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REVIEW

The key role of nitric oxide in hypoxia:

hypoxic vasodilation and energy supply-demand matching

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Abstract

SIGNIFICANCE: A mismatch between energy supply and demand induces tissue hypoxia with the potential to cause cell death and organ failure. Whenever arterial oxygen concentration is reduced, increases in blood flow - 'hypoxic vasodilation' - occur in an attempt to restore oxygen supply. Nitric oxide is a major signalling and effector molecule mediating the body's response to hypoxia, given its unique characteristics of vasodilation (improving blood flow and oxygen supply) and modulation of energetic metabolism (reducing oxygen consumption and promoting utilization of alternative pathways).

RECENT ADVANCES: This review covers the role of oxygen in metabolism and responses to hypoxia, the hemodynamic and metabolic effects of nitric oxide, and mechanisms underlying the involvement of nitric oxide in hypoxic vasodilation. Recent insights into nitric oxide metabolism will be discussed, including the role for dietary intake of nitrate, endogenous nitrite reductases, and release of nitric oxide from storage pools. The processes through which nitric oxide levels are elevated during hypoxia are presented, namely (i) increased synthesis from nitric oxide synthases, increased reduction of nitrite to nitric oxide by heme- or pterin-based enzymes and increased release from nitric oxide stores, and (ii) reduced deactivation by mitochondrial cytochrome c oxidase.

CRITICAL ISSUES: Several reviews covered modulation of energetic metabolism by nitric oxide, while here we highlight the crucial role NO plays in achieving cardiocirculatory homeostasis during acute hypoxia through both vasodilation and metabolic suppression

FUTURE DIRECTIONS: We identify a key position for nitric oxide in the body's adaptation to an acute energy supply-demand mismatch.

Keywords: Hypoxic vasodilation; nitric oxide; oxygen supply-demand balance

Introduction

Patients undergoing major surgery or suffering critical illness have increased cellular energy demands yet commonly experience challenges in ensuring an adequate oxygen supply. An oxygen supply-demand imbalance can be immediately life-threatening, such as in cardiac arrest or major haemorrhage, or it may cause more insidious damage. As organ function is critically linked to both oxygen availability (30) and adequate utilization, bioenergetic deficiency is likely to be a crucial factor underlying the pathogenesis of cell death (39) and multiple organ failure (18,202).

The main purpose of the cardiovascular system is to provide oxygen and metabolic substrates at a rate that can both meet and respond rapidly to changes in local demand. Whenever arterial oxygen concentration is reduced, increases in local blood flow - 'hypoxic vasodilation' - occur in an attempt to restore oxygen supply (102). The ability of hypoxia to increase tissue blood flow is a local response that can be demonstrated in isolated organs, thus it can occur without the involvement of neurally-mediated reflex mechanisms. A variety of mediators such as adenosine (22), ATP-sensitive potassium channels (65) and prostaglandins (173) are implicated, however significant human and animal data point towards nitric oxide (NO) as a major regulator of vascular perfusion and matching of energy supply and demand (24,25,76,119,147,190). Nevertheless, considerable controversy persists with respect to its sources and mechanisms of action.

Several reviews have covered modulation of energetic metabolism by nitric oxide (33,219,232). In this article we highlight the crucial role NO plays in achieving cardiocirculatory homeostasis during acute hypoxia through both vasodilation and metabolic suppression, and the various mechanisms through which NO production is enhanced. We identify a key position for nitric oxide in the body's adaptation to an acute energy supply-demand mismatch.

Bioenergetic reactions and the role of oxygen

Living cells constantly perform work to maintain their structures, synthesize cellular components, generate transmembrane ionic gradients, and to undertake their physiologic roles. As most of these metabolic processes are thermodynamically unfavorable, they are coupled directly or indirectly to the hydrolysis of ATP to provide the necessary free energy. ATP supply is maintained through mitochondrial and cytoplasmic reactions that proceed with and without the need for oxygen (32). With its net yield of 2 moles ATP per mole of glucose, glycolysis is generally insufficient to maintain steady-state energy levels in most cell types. Exceptionally, some cells (e.g. erythrocytes) rely solely upon glycolytic production of ATP. Many immune cells use glucose and glutamine as their primary fuel sources, although ketone bodies and fatty acids can be used to a lesser degree (168). Glucose appears to be particularly necessary for cell survival, size, activation and cytokine production. Resting lymphocytes have low-energy needs and derive most of their ATP from oxidative phosphorylation; switching to the activated state requires a dramatic increase in metabolism, which is mainly derived from rapid upregulation of glycolysis (80). Organs also vary in their predominant energy substrate. Whereas most utilize carbohydrates as their major energy source, some (e.g. cardiomyocytes) predominantly use fatty acids. Notably, this preference can change with a reduction in oxygen availability.

