet (Langley-Evans *et al*, 1994) it is possible but unlikely that the captopril treatment had any effect on the mother which changed her behaviour or physiology, and therefore had an indirect effect on the blood pressure of the pups.

Pregnant female Wistar rats were fed either an 18% (control) or a 9% casein diet throughout pregnancy. The offspring of the mothers fed a 9% protein diet weighed significantly less at birth than the control offspring, as previously shown by Langley-Evans *et al* (1996b). This supports human data which shows that maternal nutrition affects fetal growth (Margetts *et al*, 1991, Godfrey *et al*, 1996). However, these results differ from the previous studies described in this thesis where no significant difference in birth weights had been observed between the groups. This may be accounted for by the fact that the total number of pups studied in the current experiment was higher. Birth weights are generally found to be low to normal in this model indicating the moderate nature of the dietary restriction (Langley-Evans *et al*, 1996b). By 4 weeks of age the offspring of mothers fed a low protein diet weighed significantly more than the controls suggesting that energy metabolism and/or dietary intake had been programmed *in utero* to give an altered growth pattern and possibly body composition as shown previously (Chapter 3, Chapter 5: Part 1, McCarthy *et al*, 1994). Early captopril treatment appeared to alter this pattern of growth. This resulted in the male offspring treated with captopril weighing significantly less than the untreated animals (both the 18% and 9% protein exposed groups.) The female captopril treated offspring did not differ from the 18% protein untreated rats but were significantly lighter than the untreated low protein group. This is supported by studies of early captopril treatment on spontaneously hypertensive rats, where male rats treated with captopril had slower growth rates resulting in significantly lower body weights than the controls (which were littermates of the treated spontaneously hypertensive rats) at the end of treatment (Harrap *et al*, 1990). No attempt was made, in the above-mentioned paper, to discuss how captopril may affect growth. One possibility is that captopril reduces angiotensin II levels, therefore reducing the stimulation of ACTH (adrenocorticotropin hormone) production. ACTH is a hormone of the pituitary, which promotes the growth of the adrenal cortex and stimulates it to

produce steroid hormones. It is thought that angiotensin II may stimulate ACTH secretion but it is not known whether this is a direct or indirect effect (Robertson & Nicolls, 1993). A decrease in ACTH production would then lead to a reduced stimulation of hormones (such as androgens) which promote growth (Tanner, 1989). An interference with ACTH secretion would also impact upon corticosterone and CRF (corticotrophin releasing factor) secretion which regulate food intake (Liebowitz *et al*, 1984, Krahn *et al*, 1986, Heinrichs & Koob 1992, Heinrichs *et al*, 1992).

A local renin-angiotensin system has been located in the adrenal cortex, and as yet its exact actions are unknown (Robertson & Nicolls, 1993). This system has been shown to be affected by treatment with an ACE inhibitor (Yamaguchi *et al*, 1990). It is therefore possible that captopril treatment could influence the release of hormones from the adrenal cortex, which may in turn influence growth but there are no clearly identified mechanisms for this. Other possible explanations are that angiotensin II is needed for the growth and development of certain organs, particularly the kidney, where it is required for nephrogenesis (Ray *et al*, 1994), or that captopril treatment affects gut function (Golke & Unger, 1994). Gut disturbances have been noted as side affects of captopril in high doses. A decrease in food intake has also been associated with captopril treatment (Golke & Unger, 1994). There is also evidence that angiotensin II is a mitogen in bone, stimulating the synthesis of DNA in osteoblast–rich populations of cells (Hiruma *et al*, 1997).

By 12 weeks of age there was no apparent difference in body weights between the groups except for the low protein exposed captopril treated males, which were still significantly smaller than all other groups. This indicates that the differences in blood pressures, observed between the groups, are not merely a weight related phenomenon. The blood pressures were increased in the low protein group, relative to the 18% protein control group and were reduced to control levels with captopril treatment as expected, at 4 weeks of age. By 12 weeks of age the low protein exposed rats still had an elevated systolic blood pressure relative to the 18% protein exposed group but early captopril treatment

had prevented the onset of hypertension long term in these animals as shown in Spontaneously Hypertensive Rats (Harrap *et al*, 1990, Morton, 1992). Eight weeks after cessation of treatment, blood pressures of 9% casein rats treated with captopril remained significantly below those of untreated low protein exposed rats. This rat model of maternal diet induced hypertension resembles the Spontaneously Hypertensive Rat (SHR) in many aspects of its disturbances of the renin-angiotensin system, so it is helpful to look to the SHR strain for possible explanations of what may be happening in this nutritional model. One explanation for this long term effect of captopril after treatment, is that changes in vascular structure may be produced by increased arterial pressure or angiotensin II concentrations at an early age, and treatment with an ACE inhibitor may interrupt the further progression of the hypertension after treatment with the inhibitor is stopped (Harrap *et al*, 1990). Thus a relatively transient drive to increase blood pressure, mediated by the renin-angiotensin system or other vasoconstricting hormones, may become fixed as lifelong hypertension through structural adaptation. Pharmacological intervention during the early initiation phase may thus have a lifelong benefit. Studies of the vasculature in low protein exposed animals are an important further step. Folkow (1978) proposed that the structural vascular adaptation which maintained elevated blood pressure was vascular smooth muscle hypertrophy. Studies by Berry (1978) suggest that as blood pressures increase there is an increased stiffness of the arteries caused by an increased recruitment of collagen. These aspects of vascular development are currently being studied in this model. The low protein exposed rats exhibit increased aorta wall thickness and decreased compliance compared with control rats (Langley-Evans & Greenwald, personal communication). Martyn and Greenwald (1997) hypothesised that an impairment of elastin synthesis during a critical period of blood vessel development may underlie the association between intrauterine growth retardation and an increased risk of developing higher blood pressure in later life.

Increased ACE activity in later life may not be responsible at all for elevated blood pressure at this time. It could be argued that increased activity in early life may have a role in the initiation of high blood pressure which may be causing a change in

vasculature. In SHRs losartan, which is an angiotensin II receptor antagonist, has the same long term effects on blood pressure following a brief period of early treatment as the ACE inhibitors (Morton, 1992). This suggests that it is angiotensin II action at an early age, which affects long term blood pressure control. Morton also suggested a possible association between factors affecting growth and hypertension in the SHRs, which may also be relevant in this model. The low protein exposed rats tend to have an increased growth rate compared to the control rats and this was prevented with captopril treatment. It could be argued that the elevation of blood pressure in these animals is directly related to the increased growth rate. However, the low protein exposed rats do not consistently show an increase in growth rate yet still have elevated blood pressures.

Kidney size was smaller in the male offspring of the 9% protein fed mothers than the controls. This is supportive of other animal studies (Langley-Evans *et al*, 1996b, Zeman 1969) and of work in humans where fetal renal growth has been shown to be slower in babies which were small for gestational age compared to those which were appropriate for gestational age (Konje *et al*, 1996). Early captopril treatment in the low protein rats appeared to increase kidney growth which was also shown in the spontaneously hypertensive rats where early captopril treatment resulted in larger kidneys than the controls (Harrap *et al*, 1990). Angiotensin II has been associated with renal tubular necrosis (Gavras *et al*, 1971, 1975, Kremer *et al*, 1981), which may explain the smaller kidneys found in the low protein exposed rats and the fact that this reduced kidney size is not found in the animals treated with captopril. Although, plasma angiotensin II concentrations are apparently not elevated in the low protein exposed rats these animals do show increased sensitivity to angiotensin II infusion (Gardner *et al*, 1998). Although captopril treatment in the low protein exposed rats prevented the onset of hypertension in both the male and female animals, its effect on kidney size was only seen in the male rats. This may lead to the suggestion that kidney changes do not account for the changes in blood pressure, and that other mechanisms must be involved. However, gross changes in kidney size may not reflect structural changes. Langley-Evans *et al* (1999) suggest that post-natal nephrogenesis is blocked by exposure to a low protein diet *in utero*. Low

protein exposed offspring were born with an apparently normal complement of nephrons, but by 4 weeks of age they had a reduced number of nephrons compared with control rats. It is possible that early captopril treatment may have prevented this phenomenon.

Lung weight did not differ between the 18% and 9% protein exposed groups. In both males and females, captopril treatment in the 9% protein groups appeared to cause an increase in lung weight in proportion to body weight. Coughing has been reported as a side effect of captopril treatment in humans. The lungs were later dried out and the excess weight appeared to be due to fluid. It is possible that the oedema was only observed as a side-effect of captopril treatment in the low protein exposed rats, and not the control animals, because they are more susceptible to chemically induced lung injury. Previous studies have shown that the low protein exposed rats are more susceptible to sulfur dioxide-induced lung injury than the control rats (Langley-Evans *et al*, 1997).

The pharmacological effects of captopril may not be specific and it is therefore possible that its long term effects are not due to a decrease in angiotensin II concentrations in early life. Captopril also leads to increased bradykinin and prostaglandin levels and the production of nitrosthiols (Brunner *et al*, 1987, Swartz *et al*, 1980, chapter 4 part 1, Henry *et al*, 1989). However, these effects are reported only during treatment with captopril and the possible long term effects of the drug on these vasoactive agents are uncertain.

The fact that the onset of hypertension is prevented by a two week period of treatment in early life suggests that it is possible to programme aspects of hypertension during both pre and postnatal periods. It is possible that the hypertension is initiated *in utero* by increased exposure to glucocorticoids (Langley-Evans, 1997) and that the development of hypertension takes place in early postnatal life and leads to structural changes which maintain the elevated blood pressure. Rats exposed to a maternal low protein diet exhibit hypersensitivity to normal levels of circulating corticosterone in early life, via increased glucocorticoid receptor numbers (Langley-Evans *et al*, 1996c). Glucocorticoids increase

smooth muscle cell densities of AT1 receptors, therefore amplifying the effects of angiotensin II (Provencher *et al*, 1995) and also induce ACE production (Mendelsohn *et al*, 1982).

The results from the current chapter, and Chapter 3 suggest that the action of the renin-angiotensin system is upregulated by fetal exposure to a low protein diet during a critical period of postnatal development. This may then lead to alterations in the structural development of the vasculature, which maintains the elevated blood pressure after glucococorticoid sensitivity and the action of the renin-angiotensin system have normalised. Early captopril treatment may have prevented the actions of the renin-angiotensin system during a critical period, and therefore prevented the structural vascular changes and the development of hypertension occurring long-term.

In conclusion hypertension is programmed *in utero* in this model and the onset can be prevented by early postnatal captopril treatment. It is proposed that the actions of the renin-angiotensin system may have a role in the development and maintenance of high blood pressure in these rats. Due to the lack of specificity of the drug a definitive role for the renin-angiotensin system cannot be stated and it is necessary to study the effects of other anti-hypertensive drugs on this model of maternal diet-induced hypertension.

Chapter 6

Long-Term Effects of Losartan or Nifedipine Treatment on the Blood Pressures of Rats Exposed to Maternal Low Protein Diets.

6.1 Introduction

As discussed in the previous chapter, the elevation of systolic blood pressure observed in rats exposed to a maternal low protein diet *in utero* may be prevented by early treatment with captopril. Offspring of rats exposed to a 9% casein diet during pregnancy had systolic blood pressures significantly higher than control animals at 12 weeks of age. Those that received early captopril treatment (between 2 and 4 weeks of age) maintained their blood pressures at levels observed in 18% casein exposed controls.

The findings of the previous chapter may be taken to suggest a possible role for the renin-angiotensin system in the programmed development of high blood pressure, in this rat model. However, the actions of captopril are not specific to the renin-angiotensin system, and alternative mechanisms of action cannot be excluded (Brunner *et al*, 1987, Sunman *et al*, 1993). It is therefore of importance to study the effect of a more specific drug. Losartan is an angiotensin II receptor (AT_1) antagonist and its actions are thus more specifically targeted against angiotensin II (Burnier *et al*, 1991). It is also of interest to study the effect of an anti-hypertensive drug, which does not act directly by modifying the balance of the renin-angiotensin system. This will exclude the possibility that the lowering of blood pressure in early life, by any means, may prevent the onset of programmed hypertension in adulthood. Nifedipine is a calcium antagonist, which causes vasodilation but does not block the actions of the renin-angiotensin system (Krakoff 1994).

The aim of this study was to determine whether early treatment with the drugs losartan or nifedipine has the same long-term effects as captopril, in preventing the onset of hypertension in prenatally undernourished rats. Following the same protocol as the

previous chapter, losartan or nifedipine were given in the drinking water between 2 and 4 weeks of age and the long-term effects on body weight and blood pressure assessed.

6.2 Methods

The methods of animal production and dietary administration were as described in chapter 2 (sections 2.1-2.3). Losartan or nifedipine were administered in the drinking water at 100mg/l to litters (n = 2 litters per group) for a two-week period from two weeks of age. Individual doses were estimated from expected fluid intake, calculated from measured fluid intakes from chapter 5. Methods of blood pressure measurement, culling and blood and tissue collection were as described previously in chapter 2 (sections 2.4 and 2.7). Data was analysed using ANOVA and t-tests as appropriate and expressed as mean ± SEM. Analyses compared individual offspring rather that litters.

6.3 Results

Six virgin female Wistar rats were mated and given free access to a low protein (9% casein) diet throughout pregnancy. A further six were fed a control diet (18% casein) throughout gestation. One of the rats fed a low protein diet was found not to be pregnant and was therefore excluded from the study. 114 offspring (11 litters) were born (57 in each dietary group). There was no significant difference in litter size between the 9% casein group (11.4 ± 1.0 offspring) and the controls (9.5 ± 1.5 offspring). All litters were reduced to a maximum of eight shortly after birth, leaving 81 offspring (40 offspring of low protein fed mothers). Excess offspring from each litter were culled by decapitation. Birthweights did not differ significantly between the two dietary groups (5.4 ± 0.1 g, 5.5 ± 0.1 g, for the control and low protein exposed groups respectively).

6.3.1. The effects of losartan and nifedipine treatment on body weight.

At 2 weeks of age, two litters from both maternal dietary groups were treated with losartan in their drinking water at 100mg/L. Two further litters from each dietary group were treated with nifedipine in their drinking water, also at 100mg/L. Treatment continued for two weeks. Two litters were non-treated 18% casein exposed control

animals, and the remaining litter was a low protein exposed control group. By 4 weeks of age the low protein exposed animals which had not received drug treatment, weighed significantly more than the control animals (Table 6.1, $p<0.005$). However the low protein exposed rats that had received early nifedipine treatment did not differ in weight from the control rats and weighed significantly less than the low protein exposed animals which had received no drug treatment. The low protein exposed rats that had received early losartan treatment, weighed significantly less than both the 9%casein and the 18% casein exposed, untreated groups (Table 6.1). Drug treatment did not affect the body weights of the rats exposed to a maternal 18% casein (control) diet.

By 12 weeks of age, the rats exposed to a maternal 18% protein (control) diet and treated with nifedipine weighed significantly more than all other groups. This was true for both the males and females ($p=0.05$, $p=0.001$, Table 6.1). Weights did not differ between any of the other groups at 12 weeks of age (Table 6.1).

6.3.2. The effects of early losartan or nifedipine treatment on blood pressure.

Systolic blood pressures were measured at 4 and 12 weeks of age using the tail cuff method. At 4 weeks of age, the untreated low protein exposed rats had significantly elevated blood pressures (23mmHg) in comparison to the control rats ($p<0.001$, Table 6.2). ANOVAs indicated no sex differences in systolic blood pressure, so both sexes were combined in Table 6.2. Nifedipine treatment had no effect upon the blood pressures of either the low protein exposed group or the control group (Table 6.2). The low protein exposed rats that had received losartan treatment, had similar systolic blood pressures to 18% protein control animals, and, therefore, had significantly lower (29mmHg) blood pressures than the untreated low protein exposed rats ($p<0.001$, Table 6.2). Losartan treatment had no effect upon the blood pressures of rats exposed to a maternal 18% casein (control) diet (Table 6.2).

By 12 weeks of age the systolic blood pressures of all groups had risen significantly, relative to 4 weeks but the pattern between the groups remained the same ($p<0.001$,

Figure 6.1). The rats exposed to a 9% casein diet *in utero*, continued to have significantly elevated (35mmHg) systolic blood pressures relative to the 18% casein control group ($p<0.001$, Table 6.2). Early losartan treatment had no significant effects upon the blood pressures of animals exposed to the maternal control diet, whilst early nifedipine treatment was associated with raised blood pressure in the 18% protein animals (9mmHg). Early nifedipine treatment had no long term effects on the systolic blood pressures of the low protein exposed rats, whilst early losartan treatment had prevented the onset of hypertension in these animals, which had systolic blood pressures 36mmHg below untreated low protein exposed rats ($p<0.001$, Table 6.2, Figure 6.1).

Table 6.1. The effects of losartan or nifedipine treatment on the body weights of rats exposed to a maternal low protein or control diet.

Treatment	Weight at 4 weeks (g) (n)	Weight of males at 12 weeks (g) (n)	Weight of females at 12 weeks (g) (n)
18% casein (control) diet	$57 \pm 1^{\ddagger}$ (16)	$300 \pm 8^{\dagger}$ (5)	$184 \pm 2^{\dagger}$ (11)
9% casein diet	$62 \pm 1^{*}$ (8)	$320, 292^{\dagger}$ (2)	$184 \pm 4^{\dagger}$ (6)
18% casein & nifedipine	$55 \pm 2^{\ddagger}$ (11)	$330 \pm 15^{*\ddagger\S\dagger}$ (4)	$197 \pm 3^{*\ddagger\S\dagger}$ (7)
9% casein & nifedipine	$57 \pm 1^{\ddagger\S}$ (16)	$300 \pm 5^{\dagger}$ (9)	$182 \pm 9^{\dagger}$ (7)
18% casein & losartan	$58 \pm 1^{\ddagger}$ (14)	$300 \pm 7^{\dagger}$ (9)	$177 \pm 6^{\dagger}$ (5)
9% casein & losartan	$50 \pm 1^{\ddagger*\S}$ (16)	$291 \pm 4^{\dagger}$ (8)	$181 \pm 3^{\dagger}$ (8)

Six virgin female Wistar rats were mated and given free access to a low protein (9% casein) diet throughout pregnancy. A further six were fed a control diet (18% casein) throughout gestation. One of the rats fed a low protein diet was found not to be pregnant and was therefore excluded from the study. At 2 weeks of age 2 litters from both dietary groups were treated with losartan in their drinking water at 100mg/l. Two further litters from each dietary group were treated with nifedipine in their drinking water, also at 100mg/L. Treatment continued for two weeks. Body weights at 4 and 12 weeks of age were expressed as mean ± SEM. Data was analysed using 3-way-ANOVA and t-tests. $^{*}p<0.05$ where p denotes the statistical difference of the mean body weight in each group from the 18% casein control group. $^{\ddagger}p<0.05$ where p indicates the difference from the 9% casein group. $\S p<0.05$ where p shows a significant effect of losartan/nifedipine treatment relative to untreated rats of same dietary group. $^{\dagger}p<0.001$ where p denotes the difference in weight at 12 weeks compared to weight at 4 weeks. Litter n = 2 for each group.

Table 6.2. The effect of early losartan or nifedipine treatment on the blood pressures rats exposed to a maternal low protein or control diet.

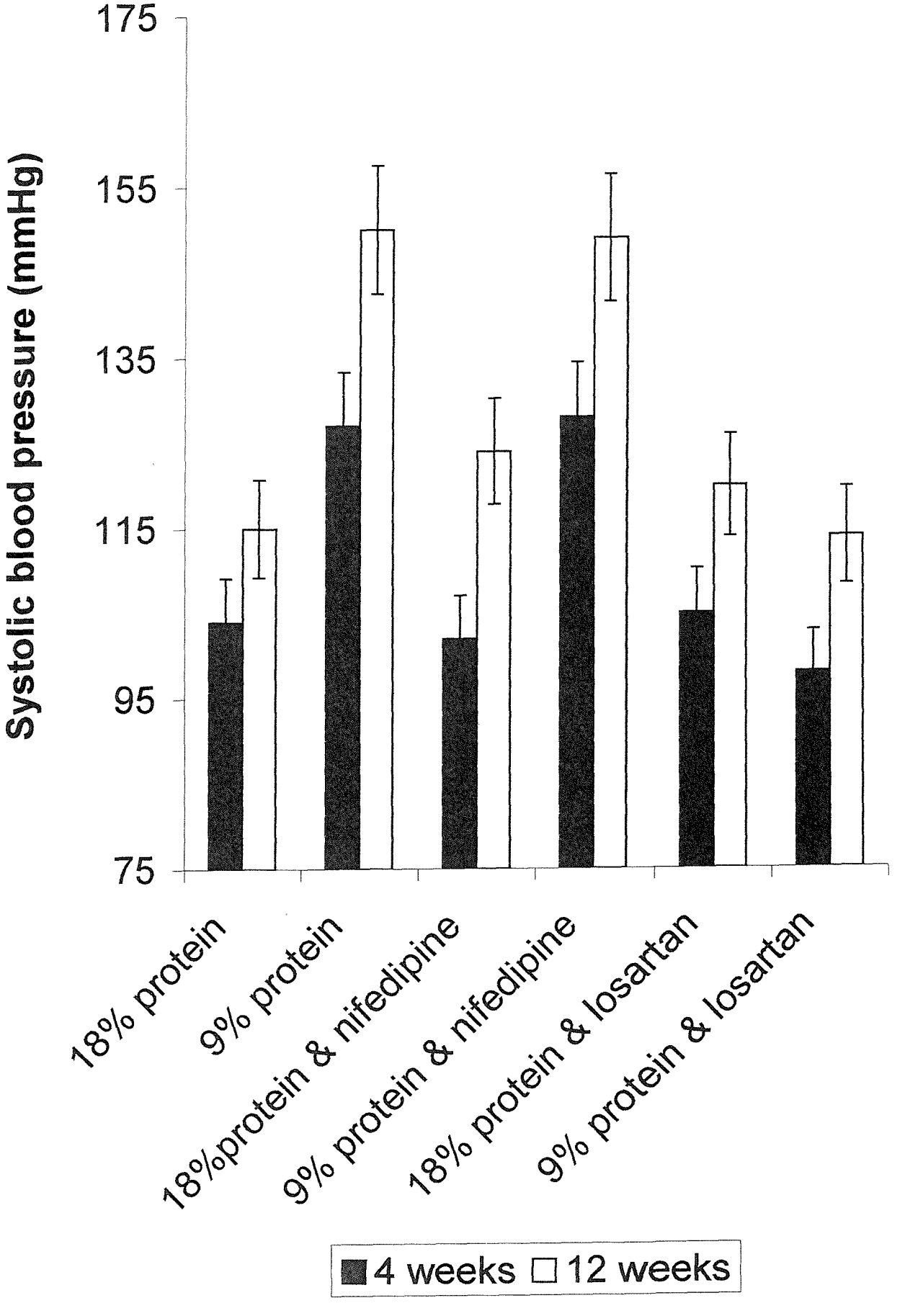
Treatment	Systolic blood pressure at 4 weeks of age (mmHg)	Systolic blood pressure at 12 weeks of age (mmHg)
18% casein (control) diet	$104 \pm 1^{\dagger}$	$115 \pm 2^{\dagger\ddagger}$
9% casein diet	$127 \pm 1^{*}$	$150 \pm 3^{*\ddagger}$
18% casein & nifedipine	$102 \pm 1^{\dagger}$	$124 \pm 1^{\dagger*\S\ddagger}$
9% casein & nifedipine	$128 \pm 2^{*}$	$149 \pm 3^{*\ddagger}$
18% casein & losartan	$105 \pm 2^{\dagger}$	$120 \pm 2^{\dagger\ddagger}$
9% casein & losartan	$98 \pm 1^{\dagger*\S}$	$114 \pm 1^{\dagger*\S\ddagger}$

For experimental detail see Table 6.1. Systolic blood pressures at 4 weeks of age were expressed as mean ± SEM. Data was analysed using 3-way-ANOVA and t-tests. $^{*}p<0.001$ where p denotes the statistical difference of the mean blood pressure in each group from the 18% casein control group. $^{\dagger}p<0.001$ where p denotes the difference from the 9% casein group. $^{\S}p<0.001$ where p shows a significant effect of drug treatment relative to untreated rats of the same dietary group. $^{\ddagger}p<0.001$ where p denotes the difference in blood pressure at 12 weeks compared to blood pressure at 4 weeks.

Figure 6.1. The effect of early nifedipine or losartan treatment on the systolic blood pressures of rats exposed to a maternal low protein diet.

For experimental detail see Table 6.1. Systolic blood pressures were measured at 4 and 12 weeks of age. Systolic blood pressures in all groups increased with age ($p<0.001$). Data was analysed using ANOVA and t-tests.

Key: 18% = 18% casein exposed group, 9% = 9% casein exposed group, 18%N =18% casein & nifedipine treatment, 9%N = 9% casein & nifedipine treatment, 18%L = 18% casein & losartan treatment, 9%L = 9% casein & losartan treatment.


Systolic blood pressure (mmHg)
175
155
135
115
95
75
18% protein
9% protein
18%protein & nifedipine
9% protein & nifedipine
18% protein & losartan
9% protein & losartan
4 weeks
12 weeks

6.4. Discussion

Early captopril treatment has been shown to prevent or delay the onset of hypertension in rats exposed to a maternal low protein diet (Chapter 4). This suggests a possible role for angiotensin II in the development of the elevated blood pressure observed in this model. However, captopril action is not specific to ACE inhibition and it was therefore necessary to carry out further studies with alternative anti-hypertensive drugs before a definitive and specific role for angiotensin II action could be confirmed. The aim of the current study was to determine the effects of early treatment with losartan (an angiotensin II receptor antagonist), or nifedipine (calcium receptor antagonist) on the long term blood pressures of rats exposed to a maternal low protein diet *in utero*.