Pathways leading to ATP synthesis have been extensively studied (145). Briefly, the glycolytic end-product pyruvate, and fatty acids enter mitochondria where they are metabolized to acetyl CoA. This enters the tricarboxylic acid (Krebs') cycle whereby oxidation reactions directly produce ATP equivalents and electron donors. Electrons are transported down the respiratory chain, generating a proton gradient across the inner mitochondrial membrane, which serves as the driving force for phosphorylation of ADP to ATP. By comparison with glycolysis, 1 mole of glucose liberates approximately 30 moles of

ATP. Oxygen is crucially required as the terminal electron acceptor by the last complex of the chain, cytochrome c oxidase (CcO, complex IV) (44). In the steady state, approximately 90% of total body O_2 consumption occurs within mitochondria (204), and is primarily directed towards oxidative phosphorylation. A constant O_2 supply is thus critical for continued cell function and survival. When compromised (e.g. supply reduced and/or demand excessively increased), a state of metabolic crisis ensues (231) with potential activation of cell death pathways.

Oxygen and nutrients required by mammalian cells to support metabolism cannot be directly obtained in sufficient quantity by diffusion alone. From an evolutionary perspective, this limitation was resolved by the development of a cardio-respiratory system whose activity is closely regulated such that, in the steady state, ventilation delivers oxygen to the alveolar capillaries at the same rate of delivery by the vasculature to the tissues, and of consumption by metabolic processes within the tissues (233). The quantity of oxygen delivered to tissues (DO_2) depends on arterial oxygen content (mainly carried by hemoglobin) and cardiac output. Regulation and distribution of cardiac output is driven by regional O_2 consumption (VO_2) that proceeds at a rate set by tissue metabolic activity. If DO_2 is reduced, VO_2 is initially maintained by increased O_2 extraction. If delivery is reduced further, a critical point is reached below which tissue extraction cannot increase any further, leading to a fall in VO_2 (212). Several complementary macro- and microcirculatory mechanisms act to prevent the onset of tissue hypoxia in the face of a reduced DO_2. These include a redistribution of blood flow to 'vital' organs, increased recruitment of perfused microvessels to facilitate O_2 availability (233), as depicted in figure 1 and, as discussed later, a decrease in tissue utilization (metabolism)

Sensing hypoxia

How eukaryotic cells sense reductions in pO_2 remains contentious. Five main mechanisms have been proposed (132) based on, respectively, heme-based proteins, O_2-sensitive ion

channels, AMP kinase, NADPH oxidase and mitochondria. Thus, hypoxia may be detected by an allosteric shift towards a deoxy- configuration in proteins capable of reversibly binding O_2 at a heme site, or by ion channels affected by local pO_2, as has been shown in carotid body cells where hypoxia can inhibit a specific K^+ current. Reduced O_2 levels increase the AMP:ATP ratio thus, at sufficient magnitude, AMP-activated protein kinases (AMPK) are induced, modulating cellular metabolism at various levels via target protein phosphorylation. With molecular O_2, NADPH oxidase or other non-mitochondrial enzymes such as xanthine oxidase or flavin-containing dehydrogenases generate superoxide ($O_2^{\cdot-}$), providing a second messenger that may regulate cellular activity through redox modifications. Finally, increased mitochondrial reactive oxygen species (ROS) production during hypoxia may result in mitochondrial O_2 sensing through changes in redox state of the electron transport chain, though without necessarily affecting respiration (50,72,96). Mitochondria may play a critical role in oxygen sensing. This model has been controversial as previous studies, which mainly relied on pharmacologic tools, produced conflicting reports (223). However, recent studies using genetic and biochemical approaches have provided evidence for a role of mitochondrial reactive oxygen species (mROS) in oxygen sensing and hypoxia-inducible transcription factor-1 (HIF-1α) activation (38). Indeed, blocking superoxide anion production by suppressing the Rieske iron-sulfur protein of complex III impairs HIF-1α induction by hypoxia, whereas hydrogen peroxide or agents that produce ROS activate HIF-1α during normoxia (95). These data indicate that mitochondria can function as O_2 sensors and stabilize HIF-1α during hypoxia by releasing ROS to the cytosol (96).