The offspring of rats fed either an 18% casein (control) diet or a 9% casein diet were treated with either losartan or nifedipine in their drinking water between 2 and 4 weeks of age, as this is the earliest period in which the young rats drink from the water bottle and the period during which captopril was given in the previous study (chapter 5). Untreated controls, from both dietary groups, were also studied throughout the same period. Body weights and systolic blood pressures were determined at 4 and 12 weeks of age.

Litter size did not differ between the two dietary groups showing that the ability of the mothers to carry a full litter was not compromised by the feeding of a moderately low protein diet, a finding consistent with Langley-Evans *et al* (1996b). Birth weights did not differ significantly in the low protein exposed groups compared to controls. As previously discussed, birth weights are generally found to be low to normal in this model. Low protein exposed rats have a tendency to exhibit increased body weights compared to control rats at 4 weeks of age. This has also been shown in previous chapters (Chapters 3, 4 & 5). Low protein exposed rats treated with losartan, however, weigh less than all other groups at 4 weeks of age. This phenomenon is also observed with early captopril treatment in this model, as well as in the SHR (Harrap *et al*, 1990). This suggests that angiotensin II may promote growth in these animals and as a consequence inhibition of angiotensin II action is growth retarding. Angiotensin II is thought to be a mitogen in

bone (Hiruma *et al*, 1997) and is also considered to be necessary for the growth and development of certain organs, for example the kidney (as discussed in Chapter 5). It is possible that decreased growth may lead to a reduced requirement for blood flow and therefore a lower blood pressure. Alternatively the level of blood flow may determine the ability of the animal to grow.

Early nifedipine treatment resulted in an increase in body weight in the control rats by 12 weeks of age. It is possible that the slight elevation in blood pressure observed in the 18% protein nifedipine treated rats may be due to the weight increase, however this wasn't consistent at each time point observed. Lower blood pressures in the losartan treated rats did not correlate with lower body weights observed in the male rats at 12 weeks of age, suggesting that this is not purely a weight related phenomenon. Although the low protein exposed rats generally appear to have increased body weights this is also not consistently related to body weight.

Early losartan treatment prevented the onset of hypertension in the rats exposed to a maternal low protein diet, as captopril did in the previous study (Chapter 5). This has also been observed in spontaneously hypertensive rats (Morton *et al*, 1992). Losartan had no effect upon the blood pressures of the control animals, as with captopril treatment (Chapter 5). Early nifedipine treatment was associated with a slightly elevated blood pressure in the rats exposed to a control diet, 8 weeks after treatment had ceased, but had no effect upon the systolic blood pressures of the low protein exposed group both during and after treatment. This provides further evidence that angiotensin II action at an early age has a long-term effect on blood pressure control, and specifically that AT_1 mediated events control the postnatal development of elevated blood pressure. It is possible that the action of angiotensin II in the 2-4 week period is a critical pre-requisite for the hypertension programmed in utero, and that programming is both pre and postnatal. The development of hypertension in this model can also be prevented by the pharmacological blockade of glucocorticoid synthesis (Langley-Evans, 1997). It is a possibility that hypertension in the low protein exposed rats has merely been delayed rather than

prevented indefinitely by these early interventions. Studies of the long term effects of early treatment with losartan or ACE inhibitors in SHRs, have shown that blood pressure starts to rise gradually after a long period without treatment, but that it doesn't return to the levels of non-treated SHRs (Morton *et al*, 1992, Adams *et al*, 1990).

A role for glucocorticoids in the programming of blood pressure, in this rat model, has been proposed as discussed in previous chapters (Chapters 3 & 5, Langley-Evans, 1997). Indeed the development of elevated blood pressure in low protein exposed rats depends upon an active maternal adrenal during pregnancy and also on intact adrenals in postnatal life (Gardner *et al*, 1997). The current results are supportive of this proposal, as glucocorticoids amplify the effects of angiotensin II by increasing smooth muscle cell densities of AT_1 receptors (Sato *et al*, 1984). Indeed preliminary data suggests that the low protein exposed rats are more sensitive to infused angiotensin II than control animals (Gardner *et al*, 1998). Glucocorticoids may also promote increased angiotensin II production as they induce synthesis of angiotensinogen and ACE activity (Mendelsohn *et al*, 1982).

Angiotensin II is believed to modulate the development of the vasculature in early life. Martyn and Greenwald (1997) hypothesise that the association between birth weight and blood pressure in later life may relate to the ability to synthesise elastin during a critical period of blood vessel development. Folkow (1978) suggested that an increase in blood pressure causes hypertrophy of vascular smooth muscle, leading to the long term maintenance of high blood pressure. Preliminary data suggests that the vasculature of low protein exposed rats differs in structure to controls, with increased smooth muscle cell size and number and reduced compliance (Langley-Evans, Martyn and Greenwald, unpublished data). Lever & Harrap (1992) proposed that changes in vascular structure may be initiated in early development by angiotensin II, growth hormone, cortisol or insulin like growth factors (IGF1 & IGF2). It can thus be argued that early losartan treatment prevents the development of long term hypertension by inhibiting angiotensin II activity, and therefore preventing structural vascular adaptation. This suggests that

programmed hyper-sensitivity to angiotensin II during a critical period in early life may determine the development of the vasculature and could lead to the long term maintenance of an elevated blood pressure. The renin-angiotensin system thus programmes blood pressure postnatally after a nutritionally initiated pre-natal exposure or increase in sensitivity to glucocorticoids. It would be difficult to assess whether angiotensin II has a pre-natal effect as ACE inhibitors have severe side effects during pregnancy and there is no evidence that angiotensin II receptor antagonists cross the placenta (Stevenson *et al*, 1995).

Angiotensin II action during the postnatal period may exert its effect on both the circulation and the kidney. Direct effects on the circulation may be achieved through an increased number of receptors or increased binding affinity in the vascular smooth muscle cells. Effects on the kidney may also be mediated through an increased number of receptors or binding affinity. Preliminary results suggest that rats exposed to a maternal low protein diet have an increased expression of AT_1 receptors in the kidney compared with control animals (Trowern, personal communication). Angiotensin II may also affect the blood pressure by influencing the development of the kidney. Angiotensin II has been associated with renal tubular necrosis (Gavras *et al*, 1971, 1975, Kramer *et al*, 1981) and may also be involved in nephrogenesis and determining the number of nephrons in the kidney. Low protein exposed rats have a decreased number of nephrons compared with control rats and exhibit impaired renal function (Langley-Evans *et al*, 1999a, 1999b). Angiotensin II is also associated with progressive glomerular damage (Yamoto *et al*, 1990). Ketteler *et al* (1995) hypothesise that angiotensin II induces TGFβ expression which may exert effects on extracellular matrix accumulation, cell proliferation and hypertrophy (Gibbons *et al*, 1990).

Although results from the present study strongly suggest a role for angiotensin II in the development of hypertension in this model, evidence from earlier studies shows that circulating angiotensin II levels do not differ in the low protein exposed rats compared to the controls. However, it is possible that local renin-angiotensin systems, for example in

the vascular smooth muscle, may differ between the two dietary groups or that angiotensin II receptors may be up-regulated in the low protein exposed rats. Further research is continuing in this area considering angiotensin II receptor expression and binding in both the smooth muscle cells and in the kidneys.

In conclusion this study provides further evidence for a role for angiotensin II in the development of hypertension in this model. Further research is necessary to define the mechanism involved in more detail.

Chapter 7

General discussion

A growing body of evidence indicates that hypertension and cardiovascular disease may be partially determined by the environment in which the developing embryo and fetus finds itself, including maternal nutrition. Epidemiological data suggest that intrauterine growth retardation is related to an increased risk of hypertension in later life (Barker *et al*, 1993). Robinson *et al*, (1994) suggest that maternal nutrition is a primary determinant of fetal growth. The hypothesis proposed by Barker (1994) is that the nutritional profile of the mother permanently programmes the metabolic and physiological functions of the fetus. More recent studies have suggested a possible relationship between maternal diet during pregnancy, placental and fetal growth (Godfrey *et al*, 1996) and blood pressure in the offspring (Campbell *et al*, 1996). A high carbohydrate intake in early pregnancy and a low protein intake in late pregnancy were associated with lower birth weight and placental weight (Godfrey *et al*, 1996). Similarly a high carbohydrate and low protein diet in late pregnancy was associated with an increased risk of high blood pressure in adult life (Campbell *et al*, 1996).

The findings from human studies are supported by work in animal models (Langley-Evans *et al*, 1996g). Rats fed a low protein diet during pregnancy, where the energy content of the diet is balanced with increased carbohydrate, gain less weight in the final week of pregnancy, possibly relating to impaired fetal growth at this time (Chapter 3). Disproportionate growth patterns have been observed in these fetuses during mid to late gestation (Langley-Evans *et al*, 1996b). Birth weights are low to normal in the low protein (9% casein) exposed offspring in comparison with the controls (Chapter 3, Langley-Evans *et al*, 1996b). The postnatal growth pattern of the low protein exposed animals is altered, in as much as they have a tendency to start life smaller and gain more weight than the control rats (Chapter 3, Langley-Evans *et al*, 1996g, Anguitta *et al*, 1992). When allowed to select their own macronutrient diet, the offspring of rats fed a 9% casein diet during pregnancy tend to consume more fat and gain more weight than

control rats (McCarthy *et al*, 1994). When analysing regional adipose tissue weights these same animals are found to have significantly higher intraabdominal fat masses compared with control rats (McCarthy *et al*, 1994). Systolic blood pressure is elevated in the maternal low protein exposed rats (Chapter 3, Langley & Jackson, 1994) and this is observed from an early age and is apparently life-long (Langley-Evans & Jackson, 1995). This model is currently being used to investigate the possible mechanisms underlying the programming of hypertension *in utero*.

Preliminary evidence for a possible role for the renin-angiotensin system in the maintenance of the hypertensive state in this animal model, came from studies by Langley & Jackson (1994) and Langley-Evans & Jackson (1995). These studies showed that the rats exposed to a maternal low protein diet *in utero* had elevated ACE (angiotensin converting enzyme) activity in comparison with controls, and that the blood pressures of these animals could be returned to control levels by treatment with captopril, which is an ACE inhibitor. This effect was apparently reversible as blood pressure returned to previous elevated levels after treatment was removed (Langley-Evans & Jackson, 1995).

The aim of this thesis was to further explore the role of the renin-angiotensin system in this rat model of maternal-diet-induced hypertension. The results lend support to the theory that maternal diet programmes the metabolic competence of the offspring. The level of protein restriction used in the prenatal diet in this rat model is moderate. A 9% protein diet is considered adequate for a non-pregnant adult rat, while a 12% protein diet is considered to be the requirement for pregnancy (Clarke *et al*, 1978). The birth weights of the low protein exposed rats in the present studies were shown to be low to normal as has been previously shown in this model (Langley-Evans *et al*, 1996). In the human population it is disproportionate growth of the fetus that is related to the risk of hypertension as opposed to birth weight *per se* (Barker *et al*, 1993) and previous studies provide evidence for such disproportionate fetal growth in the animal model (Langley-Evans *et al*, 1996b). Postnatally there is an indication of some 'catch-up' in growth as, at

weaning, body weights of the low protein exposed rats were greater than those of the control group as has been previously shown (Langley-Evans *et al*, 1996g, McCarthy *et al*, 1994, Anguitta *et al*, 1993). In the human population hypertension is closely associated with obesity (JNCR, 1993). Hypertension and cardiovascular disease are also associated with non-insulin dependent diabetes melitus (NIDDM) in the human population (Pi-Sunyer, 1991). Rats exposed to a maternal low protein diet exhibit abnormal insulin responses that indicate possible insulin resistance, which is a risk factor for NIDDM (Pickard *et al*, personal communication). Similar rat models have been used to study the effects of exposure to a low protein diet *in utero* on pancreatic growth and function in the offspring (Snoek *et al*, 1990, Dahri *et al*, 1991, Desai *et al*, 1994). Pancreas weight was found to be lower in rats exposed to a maternal low protein diet (Desai *et al*, 1994). Snoek *et al* (1990) reported a reduction in β-cell proliferation and islet size, as well as a reduction in islet vascularisation and pancreatic insulin content in rats exposed to a maternal low protein diet *in utero*. A subsequent study also showed impaired glucose tolerance and a lower insulin response to glucose in the same rat model (Dahri *et al*, 1991). These rat models differ from the model described in this thesis. Although the diets given during pregnancy contain similar amounts of protein, the fat and carbohydrate contents differ between the diets. These differences in dietary exposure affect the outcome of disease in these models, indeed, elevated blood pressure is not consistently observed in the model used by Desai *et al*, as it is in the model described in the current studies (Langley-Evans, 1998).

The systolic blood pressures of the rats exposed to a maternal low protein diet were consistently elevated compared to the controls and this was evident from 4 weeks of age (Chapters 3, 4, 5 & 6). This was associated with elevated pulmonary ACE activity from 4 weeks of age (Chapter 3: part 2). Signs of an increase in ACE activity were observed from the fetal stage with no apparent alterations in maternal pulmonary ACE activity. Plasma renin activity, angiotensinogen concentrations and angiotensin II levels were found to be unchanged in the low protein exposed rats in comparison with the controls and urinary prostaglandin concentrations were significantly elevated (Chapter 3: part 2).

These results appear surprising in association with an increase in blood pressure, however they do show that there are disturbances in the renin-angiotensin and prostaglandin systems in this model. Elevated urinary prostaglandin excretion may be explained by the decreased renal PGDH activity observed in the low protein rats, although how, or if, this interacts with the renin-angiotensin system is unknown at this time. In humans, renin concentrations have been shown to be lower in adults who were of low birth weight in comparison to those of high birth weight (Martyn *et al*, 1996). Urinary prostaglandin levels were negatively correlated with both head circumference and ponderal index in 10-year-old children (Chapter 4: part 2). These results again show similarities between the pathophysiological process in the human population and the rat model used to study the underlying mechanism.

Captopril treatment was shown to lower both the blood pressure and the pulmonary ACE activity in the low protein exposed rats at 12 weeks of age, but had no effect on the control animals. A previous study by Langley-Evans & Jackson (1995) has suggested that this process is reversible and that after the removal of captopril treatment the blood pressures of the adult animals return to the elevated levels. A brief period of early treatment with captopril prevented the development of the hypertensive-state in these rats, in a manner analogous to observations in spontaneously hypertensive rats (Harrap *et al*, 1990). As previously discussed in Chapter 5, captopril treatment is not specific and also affects the prostaglandins and the kinins. Captopril is also an antioxidant and can modulate local effects of nitric oxide on blood pressure (Benzies & Tomlinson, 1998). However, prostaglandins and kinins interact with the renin-angiotensin system in the control of blood pressure, and the main mechanism of action of captopril is still considered to be through reducing ACE activity. The present rat model of hypertension resembles the spontaneously hypertensive rat (SHR) in its patterns of disturbance of the renin-angiotensin system, so it is helpful to look at the SHR model for possible explanations of the programming mechanism. The long-term effect of captopril after early treatment may be explained in terms of prevention of structural vascular changes, normally produced by increased arterial pressure or angiotensin II concentrations at an

early age. Preliminary data suggests that the vasculature of the low protein exposed rats differs in structure to that of the control rats. Increased smooth muscle cell size and number and also reduced elasticity or compliance have been observed in this model and are being explored in greater detail by others (Langley-Evans *et al*, unpublished data). Intervention with an ACE inhibitor may interrupt the progression of the hypertension, an effect which outlasts the period of intervention and persists after treatment with the inhibitor is stopped (Harrap *et al*, 1990). The lack of specificity of the drug captopril means that a role for angiotensin II in the development of the hypertensive-state was not definitively demonstrated by this study and further studies with other anti-hypertensive drugs were required.

In SHRs, a brief period of treatment in early life with losartan, which is an angiotensin II antagonist, has the same long term effects on blood pressure as the ACE inhibitors do (Morton, 1992). This supports the hypothesis that it is angiotensin II action, and not other captopril-target systems, which modify long term blood pressure control at an early age. Studies of early treatment with losartan and nifedipine in the low protein exposed rats confirmed that an angiotensin II antagonist also prevented the onset of hypertension in this model, whilst alternative anti-hypertensive drugs had no effect. These results strongly suggest a specific role for angiotensin II in the development of hypertension programmed by fetal nutrition.

Angiotensin II concentrations, however, were not found to be elevated in the low protein exposed animals. Recent studies by Gardner *et al* (1998) have shown that these animals have increased sensitivity to angiotensin II. Intravenous injections of angiotensin II (1 - 40ng) were given to both the 9% protein exposed rats and the 18% protein controls. At low doses of angiotensin II there was a greater pressor response in the low protein exposed rats than the controls (Gardner *et al*, 1998). Preliminary results suggest that this is due to an increased expression of AT_1 receptors, as has been shown in the kidneys of low protein exposed rats compared with control animals (Trowern, personal communication). Angiotensinogen levels were shown not to be limiting in this model, so

this does not explain the increased ACE activity with no apparent increase in angiotensin II concentrations. It could be argued that the increased ACE activity is merely a consequence of excess glucocorticoids in early life and has little effect on blood pressure in this model (Mendlesohn *et al*, 1982). It is likely that the glucocorticoids are also affecting local renin-angiotensin systems and angiotensin II receptor expression (Sato *et al*, 1994) and it is suggested that these may be of importance to the mechanisms by which blood pressure is elevated at an early stage. It is argued that increased blood pressure is initiated by glucocorticoid action which leads to the amplification of hypertension by the renin-angiotensin system, causing structural vascular changes which maintain the elevated blood pressure in later life.

The glucocorticoids have various influences on the renin-angiotensin system and have been well characterised as having a programming effect upon the developing fetus in this model. Fetal protection from maternal glucocorticoids is normally effected by placental 11β-hydroxysteroid dehydrogenase, which converts physiological glucocorticoids to inactive products (Benediktsson *et al*, 1993). A low protein diet in the mother is associated with decreased placental activity of 11βHSD, which leads to increased exposure of the fetus to glucocorticoids (Langley-Evans, 1997). The late gestational growth retardation observed in these rats may be partially due to over-exposure to glucocorticoids (Langley-Evans, 1997). Preliminary studies suggest that glucocorticoid receptors are upregulated in the lung of neonates exposed to a maternal low protein diet, which would tend to exacerbate the effects of high local hormone concentrations (Bertram, Personal communication). Other hormones may influence intrauterine growth retardation, for example insulin-like-growth-factors are thought to exert control over the availability of substrate across the placenta (Harding *et al*, 1994, Lui *et al*, 1994) and have been shown to be positively associated with fetal weight (Owens *et al*, 1994, Harding *et al*, 1994). Androgens have been shown to programme the expression of hepatic androgen metabolising enzymes (Gustafsson & Stenburg, 1974). It is suggested that the excess glucocorticoids in the fetus lead to a resetting of the HPA-axis. High glucocorticoid levels from the adrenal gland have a negative feedback affect on the

pituitary and the hypothalamus (Dallman *et al*, 1993). In early adult life the low protein exposed rats have normal circulating concentrations of corticosterone but a blunted diurnal secretion pattern of ACTH, and they are more sensitive to corticosterone (Langley-Evans *et al*, 1996c). Glucocorticoids also influence the expression of angiotensin II receptors (Sato *et al*, 1994), the synthesis of angiotensinogen and ACE activity (Mendelsohn *et al*, 1982).

As well as being influenced by glucocorticoids and the resetting of the HPA-axis, it is possible that the renin-angiotensin system could be programmed by abnormal placental development, leading to impaired placental perfusion and a decrease in the oxygen available to fetal tissue. Placental PGDH activity was elevated in the low protein exposed rats, which may lead to a decrease in local PGE_2 concentrations and therefore a decrease in placental tissue perfusion. Tissue ACE activity is dependent upon oxygen, and a reduction in oxygen perfusion of the placenta is associated with a marked decrease in ACE activity (Lumbers, 1995). This could have adverse affects on the functioning of the fetoplacental renin-angiotensin system. The fetal renin-angiotensin system is involved in the control of fetal renal function and the regulation of the fetal arterial pressure (Lumbers, 1995), so it is possible that disturbances at this time could lead to long term dysfunction of the renin-angiotensin system. It is unlikely, however, that this possible decrease in oxygen would contribute greatly to the programming of hypertension as severe restrictions of placental blood flow, by uterine artery ligation in guinea pigs, have shown only small increases in blood pressure in later life (Persson & Jansson, 1992). Housing rat dams in hypoxic conditions (11% oxygen) during pregnancy, had no effect upon the later blood pressure of the offspring (Langley-Evans, 1998). Specific amino acid deficiencies caused by the restricted protein intake may also effect placental and fetal development. For example, in theory, 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) did not show a decrease in maternal serum or fetal free amino acid pool arginine concentrations in rats fed a maternal low protein diet. Threonine concentrations

were reduced in maternal serum and the fetal free amino acid pool in the same study (Rees *et al*, 1999). This may be due either to an increased drive to produce glycine, or an increased requirement to metabolise 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 metabolised to homocysteine, which leads to increased growth when added to embryo cultures *in vitro* (VanAerts *et al*, 1994).

Late gestation growth retardation is likely to have a severe effect on the development of the kidney, as it is in a period of rapid growth and its development takes place largely in late pregnancy. Slowed kidney growth and reduced nephron number have been shown in rats and humans, in association with intrauterine growth retardation (Hinchliffe *et al*, 1992, Langley-Evans 1999, Langley-Evans *et al*, 1999, Chapter 4, Zeman 1969). Over-exposure of the fetus to glucocorticoids in late gestation may also affect the development of the kidney. Administration of dexamethasone during pregnancy impairs nephron formation in rats and elevates fetal glomerular filtration rate and sodium reabsorpsion in sheep (Celsi *et al*, 1997), suggesting that the kidney is sensitive to glucocorticoid action during development. Impaired kidney development is likely to lead to renal dysfunction, such as decreased renal blood flow, increased filtration fractions (Langley-Evans *et al*, 1999), and impaired glomerular filtration (Hall & Zeman, 1968) as well as causing disturbances of the renin-angiotensin system. All of these factors may contribute towards the control of blood pressure. It is likely that the renin-angiotensin system interacts with the prostaglandins and the kinins within the kidneys to raise the blood pressure. Studies with early captopril or losartan treatment support the hypothesis that the renin-angiotensin system could also modify local vascular structure (Harrap *et al*, 1990) and indirectly affect growth (Robertson & Nicolls, 1993).

The results from this thesis have further characterised the rat model of maternal diet induced hypertension, showing the similarities between it and the human

pathophysiological process and demonstrating it to be an appropriate model in which to study the fetal origins of hypertension. The results have also shown that there is growing evidence for the involvement of the renin-angiotensin system in the development of the high blood pressure in this model. In summary, it is proposed that an over exposure of the fetus to glucocorticoids programmes the HPA-axis and glucocorticoid sensitivity is increased (Langley-Evans *et al*, 1996c, 1996f). This then enhances the actions of angiotensin II and this is the primary driver for the elevated blood pressure (overview shown in figure 7.1). The elevated blood pressure may then be amplified and maintained by structural vascular changes caused by increased sensitivity to angiotensin II. Treatment with an ACE inhibitor or angiotensin II receptor antagonist in early life prevents the onset of elevated blood pressure long term showing that this is a preventable process. This may have implications for public health in the future. Further research may help determine whether interventions in humans are a possibility and whether they are justified and safe.

Future work should further investigate the hypothesis that angiotensin II has a specific role in the development of elevated blood pressure in this model. It is not clear from the present results how angiotensin II acts to elevate blood pressure in the low protein exposed rats, as increased circulating concentrations have not been shown in these animals. Elevated angiotensin II at certain critical periods of development cannot be ruled out at present. It is, therefore, suggested that making a series of circulating angiotensin II measurements, particularly during the period of 0-4 weeks of age may be useful in this model.

Angiotensin II sensitivity should also be assessed in the low protein exposed rats. Both the AT_1 and AT_2 receptors could be characterised particularly in the kidney and vascular smooth muscle by performing investigations of expression and binding affinity.

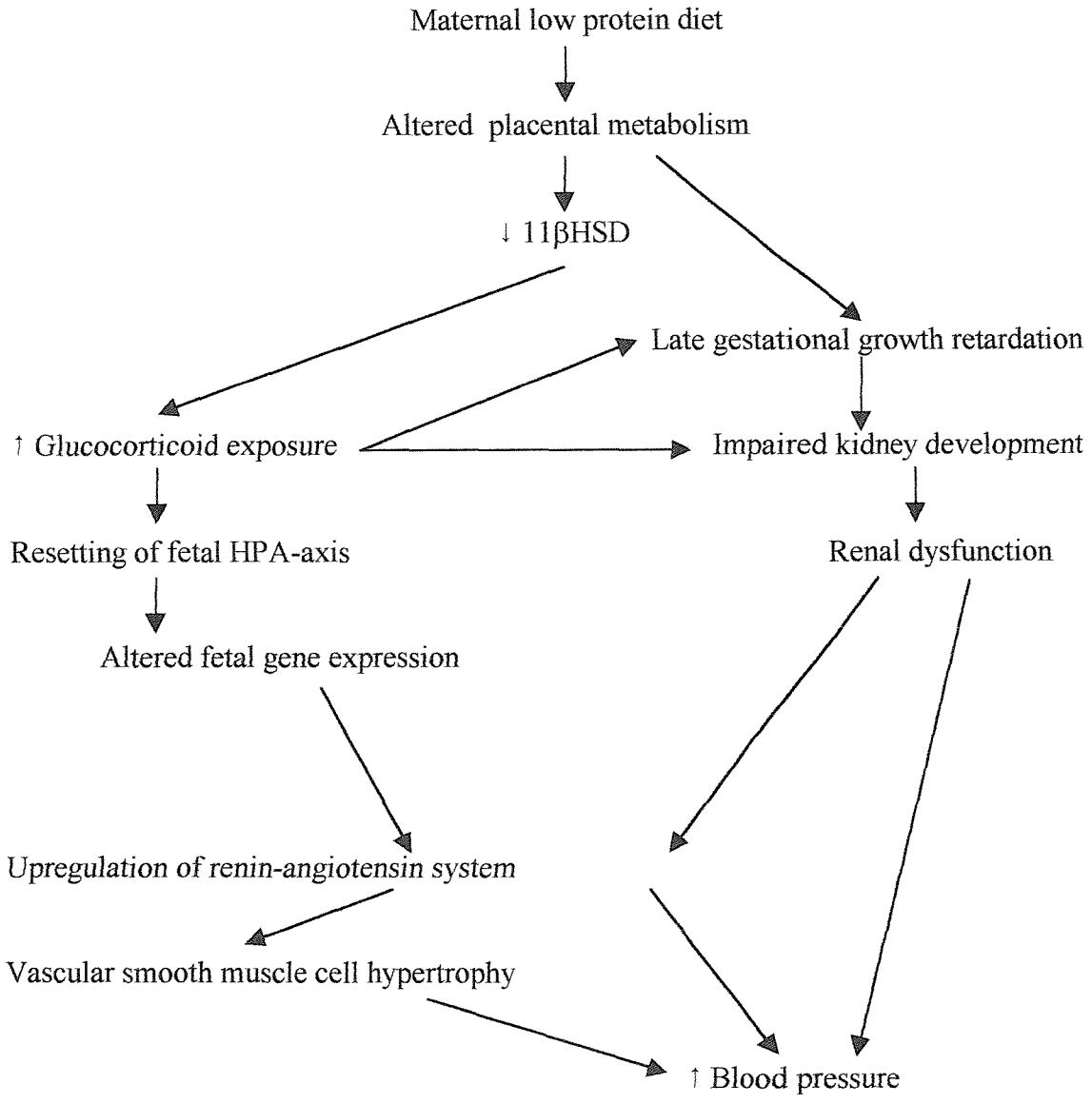
Angiotensin II effects on the vasculature during the period of 0-4 weeks of age may be determined by comparing the vascular structure in the low protein exposed rats with that

of losartan treated low protein exposed rats. For example vascular smooth muscle cell size and density, and elasticity and compliance may be studied. Collagen and elastin content of the blood vessels could also be measured.

The effect of angiotensin II on renal development during this period may also be investigated using rats treated with losartan. Nephron number and renal function could be studied in these rats compared with untreated low protein exposed rats.

In order to assess whether angiotensin II has a role in the development of elevated blood pressure in humans, it may be worth measuring circulating angiotensin II concentrations in a group of pre-pubertal children, whose birth characteristics are known. If the hypothesis that angiotensin II is indeed involved in the human pathophysiological process it could be possible to establish a critical period in humans when intervention may be achievable.

Figure 7.1 Possible mechanisms involved in the programming of hypertension in the low protein rat model.



References

Adams MA, Bobik A, Korner PI. Enalapril can prevent vascular amplifier development in spontaneously hypertensive rats. *Hypertension.* 1990; **16**: 252-260.

Allen RKA. A review of angiotensin converting enzyme in health and disease. *Sarcoidosis.* 1991; **8**: 95-100.

Anderson PW, Do YS, Hsueh WA. 1993. Angiotensin II causes mesangial cell hypertrophy. *Hypertension.* 1993; **21**: 29-35.

Anguitta RM, Sigulem DM, Sawaya AL. Intrauterine food restriction is associated with obesity in young rats. *J Nutr*, 1993;**123**:1421-1428.

Bader M, Paul M, Fernandez-Alfonso M, Kaling M, Ganten D. Textbook of hypertension. Swales JD (Ed). Blackwell Scientific Publications 1994. 214-232.

Barker DJP, Osmond C. Infant mortality, childhood nutrition and ischaemic heart disease in England and Wales. *Lancet* 1986; **i**: 1077-81.

Barker DJP, Osmond C, Law CM. The intrauterine and early postnatal origins of cardiovascular disease and chronic bronchitis. *J. Epidemiol. Community Health* 1989a; **43**:237-40.

Barker DJP, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. *Lancet* 1989b: **ii**: 577-80.

Barker DJP, Bull AT, Osmond C, Simmonds SJ. Fetal and placental size and risk of hypertension in adult life. *BMJ* 1990: **301**: 259-62.

Barker DJP, Godfrey KM, Osmond C, Bull A. The relation of fetal length, ponderal index and head circumference to blood pressure and the risk of hypertension in adult life. *Peadiatr. perinat. Epidemiol.* 1992; **6**: 35-44.

Barker DJP, Gluckman PD, Godfrey KM, Harding JE, Owens JA, Robinson JS. Fetal nutrition and cardiovascular disease in adult life. *Lance*t 1993;**314**:938-941

.

Barker DJP. Mothers, babies and disease in later life. London: BMJ publishing group. 1994.

Barker DJP. Fetal origins of coronary heart disease. BMJ 1995; 311: 171-174.

Barraclough CA. Production of anovulatory, sterile rats by single injections of testosterone proprionate. *Endocrinology* 1961; **68**: 62-67.

Barrett G, Morgan T, Smith M, Aldred P. Effect of mineralocorticoids and salt loading on renin release, renal renin content and renal renin mRNA in mice. *Clin. Exp.Pharmacol. Physiol.* 1989; **16**: 631-639.

Bartley M, Power C, Blane D, Davey Smith G, Shipley M. Birth weight and later socioeconomic disadvantage : Evidence from the 1958 British cohort study. *BMJ* 1994; **309**:1475-9.

Benediktsson R, Lindsay RS, Noble J, Seckl JR, Edwards CRW. Glucocorticoid exposure *in utero* : new model for hypertension. *Lancet* 1993; **341**: 339-41.

Benzie IFF, Tomlinson B. Antioxidant power of angiotensin-converting enzyme inhibitors in vitro. *Br.J.Clin.Pharmacol.* 1998; **45**: 168-169.

Benzoni D, Vincent M, Sassard J. Urinary 6-ketoprostaglandin F1α in genetically hypertensive rats of the Lyon strain. *Clin. Sci.* 1984; **66**: 453-457.

Berne R, Levy M (Editors). Physiology. The CV Mosby company.

Berry C. Hypertension and arterial development: Long term considerations. *Br. Heart. J.* 1978; **40**: 709-717.

Bohlender J, Menard J, Wagner J, Luft FC, Ganten D. Human renin-dependent hypertention in rats transgenic for human angiotensinogen. *Hypertension.* 1996; **27**: 535-540.

Bookstein JJ, Abrams HL, Beunger RE, Lecky J, Franklin SS, Reiss MD *et al.* Radiologic aspects of renovascular hypertension: II. The role of uroFigurey in unilateral renovascular disease. *JAMA* 1972; **220**: 1225-1230.

Brasal JO, Boyd D. Influence of thyroid hormone on fetal brain growth and development. Fisher DA, Burrows GN (Eds). Perinatal thyroid physiology and disease. New York, Raven Press 1975.

Broughton-Pipkin F. The hypertensive disorders of pregnancy. *BMJ.* 1995; **311**: 609-613.

Brunner HR, Waeber B, Nussberger J. Pharmocology of converting enzyme inhibitors. *Clinical and experimental hypertension.* 1987;**A**9:275-288

Bunag R. Validation in awake rats of a tail cuff method for measuring systolic blood pressure. *J. Applied Physiol.* 1973; **34**: 279-81.

Burneir M, Roch-Ramel F, Brunner HR. Renal effects of angiotensin II receptor blockade in normotensive subjects. *Kidney Int.* 1996; **49**: 1787-1790.

Calder P, Yacoob P. The level of protein in the diet of pregnant rats affects lymphocyte proliferation and natural killer cell activity at weaning. *Proc. Nutr. Soc.* 1999; in press.

Cambien F, Poirier O, Lecerf L, Evans A, Cambou JP, Arveiler D et al. Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. *Nature* 1992; **359**: 641-4

Campbell DJ. Circulating and tissue angiotensin systems. *J. Clin. Invest.* 1987; **79**: 1-6.

Campbell DM, Hall MH, Barker DJP, Cross J, Shiell AW, Godfrey KM. Diet in pregnancy and the offspring's blood pressure 40 years later. *Brit. J. Obs. Gynae.* 1996; **103**: 273-80.

Celsi G, Kistner A, Eklof A-C, Ceccatelli S, Aizman R, Jacobson S. Fetal and neonatal physiology symposium, Cambridge. 1994; 24.

Christensen K, Vaupel JW, Holm NV, Yashin AI. Mortality amoung twins after age 6: fetal origins hypothesis versus twin method. *BMJ* 1995; **310**: 432-436.

Clarke HE, Coates ME, Eva JK et al. Dietary Standards for laboratory animals: Report of the Laboratory Animals Centre Diets Advisory Committee. *Laboratory Animals.* 1978; **11**: 1-28.

Clark PM, Hindmarsh PC, Shiell AW, Law CM, Honour JW, Barker DJP. Size at birth and adrenocortical function in childhood. *Clin. Endocrin.* 1996; **45**: 721-726.

Crowe C, Dandekar P, Fox M, Dhingra K,Bennet L, Hanson MA. The effects of anaemia on heart, placenta and body weight, and blood pressure in fetal and neonatal rats. *J.Physiol.* 1995; **488**: 515-519.

Curhan GC, Willett WC, Rimm EB, Spiegelman D, Ascherio AL, Stampfer MJ. Birth weight and adult hypertension, diabetes mellitus and obesity in US men. *Circulation* 1996; **94**: 3246-3250.

Currie MG, Needleman P. Renal arachidonic acid metabolism. *Ann. Rev. Physiol.* 1984; **46**: 327-341.

Dahl LK. Possible role of salt intake in the development of essential hypertension. Cottier P, Bock KD (Eds). Essential hypertension: an international symposium. Berlin: Springer Verlag 1960: 61-75.

Dahri S, Snoeck A, Reusens-Billin B, Remacle C, Hoet JJ. Islet function in the offspring of mothers on low-protein diet during gestation. *Diabetes* 1991; **40**, suppl 2: 115-120.

Dallman MF, Strack AM, Akana SF, Bradbury MJ, Hanson ES, Scribner KA, Smith M. Feast and famine: Critical role of glucocorticoids with insulin in daily energy flow. *Fron Neuro* 1993;**14**:303-347

Department of Health. Nutritional aspects of cardiovascular disease. 1994 Report on health and social subjects 46, HMSO.

Desai M, Crowther NJ, Ozanne SE, Lucas A, Hales CN. Adult glucose and lipid metabolism may be programmed during fetal life. *Biochem. Soc. Trans.* 1995; **23**: 331-335.

Desai M, Crowther NJ, Lucas A, Hales CN. Organ-selective growth in the offspring of protein-restricted mothers. *Br. J. Nutr.* 1996; **76**: 591-603.

De Thomas ME, Mercuri O, Rodrigo A. Effects of dietary protein and EFA deficiency on liver Δ5, Δ6 and Δ9 desaturase activities in the early developing rat. *J. Nutr.* 1980; **110**: 595-599.

De Thomas ME, Mercuri O, Serres C. Effect of cross-fostering rats at birth on the normal supply of essential fatty acids during protein deficiency. *J.Nutr.* 1983; **113**: 314-319.

Dibona GF. The function of renal nerves. *Rev. Physiol. Biochem. Pharmacol.* 1982; **94**: 76-181.

Diz DI, Baer PG, Nasjletti A. Angiotensin II-induced hypertension in the rat. Effects on the plasma concentration, renal excretion, and tissue release of prostaglandins. *J. Clin. Invest.* 1983; **72**: 466-477.

Divon MY, Haglund B, Nisell H, Otterbland PO, Westgren M. Fetal and neonatal mortality in the postterm pregnancy: the impact of gestational age and fetal growth restriction. *Am. J. Obstet. Gynecol.* 1998; **178**: 726-731.

Dobbing J. Nutritional growth restriction and the nervous system. Davison AN, Thomson RHS (Eds). The molecular basis of neuropathology. London, Edward Arnold 1981; 221-223.

Dunn MJ. Renal prostaglandin production in the Japanese (Kyoto) spontaneously hypertensive rat. *Clin. Sci. Mol. Med.* 1978: **55**: 191s-193s.

Dzau VJ. Significance of the vascular renin-angiotensin pathway. *Hypertension* 1986;**8**: 553-9.

Dzau VJ, Pratt RE. Renin-angiotensin system: biology, physiology and pharmacology. Haber E, Morgan H, Katz Z, Fozzard H (Eds). Handbook of experimental cardiology. New York: Raven Press 1986; 1631-1661.

Edwards CRW, Benediktsson R, Lindsay RS, Seckl JR. Dysfunction of placental glucocorticoid barrier: link between fetal environment and adult hypertension. *Lancet* 1993; **341**: 355-357.

Farrario CM, Flack JM. Pathologic consequences of increased angiotensin II activity. *Cardiovasc. Drugs. Ther.* 1996; **10**: 511-518.

Farrow PR, Wilkinson R. Reversible renal failure during treatment with captopril. *BMJ* 1979; **1**:1680.

Folkow B. Cardiovascular structural adaptation: its role in the initiation and maintenance of primary hypertension. *Clin. Sci. Mol. Med.* 1978; **55**: 3s-22s.

Forrester TE, Wilks RJ, Bennet FI, Simeon D, Osmond C, Allen M, Chung AP, Scott P. Fetal growth and cardiovascular risk factors in Jamaican schoolchildren. *BMJ* 1996; **312**: 156-160.

Frelson JL, Guidicelli JF. Compared myocardial and vascular effects of captopril and hidydralazine during hypertension development in spontaneously hypertensive rats. *Br. J. Pharmacol.* 1983; **80**: 530-543.

Gardner DS, Jackson AA, Langley-Evans SC. The effect of prenatal diet and glucocorticoids upon growth and systolic blood pressure in the rat. Proc. Nutr. Soc. 1998; 57: 235-240.

Gavras H, Brown JJ, Lever AF, MacAdam RF, Robertson JIS. Acute renal failure, tubular necrosis, and myocardial infarction induced in the rabbit by intravenous angiotensin II. *Lancet* 1971;**ii**:19-22.

Genser G, Rymark P, Isberg PE. Low birthweight and risk of high blood pressure in adulthood. *BMJ* 1988; **296**: 1498-1500.

Gibbons GH, Pratt RE, Dzau VJ. Vascular smooth muscle hypertrophy vs. hyperplasia: autocrine tranforming growth factorβ1 expression determines growth response to angiotensin II. *J. Clin. Invest.* 1992; **90**: 456-461.

Gleiberman L. Blood pressure and dietary salt in human populations. *Ecol.Fd.Nutr.* 1973; **2**: 143-156.

Godfrey KM, Forrester T, Barker DJP, Jackson AA, Landman JP, Hall J St E, Cox V, Osmond C. Maternal nutritional status in pregnancy and blood pressure in childhood. *Br. J. Obstet. Gynaecol.* 1994; **101**: 398-403.

Godfrey K, Robinson S, Barker DJP, Osmond C, Cox V. Maternal nutrition in early and late pregnancy in relation to placental and fetal growth. *BMJ* 1996; **312**: 410-4.

Godfrey K, Barker DJ, Robinson S, Osmond C. Maternal birthweight and diet in pregnancy in relation to the infants thinness at birth. *Br.J.Obstet.Gynaecol.* 1997; **104**: 663-667.

Gohlke P, Lamberty V, Kuwer I, Bartenbach S, Schnell A, Linz W et al. Long-term low-dose angiotensin converting enzyme inhibitor treatment increases vascular cyclic guanosine 3',5'-monophosphate. *Hypertension* 1993;**22**:682-687

Gohlke P, Unger T. Textbook of hypertension. Swales JD (Ed). Blackwell Scientific Publications 1994. 1115-1127.

Gomez RA, Lynch KR, Chevalier RI, Everett AD, Johns DW, Wilfong N *et al.* Renin and angiotensinogen gene expression and intrarenal renin distribution during ACE inhibition. *Am. J. Physiol.* 1988; **254**: F900-F906.

Goodfriend TL, Elliot ME, Catt KJ. Drug therapy: Angiotensin receptors and their antagonists. *N. Engl. J. Med.* 1996; **334**: 1649-54.

Gordon RD, Stowasser M, Klemm SA, Tunny TJ. Textbook of hypertension. Swales JD (Ed). Blackwell Scientific Publications 1994. 865-892.

Gordon T, Kannel WB. Drinking and its relation to smoking, BP, blood lipids and uric acid. *Arch. Intern. Med.* 1983; **143**: 1366-1374.

Gould AB, Green D. Kinetics of the human renin and human substrate reaction. *Cardiovasc. Res.* 1071; **5**: 86-89.

Grady EF, Sechi LA, Griffin CA, Schambelan M, Kalinyak JE. Expression of AT2 receptors in the developing rat fetus. *J. Clin. Invest.* 1991; **88**: 921-933.

Granstrom E, Kindahl H. *Advances in prostaglandin research.* **5**; 119-210, JC Frolich, ed., Raven Press, New York, 1978.

Granstrom E, Samuelsson B. *Advances in prostaglandin and thromboxane research. 5*; 1-13, JC Frolich, ed., Raven press, New York, 1978.

Griendling KK, Alexander RW. Textbook of hypertension. Swales JD (Ed). Blackwell Scientific Publications 1994. 244-253.

Grobbee DE, Hofman A. Does sodium restriction lower blood pressure? BMJ 1986; **293**:27-29.

Gustafsson JA & Stanburg A. Neonatal programming of androgen responsiveness of the liver of adult rats. *J Biol Chem* 1974;**249**:719-723.

Guyton AC. Blood pressure control - special role of the kidneys and body fluids. *Science* 1991; **252**:1813.

Hackenthal E, Paul M, Ganten D, Taugner R. Morphology, physiology and molecular biology of renin secretion. *Physiol. Rev.*1990;**70**: 1067-1116.

Hall JE. Regulation of glomerular filtration rate and sodium excretion by angiotensin II. *Fase*b 1986;**45**:1431-37.

Hall SM, Zemen FJ. Kidney function of the progeny of rats fed a low protein diet. *J Nutr* 1968;**95**:49-54.

Harding JE, Johnston BM. Nutrition and fetal growth. *Reprod. Fertil. Dev.* 1995; **7**: 539-547.

Harding, JE, Liu L, Evans PC, Gluckman PD. Insulin-like growth factor 1 alters feto-placental protein and carbohydrate metabolism in fetal sheep. *Endocrinology* 1994; **134**,no.3: 1509-1514.

Harrap SB, Van der Merwe WM, Griffin SA, MacPherson F, Lever AF. Brief angiotensin converting enzyme inhibitor treatment in young spontaneously hypertensive rats reduces blood pressure long term. *Hypertension* 1990;**16**:603-614.

Harris GW, Levine S. Sexual differentiation of the brain and its experimental control. *J. Phys.* 1962; 42P-43P.

Hassam AG, Sinclair AJ, Crawford MA. The incorporation of orally fed radioactive gamma-linolenic acid and linoleic acid into the liver and brain lipids of suckling rats. *Lipids.* 1975; 7: 417-420.

Hayakiri M, Khondo Y, Izumi H. A rapid and simple spectrophotometric assay of angiotensin converting enzyme. *Anal. Biochem* 1987; **84**: 361-369.

Heinrichs SC, Koob GF. Corticotrophin-releasing factor modulates dietary preference in nutritionally and physically stressed rats. *Psycopharmacology.* 1992; **109**: 177-184.

Heinrichs SC, Cole BJ, Pich EM, Menzaghi F, Koob GF, Hauger RL. Endogenous corticotrophin-releasing factor modulates feeding induced by neuropeptide Y or a tail pinch stressor. *Peptides.* 1992; **13**: 879-884.

Henry PJ, Drummer OH, Horowitz JD. S-nitrosthiols as vasodilators: implications regarding tolerance to nitric oxide-containing vasodilators. *Br. J. Pharmacol.* 1989; **98**: 757-766.

Hilbert P, Lindpaintner K, Beckmann JS, Serikawa T, Soubrier F, Dubay C *et al.* Chromosomal mapping of two genetic loci associated with blood pressure regulation in hereditary hypertensive rats. *Nature* 1991; **353**:521-529.

Hinchliffe SA, Lynch MRJ, Sargent PH. The effect of intrauterine growth retardation on the development of renal nephrons. *Br. J. Obstet. Gynaecol.* 1992; **99**: 296-391.

Hiruma Y, Inoue A, Hirose S, Hagiwara H. Angiotensin II stimulates the proliferation of osteoblast-rich populations of cells from rat calvarie. *Biochem. Biophys. Res. Commun.* 1997; **230**: 176-178.

Hricik DE, Browning PJ, Kopelman R, Goorno WE, Madias ME, Dzau VJ. Captopril-induced functional renal insufficiency in patients with bilateral renal artery stenosis in a solitary kidney. *N. Engl. J. Med.* 1983; **308**: 373-376.

Hunt SC, Williams RR, Barlow GK. A comparison of posistive family history definitions for defining the risk of future disease. *J. Chron. Dis.* 1986; **39**:809-821.

Itoh H, Mukoyama M, Pratt RE, Gibbons GH, Dzau VJ. Multiple autocrine growth factors modulate vascular smooth muscle cell growth response to angiotensin II. *J. Clin. Invest.* 1993; **91**: 2268-2274.

Jackson EK, Branch RA, Oates JA. Participation of prostaglandins in the control of renin release. *Adv. Prostagl. Thrombox. Res* 1982; **10**:255-276.

Jeunemaitre X, Soubrier F, Kotelevtsev YV, Lifton RP *et al.* Molecular basis of human hypertension: role of angiotensinogen. *Cell* 1992; **71**: 169-80.

The fifth report of the Joint National Committee (JNCR) on detection, evaluation and treatment of high blood pressure. *Arch. Intern. Med.* 1993; **153**: 154-83.

Ketteler M, Noble NA, Border WA. Tranforming growth factor-β and angiotensin II: The missing link from glomerular hyperfiltration to glomerulosclerosis? *Annu. Rev. Physiol.* 1995; **57**: 279-295.

Kirchheim H, Ehmke H, Fischer S, Lowe W, Persson P. Sympathetic modulation of the pressure-dependant renin release in concious dogs. *Clin. Exp. Hypertens.* 1987; **A9** suppl 1: 167-180.

Kleinman HK, Ponce ML. The good side of side effects: Captopril blocks vessel and tumour growth. *J. Clin. Invest.* 1996; **98**: 599.

Klickstein LB, Kaempfer CE, Wintroub BU. The granulocyte angiotensin system: angiotensin I converting activity of cathepsin G. *J.Biol.Chem.* 1982; **257**: 1504-1506.

Konieczkowski M, Dunn MJ, Stork JE, Hassid A. Glomerular synthesis of prostaglandins and thromboxane in spontaneously hypertensive rats. *Hypertension.* 1983; **5**:446-452.

Konje JC, Bell SC, Morton JJ, Chazal R, Taylor DJ. Human fetal kidney morphometry during gestation and the relationship between weight, kidney morphometry and plasma active renin concentration at birth. *Clin Sci* 1996;**91**:169-175.

Krahn DD, Gosnell BA, Grace M, Levine AS. CRF antagonist partially reverses CRF- and stress-induced effects on feeding. *Brain Res. Bull.* 1986; **17**: 285-289.

Krakoff LR. Management of the hypertensive patient. Churchill Livingstone 1994: 175-194.

Kremer D, Lindop G, Brown WCB, Morton JJ, Robertson JIS. Angiotensin induced myocardial necrosis and renal failure in the rabbit. *Cardio Res* 1981;**15**:43-46

Lahera V, Duran F, Cachofeiro V, Canizo FJ, Tresguerres JAF, Rodriguez FJ. Different excretion pattern of urinary PGE2 and 6-keto-PGF1α in two kidney one clip goldblatt rats. *Prostagl. Leuk. Med.* 1986; **24**: 35-41.

Langley SC, Jackson AA. Increased Systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diet. *Clin. Sci.* 1994; **86**: 217-22.

Langley-Evans SC, Jackson AA. Captopril normalises systolic blood pressure in rats with hypertension induced by fetal exposure to maternal low protein diets. *Comp. Biochem. Physiol.* 1995; **110A**: 223-28.

Langley-Evans SC, Clamp AG, Grimble RF, Jackson AA. Influence of dietary fats upon systolic blood pressure in the rat. *Int. J. Food Sci. Nutr.* 1996a; **47**: 417-425.

Langley-Evans SC, Gardner DS, Jackson AA. Association of disproportionate growth of fetal rats in late gestation with raised systolic blood pressure in later life. *J Rep Fert* 1996b;**106**:307-312.

Langley-Evans SC, Gardner DS, Jackson AA. Evidence of programming of the hypothalmic-pituitary-adrenal axis by maternal protein restriction during pregnancy. *J. Nutr.* 1996c; **126**: 1578-1585.

Langley-Evans SC, Phillips GJ, Benediktsson R, Gardener DS, Edwards CRW, Jackson AA, Seckl JR. Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension in the rat. *Placenta* 1996d; **17**: 169-172.

Langley-Evans SC, Phillips GJ, Jackson AA. Fetal exposure to low protein maternal diet alters the susceptibility of young adult rats to sulfur dioxide-induced lung injury. *J. Nutr* 1996e; **127**: 202-209.

Langley-Evans SC, Phillips GJ, Gardner DS, Jackson AA. The role of glucocorticoids in programming of maternal diet induced hypertension in the rat. *J. Nutr. Biochem.* 1996f; **7**: 173-178.

Langley-Evans SC, Welham SJM, Sherman RC, Jackson AA. Weanling rats exposed to maternal low-protein diets during discrete periods of gestation exhibit differing severity of hypertension. *Clin. Sci.* 1996g; **91**: 607-615.

Langley-Evans SC. Intrauterine programming of hypertension by glucocorticoids. *Life Sci* 1997;**60**:1213-1221.

Langley-Evans SC, Gardner DS, Welham SJM. Intrauterine programming of cardiovascular disease by maternal nutritional status. *Nutrition* 1998; **14**: 39-47.

Langley-Evans SC, Nwagwu M. Impaired growth and increased glucocorticoid-sensitive enzyme activities in tissues of rat fetuses exposed to maternal low protein diets. *Life Sci.* 1998; **63**: 605-615.

Langley-Evans SC, Welham SJM, Jackson AA. Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. *Life Sci.* 1999; **64**: 965-974.

Langley-Evans SC. Intrauterine programming of hypertension by maternal undernutrtion: a comparison of two low protein diet protocols. *Int. J. Food. Sci. Nutr.* 1999; submitted.

Larragh JH. The renin system and the renal regulation of blood pressure. Seldin DW, Giebisch G (Eds). The kidney; physiology and pathophysiology. New York: Raven Press 1992; 1411-1453.

Law SC, Barker DJP, Bull AR, Osmond C. Maternal and fetal influences on blood pressure. *Arch. Dis. Child.* 1991; **66**: 1291-1295.

Law CM, Gordon GS, Shiell AW, Barker DJP, Hales CN. Thiness at birth and glucose tolerance in seven-year-old children. *Diabetic Medicine* 1995; **12**: 24-29.

Law CM, Shiell AW. Is blood pressure inversely related to birth weight? The strength of evidence from a systematic review of the literature. *J. Hypertens.* 1996;**14**: 935-941.

Lee RMKW, Berecek KH, Tsoporis J, McKenzie R, Triggle CR. Prevention of hypertension and vascular changes by captopril treatment. *Hypertension.* 1991; **17**: 141-150.

Levens NR. Role of the intrarenal renin-angiotensin system in the control of renal function. *Circ. Res.* 1981; **48**: 157.

Lever AF, Harrap SB. Essential hypertension: A disorder of growth with origins in childhood. *J. of Hyper.* 1992;**10**: 101-120.

Levy L, Jackson AA. Modest restriction of dietary protein during pregnancy in the rat: fetal and placental growth. J. Dev. Physiol. 1993; 19: 113-118.

Liebowitz SF, Rowland CR, Horr L, Squillar V. Noradrenergic feeding elicited via the paraventricular nucleus is dependent upon circulating corticosterone. *Physiol. Behav.* 1984; **32**: 857-864.

Liu L, Harding JE, Evans PC, Gluckman PD. Maternal insulin-like growth factor-1 infusion alters feto-placental carbohydrate and protein metabolism in pregnant sheep. *Endocrinol.* 1994; **135**: 895-900.

Lucas A. Early diet, later consequences. Conning D (Ed). *BNF* 1991; 24-29.

Lucas A, Morley R. Does early nutrition in infants born before term programme later blood pressure? *BMJ* 1994; **309**: 304-8.

Lucas A, Baker BA, Desai M, Hales CN. Nutrition in pregnant and lactating rats programs lipid metabolism in the offspring. *Br.J.Nutr* 1996; **76**: 605-612.

Lumbers ER. Functions of the renin-angiotensin system during development. *Clin. Exp. Pharm. Physiol.* 1995; **22**: 499-505.

Lumbers ER, Kingsford NM, Menzies RI, Stevens AD. The acute effects of captopril, an angiotensin converting enzyme inhibitor, on the pregnant ewe and her fetus. *Am. J. Physiol.* 1992; **262**:R754-R760.

Lumbers ER, Reid GC. The actions of vasoactive compounds in the foetus and the effect of perfusion through the placenta on their biological activity. *Aus. J. Exp.Biol. Med. Sci.* 1979; **56**: 11-24.

MacMahon S, Peto R, Cutler J. Blood pressure, stroke and coronary heart disease. *Lancet* 1990; **335**: 763-74.

Manatunga AK, Reister TK, Miller JZ, Pratt JH. Genetic influences on the urinary excretion of aldosterone in children. *Hypertension* 1992; **19**:192-197.

Margetts BM, Rowland MGM, Foord FA, Cruddas AM, Cole TJ, Barker DJP. The relation of maternal weight to the blood pressures of Gambian children. *Int. J. Epidemiol.* 1991; **20**: 938-43.

Matineau A, Robillard M, Falardeau P. Defective synthesis of vasodilator prostaglandins in the spontaneously hypertensive rat. *Hypertension* 1984; **6** suppl 1: I161-I165.

Martyn CN, Greenwald SE. Impaired synthesis of elastin in walls of aorta and large conduit arteries during early development as an initiating event in pathogenesis of systemic hypertension. *Lancet*. 1997; **350**: 953-955.

Martyn CN, Lever AF, Morton JJ. Plasma concentrations of inactive renin in adult life are related to indicators of feotal growth. *J.Hypertens*. 1996; **14**: 881-886.

Materson BJ. Management of hypertension. Hollenburg NK (Ed). Butterworths publishers 1987:1-24.

Matthes JWA, Lewis PA, Davies DP, Bethel JA. Relation between birthweight at term and systolic blood pressure in adolescence. *BMJ* 1994; **308**: 1074-1077.

McCarthy HD, Pickard C, Speed J, Jackson AA. Sexual dimorphism of macronutrient selection and regional adipose tissue accumulation following in utero exposure to a maternal low protein diet. *Proc. Nutr. Soc.* 1994; **53**: 172A.

Meade TW, Imeson JD, Gordon D, Peart WS. The epidemiology of plasma renin. *Clin. Sci.* 1983; **64**: 273-280.

Mendelsohn FAO, Lloyd CJ, Kachel C, Funder JW. Induction by glucocorticoids of angiotensin converting enzyme production from bovine endothelial cells in culture and rat lung in vivo. *J Clin Invest* 1982;**70**:684-692.

Mercuri O, de Thomas ME, Itarte H. Prenatal protein depletion and delta9, delta 6 and delta 5 desaturase. *Lipids* 1979; **14**: 822-825.

Merlet-Benichou C, Gilbert T, Muffat-Joly M, Lelievre-Pegorier M, Leroy B. Intrauterine growth retardation leads to a permanent nephron deficit in the rat. *Pediatr. Nephrol.* 1994; **8**: 175-180.

Moncada S, Vane JR. Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A2 and prostacyclin. *Pharmacol. Rev.* 1979; **30**: 293-331.

Moore SE, Cole TJ, Poskitt EM, Sonko BJ, Whitehead RG, McGregor IA, Prentice AM. Season of birth predicts mortality in rural Gambia. *Nature* 1997; **388**: 434.

Morris BJ, Griffiths LR. Frequency in hypertensives of alleles for a RFLP associated with the renin gene. *Biochem. Biophys. Res. Commun.* 1988; **150**:219-224.

Morris HG, Sherman NA, Shepperdson FT. *Prostaglandins* 1981; **21**: 771-778.

Morton JJ, Beattie EC, MacPherson F. Angiotensin II receptor antagonist Losartan has persistent effects on blood pressure in the young spontaneously hypertensive rat: Lack of relation to vascular structure. *J. Vasc Res* 1992; **29**: 264-269.

Morton JJ, Wallace ECH. The importance of the renin-angiotensin system in the development and maintenance of hypertension in the two-kidney, one-clip hypertensive rat. *Clin. Sci.* 1983; **64**: 359-370.

Murphy TJ, Alexander RW, Griendling KK, Runge MS, Bernstein KE. Isolation of a cDNA encoding the vascular type-I angiotensin II receptor. *Nature* 1991; **351**: 233-236.

Nagai K, Nabekura H, Mibe M, Ohshige T, Mori N. Prostaglandin dehydrogenase activity in placenta and maternal lung, kidney and gastric mucosa during rat pregnancy. *Mol. Cell Endo.* 1991; **80**: 153-163.

Naismith DJ & Morgan BLG. The biphasic nature of protein metabolism during pregnancy in the rat. *Br J Nutr* 1976;**36**:563-566

Narce M, Poisson JP, Belleville J, Chanussot. Depletion of Δ9 desaturase (EC 1.14.99.5) enzyme activity in growing rat during dietary protein restriction. *Brit. J. Nutr.* 1992; **68**, 627-637.

Narce M, Asdrubal P, Delachambre MC, Vericel E, Largarde M, Poisson JP. Age-related changes in linoleic acid bioconversion by isolated hepatocytes from spontaneously hypertensive and normotensive rats. *Molecular and Cell. Biochem.* 1994; **141**: 9-13.

Nasjletti A, Baer PG. Prostanoids in clinical and experimental hypertension. Halushka PV, Mais DE (Eds). Ecosanoids in the cardiovascular and renal systems. MTP Press Limited, UK 1988. 159-175.

National Research Council. Nutrient requirement of the rat. Nutrient requrements of domestic animals. National Academy of Sciences, Washington DC; 1978.

Ng KKF, Vane JR. Conversion of angiotensin I to angiotensin II *Nature* 1967; **216**: 762-6.

Owens JA, Kind KL, Carbone F, Robinson JS, Owens PC. Circulating insulin-like growth factors-I and -II and substrates in fetal sheep following restriction of placental growth. *J. Endocrinology* 1994; **140**: 5-13.

Penny WJ, Lewis MJ. Nifedipine is excreted in human milk. *Eur. J. Clin. Pharm.* 1989; **36**: 427-428.

Persson E, Jansson T. Low birth weight is associated with elevated adult blood pressure in the chronically catheterised guinea-pig. *Acta. Physiol. Scan.* 1992; **115**: 195-196.

Pfeffer JM, Pfeffer MA, Frohlich ED. Validity of an indirect tail-cuff method for determining systolic arterial pressure in unanesthetized normotensive and spontaneously hypertensive rats. *J. Lab. Clin. Med.* 1971; **78**: 957-962.

Phillips GJ, Langley-Evans SC, Benediktsson R, Seckl JR, Edwards CRW, Jackson AA. The role of dietary protein restriction during pregnancy on the activity of placental 11β-hydroxysteroid dehydrogenase. *Proc. Nutr. Soc.* 1994; **53**: 169A.

Pi-Sunyer FX. Health implications of obesity. *Am. J. Clin. Nutr.* 1991; **53** (suppl 6): 1595S-1603S.

Provencher PH, Saltis J, Funder JW. Glucocorticoid but not mineralocorticoids modulate endothelin-I and angiotensin II binding in SHR vascular smooth muscle cells. *J. Steroid. Biochem. Mol. Biol.* 1995; **5**: 219-225.

Raimbach SJ, Thomas AL. Renin and angiotensin converting enzyme concentrations in the fetal and neonatal guinea pig. *J. Physiol.* 1990; **423**: 441-451.

Rapp JP, Wang S-M, Dene H. A genetic polymorphism in the renin gene of Dahl rats cosegregates with blood pressure. *Science* 1989; **243**: 542-544.

Ravelli GP, Stein ZA, Susser MW. Obesity in young men after famine exposure in utero and early infancy. *New Eng. J. Med.* 1976; **295**: 349-353.

Reaven GM, Hofman BB. A role for insulin in the aetiology and course of hypertension? *Lancet* 1987; **2**: 435-437.

Rees WD, Hay SM, Buchan V, Antipas C, Palmer RM. The effects of maternal protein restriction on the growth of the rat fetus and its amino acid supply. *Br. J. Nutr.* 1999; **81**: 243-50.

Rich-Edwards J, Stampfer M, Manson J, Rosner B, Colditz G, Willett W, Speizer F, Hennekens C. Birthweight, breastfeeding and the risk of coronary heart disease in the nurses health study. *Am. J. Epidemiol.* 1995; **141**: s78.

Robertson & Nicolls (eds). The renin-angiotensin system (Gower Press) 1993.

Robinson JS, Owens JA, De Barro T, Lok F, Chidzanju S. Maternal nutrition and fetal growth. Ward RHT, Smith SR, Donnai D (Eds). Early fetal growth and development. 1994; 317-328.

Sato A, Suzuki H, Murakami M, Nakazato Y, Iwaita Y, Saruta T. Glucocorticoid increases angiotensin II type 1 receptor and its gene expression. *Hypertension* 1994;**23**:25-30.

Schalekamp MADH, Derkx FHH, Van den Meiracker AH. Renin inhibitors, angiotensin converting enzyme inhibitors and angiotensin II receptor antagonists: relationships between blood pressure responses and the effects on the renin-angiotensin system. *J. Hypertens.* 1992; **10**: s157-164.

Schelling P, Fischer H, Ganten D. Angiotensin and cell growth: a link to cardiovascular hypertrophy? *J. Hypertens.* 1991; **9**: 3-15.

Scholkens BA, Linz W, Han YK. Heart and vascular wall as targets for tissue converting enzyme inhibition. *Clin. Exp. Hypertens.* 1987; **9**: 427-433.

Schunkert H, Inglefinger JR, Hirsch AT, Pinto Y, Remme WJ, Jacob H, Dzau VJ. Feedback regulation of angiotensin converting enzyme activity and mRNA levels by angiotensin II. *Circ. Res* 1993; **72**: 312-8

Seckl JR. Glucocorticoids, feto-placental 11β-hydroxysteriod dehydrogenase type 2, and the early origins of adult disease. *Steroids*. 1997; **62**: 89-94.

Semple PF. Putative mechanisms of cough after treatment with angiotensin converting enzyme inhibitors. *J. Hypertens. Suppl*. 1995; **13**: S17-21.

Shaper AG, Pocock SJ, Walker M, Cohen NM, Wale CJ, Thomson AG. British Regional Heart study: cardiovascular risk factors in middle aged men in 24 towns. *BMJ* 1981; **283**: 179-86.

Shapiro R, Riordan JF. Inhibition of angiotensin converting enzyme: dependance on chloride. *Biochemistry* 1984; **23**: 5234-5240.

Shubiger G, Flury G, Nussberger J. Enalapril for pregnancy-induced hypertension: Acute renal failure in a neonate. *Ann. Intern. Med.* 1988; **108**: 215-216.

Sloop TC, Clark JC, Rumbaugh RC, Lucier GW. Imprinting of hepatic estrogen-binding proteins by neonatal androgens. *Endocrinology* 1983; **112**: 1639-1645.

Smart JL. Critical periods and brain development. The childhood environment and adult disease. John Wiley & Sons 1991.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD *et al.* Measurement of protein using bicinchonic acid. *Anal. Biochem.* 1985; **150**: 76-85.

Snoeck A, Remacle C, Reusens B, Hoet JJ. Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. *Biol. Neonate* 1990; **57**:107-118.

Soubrier F, Jeunemaitre X, Rigat B, Houot Am, Cambien F, Corvol P. Similar frequencies of renin gene restriction fragment length polymorphisms in hypertensive and normotensive subjects. *Hypertension* 1990; **16**: 712-717.

Stamler J, Stamler R. Blood pressure, systolic and diastolic, and cardiovascular risks. *Arch. Intern. Med.* 1993; **153**: 590-615.

Stewart PM, Corrie JET, Shackleton CHL, Edwards CRW. Syndrome of apparent mineralocorticoid excess: a defect in the cortisol-cortisone shuttle. *J. Clin. Invest.* 1988; **82**: 340-349.

Stein CE, Fall CHD, Osmond C, Cox V, Barker DJP. Fetal growth and coronary heart disease in South India. *Lancet* 1996; **348**: 1269-1273.

Stryer L. Biochemistry. 1988. WH Freeman and company / New York.

Stevenson KM, Gibson KJ, Lumbers ER. Comparison of transplacental transfer of enalapril, captorpil and losartan in sheep. *Br. J. Pharmacol.* 1995; **114**: 1495-1501.

Sunman W, Sever PS. Non-angiotensin effects of angiotensin converting enzyme inhibitors. *Clin. Sci.* 1993; **85**: 661-670.

Swales JD (Editor). Text book of hypertension. 1994. Blackwell Scientific Publications.

Swartz SL, Williams GH, Hollenberg NK, Levine L, Dluhy RG, Moore TJ. Captopril-induced changes in prostaglandin production. *J. Clin. Invest.* 1980; **65**: 1257-64.

Tai H, Yuan B, Sun M. Metabolism of prostaglandins in spontaneously hypertensive rats. *Life Sci*;**24:**1275-1280.

Tang SS, Loscalzo J, Dzau VJ. Tissue plasminogen activator activates renin angiotensin in vitro. *J.Vasc.Med.Biol.* 1989; **1**: 67-74.

Tanner JM. Fetus into man. Castlemead publications. 1989.

Tanner JM, Thomson AM. Standards for birth weight at gestation periods from 32 to 42 weeks allowing for maternal height and weight. *Arch. Dis. Child.* 1970; **45**: 566-569.

Tewksbury DA, Dart RA. High molecular weight angiotensinogen levels in hypertensive pregnant women. *Hypertension.* 1982; **4**: 729-734.

Thurston H, Bing RF, Pohl JEF, Swales JD. Renin subgroups in essential hypertension: an analysis and critique. *Q. J. Med.* 1978; **66**: 325-337.

Timmermans PBMWM, Wong PC, Chiu AT. Angiotensin II receptors and angiotensin II receptor anagonists. *Pharm. Rev.* 1993: **45**: 206-245.

Tsai MY, Brown DM. Effect of dexamethasone on fetal lung 15-hydroxy-prostaglandin dehydrogenase: possible mechanism for the prevention of patent ductus ateriosus by maternal dexamethasone therapy. *Prost. Leuk. Med.* 1987; **27**: 237-245.

Vos PF, Boer P, Braam B, Koomans HA. Efficacy of intrarenal ACE-inhibition estimated from the renal response to angiotensin I and II in humans. *Kid. Int* 1995; **47**: 274-81.

Wagner C, Kurtz A. Regulation of renal renin release. *Curr. Opin. Nephrol. Hypertens.* 1998; **7**: 437-441.

Walton A, Hammond J. The maternal effects on growth and conformation in Shire horse-Shetland pony crosses. *Proc. Royal Soc. B.* 1938; **125**:311-335.

Watson RDS, Stallard TJ, Flinn RM. Factors determining direct arterial pressure and its variability in hypertensive man. *Hypertension* 1980; **2**:303.

Whincup P, Cook D, Papacosta O, Walker M. Birth weight and blood pressure: Cross sectional longitudinal relations in childhood. *BMJ* 1995; **311**: 773-776.

Widdowson EM, McCance RA. The effects of finite periods of undernutrition at different ages on the composistion and subsequent development of the rat. *Proc. R. Soc. Lond.* 1963; **158**: 329-342.

Widdowson EM, McCance RA. A review: new thoughts on growth. *Pediatr. Res.* 1975; **9**: 154-156.

Wilkinson R. Textbook of hypertension. Swales JD (Ed). Blackwell Scientific Publications 1994. 831-857.

Winick M, Noble A. Cellular response in rats during malnutrition at various ages. *J. Nutr.* 1966; **89**: 301-306.

Woodall SM, Johnston BM, Breier BH, Gluckman PD. Chronic maternal undernutrition in the rat leads to delayed postnatal growth and elevated blood pressure in the offspring. *Pediatr Res* 1996;**40**:438-443.

Yamaguchi T, Naito Z, Stoner DG, Franco-Saenz R, Mulrow PJ. Role of the adrenal renin-angiotensin system on adrenocorticotrophic hormone and potassium-stimulated aldosterone production by rat adrenal glomerulosa cells in monolayer culture. *Hypertension* 1990;**16**:635-41.

YamotoT, Noble NA, Millar DE, Border WA. Sustained expression of transforming growth factor-β1 underlies development of progressive kidney fibrosis. *Kidney Int.* 1994; **45**: 916-927.

Zeman FJ. Effects of maternal protein restriction on the kidney of newborn young of rats. *J. Nutr.* 1968; **94**: 111-116.

Zia P, Zipser R, Speckart PF, Horton R. The measurement of urinary prostaglandin E in normal subjects and in high renin states. *J.Lab. Clin.Med.* 1978; **92**: 415-422.

Appendices

Appendix 1

Composition of CRMX laboratory chow diet

Nutrient	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

	Dietary composition (% by weight)	
	18% protein	9% protein
Casein	18	9
Snowflake (cornstarch)	42.5	48.5
Solkafloc (cellulose)	5	5
Sucrose	21.3	24.3
Choline	0.2	0.2
Mineral mix	2	2
Vitamin mix	0.5	0.5
DL-methionine	0.5	0.5
Corn oil	10	10

Appendix 3

Angiotensin II inhibitor

1. Dissolve 0.5g 0-phenanthroline (1,10-phenantholine monohydrate) in 1ml absolute alcohol in a small beaker.
2. Transfer to a 100ml volumetric flask.
3. Wash beaker with a further 1ml alcohol and also add to the volumetric flask.
4. Dissolve 4.64g EDTA (disodium salt) in approximately 60ml boiled distilled water in a beaker (heat gently).
5. Add to 0-phenanthroline slowly with continual mixing.
6. Allow to cool and add 0.2g neomycin sulphate and make up to 100mls with boiled distilled water.

Use 0.5ml of inhibitor for a 10ml blood sample.

Appendix 4

Angiotensin converting enzyme assay reagents

20mM K_2HPO_4, pH 8.3, 0.6M NaCl

3mM Hippuryl-Histidyl-Leucine (HHL)

3% Cyanuric chloride in 1,4 dioxane

Standards 0-500µg/ml Hippuric acid

Appendix 5

Protein assay

Standard: 1mg/ml B.S.A. made up in NaOH (or buffer). Range 0.2mg/ml to 1mg/ml.

BCA solution: 50ml Bicinchoninic acid solution (Sigma: B-9643)

1ml 4% copper sulphate.

Mix these solutions together for 5 minutes then use directly in the assay.

Appendix 6

Plasma renin activity assay

Chemicals

Tris (BDH)

5M Hydrochloric acid

EDTA disodium salt (BDH)

Dextran T70 (Pharmacia)

Norit SX-1 charcoal

Human albumin (Sigma)

[I^{125}] Ile^5 angiotensin I (Dupont)

Angiotensin I antiserum (BPU)

Ile^5 angiotensin I (MRC standards division)

Neomycin sulphate N-1876

Reagents

Tris/EDTA buffer - 3M/200mM, pH7.0 at 37^0C

Tris/HCl buffer - 50mM, pH7.4, at 22^0C

Dextran/charcoal - 2L Tris/HCl buffer. Mix 0.282g dextran into 50mls of buffer. Mix 15g charcoal into rest of buffer. Add two mixtures together and mix.

Antibody trapping mixture - Mix antiserum with 3M Tris (1:150). Take 1ml + add 2ml 3M Tris/EDTA + 6 ml 50mM Tris/HCl.

Angiotensin I standard - 100ng/ml 50mM Tris buffer

[I^{125}]-angiotensin I Label - 5pg/50µl Tris/HCl buffer

Appendix 7

Angiotensin II radioimmunoassay

Chemicals

o-phenanthroline (BDH), neomycin sulphate (Sigma), EDTA disodium salt (BDH), ethanol, Tris (BDH), 5M HCl, human albumin (Sigma), bovine albumin (Sigma), dextran T70 (Pharmacia), Norit SX-1 charcoal, SEP-PAK C18 cartridges (Waters), methanol, Hypertensin (val^5 angiotensin II amide) (Ciba), [I^{125}]-Ile^5 angiotensin II (Dupont), angiotensin II antiserum (BPU).

Reagents

Inhibitor solution - 0.1M EDTA / 0.05M o-phenanthroline

Tris/HCl buffer - 50mM, pH7.4, 22^0C.

Dextran/charcoal - 2L Tris/HCl buffer. Mix 0.282g dextran into 50mls of buffer. Mix 15g charcoal into rest of buffer. Add two mixtures together and mix.

Angiotensin II antiserum - 1:10, 000 in Tris buffer.

Angiotensin II standard - 2ng/ml Tris buffer.

I-125 angiotensin II - 2.5pg/50µl Tris buffer.

Extraction of angiotensin II from plasma

Place SEP-PAK columns on Vac. Elute sps 24 with LP3 tubes for eluate collection. Wash column with 5ml methanol followed by distilled water at 10psi. Load plasma onto columns, and wash through with 5ml distilled water. Elute columns with 80% methanol in distilled water (2ml). Remove tubes containing eluates and place in vacuum centrifuge at 37^0C. Dry samples and store at -20^0C.

Appendix 8

Amersham Prostaglandin Enzymeimmunoassay systems

Components of the assay systems

Microtitre titre plate - 96 wells, coated with IgG.

Prostaglandin conjugate

Standard

Antibody

Assay buffer concentrate

TMB (3,3',5,5'-tetramethylbenzidine) substrate

Wash buffer concentrate

Appendix 9

Creatinine assay

Chemicals and reagents

Fullers earth (Sigma)

Oxalic acid 18g/dl, saturated

Alkaline Picrate - equal volumes of each 0.6N NaOH + 1% 0.04M Picric acid (prepare just before use).

Standards - Stock =100mg creatinine (Sigma) + 0.8ml concentrated HCl made up to 100ml with distilled water. Standards 0-400μg/ml.