The hypoxia-inducible transcription factor 1 (HIF-1) pathway is central to the body's innate response to the stressful condition of hypoxia. HIF is a heterodimer composed of α and β subunits that induces expression of multiple genes that promote adaptation and survival (214). The β subunit is constitutively expressed, while α subunit expression is tightly regulated by the local oxygen tension through the action of prolyl hydroxylase (PHD). When O_2 tension falls below a critical threshold, proline residues cannot be hydroxylated. This

prevents ubiquitination, allowing the α-subunit to accumulate and hetero-dimerize with HIF-1β. The heterodimer can then bind to specific DNA regions within the nucleus, exerting its regulatory activities. The PHDs are considered effective O_2 sensors in their own right as their Km values for oxygen are above atmospheric O_2 concentrations (110). This allows small changes in O_2 supply to affect the enzyme's activity.

For responses to occur, a decrease in PO_2 must be detected by an O_2 sensor that activates signalling pathways triggering functional responses. In general, adaptation to acute changes in O_2 concentration (lasting from seconds to minutes) principally occur as a result of alterations of pre-existing proteins (e.g. involving phosphorylation or changes in redox state), whereas chronic changes (lasting from minutes to hours or longer) mainly occur through altered gene expression.

Responding to hypoxia

(a) Transcriptomic

Given oxygen's essential role in cellular metabolism, a wide array of responses has evolved to cope with situations of oxygen supply-demand mismatch. Functional adaptation occurs at systemic, tissue and cellular levels, ultimately leading to a new phenotype that enhances the likelihood of survival (211). Such adaptation depends both on the modulation of activity of various enzymatic systems by metabolic messengers (e.g. pH, phosphate potential, redox potential), and on altered gene transcription with increased expression of genes encoding, for example, growth factors (e.g. VEGF, PDGF-β), cytokines (e.g. IL-1, IL-8), endothelin and adhesion molecules (e.g. VCAM-1, ICAM-1).

The AMP kinase (AMPK) system is a well-conserved pathway for maintaining the balance between energy production and utilization (103). Triggered by an increase in AMP:ATP ratio, this system switches on an energy-preserving phenotype, both rapidly through

phosphorylating metabolic enzymes, and by a longer-term adaptation through regulating gene expression via phosphorylation of transcription factors and co-activators. AMPK targets include carbohydrate homeostasis, lipid metabolism, protein synthesis, mitochondrial biogenesis, cell signalling, proliferation, gene expression and transmembrane ion transport (128).

Responses to hypoxia that involve induction or repression of gene expression are mainly mediated by HIF-1 (214). Three isoforms of HIFα have been characterized, of which HIF-1α and HIF-2α are the most structurally similar and best studied. HIF-3α can be found as multiple splice variants, some of which can even inhibit activity of HIF-1α and HIF-2α (169). While HIF-1α is expressed ubiquitously in all cells, the other isoforms are only selectively expressed in certain tissues, such as vascular endothelium, lungs and kidney. Activation of HIF-1 and HIF-2 can regulate expression of many other genes induced by hypoxia, such as vascular endothelial growth factor (VEGF), a potent angiogenic factor that contributes to long-term adaptation to hypoxia through new blood vessel formation (130). However, each HIF isoform may have their unique targets offering different adaptive pathways to hypoxia (160); HIF-1 preferentially induces genes coding for the glycolytic pathway whereas HIF-2 is involved in regulation of genes important for cell cycle progression and induction of erythropoietin (161). The two HIF isoforms also have distinct and somewhat opposing roles to NO regulation in macrophages. While HIF-1 promotes iNOS expression and increases NO production, HIF-2 promotes arginase expression, reducing the amount of arginine available for NO synthesis (229). This may offer a balancing regulatory mechanism for NO homeostasis.

Apart from O_2 tension many other factors govern HIFα stability, including microRNAs and post-translational modifications such as acetylation (91). In addition to O_2, PHDs require Fe^{2+}, 2-oxoglutarate and ascorbate to exert their activity, but may be inhibited by NO, Krebs'

cycle intermediates and reactive oxygen species (126). To date, >200 HIF gene targets have been identified, including those encoding for proteins involved in angiogenesis, energy metabolism, erythropoiesis, cell proliferation and viability, vascular remodelling, and vasomotor responses (214).

MicroRNAs, specific small, non-coding RNA sequences, also appear to be involved in the hypoxic response (140). These 19- to 24-ribonucleotide sequences, once transferred to the cytoplasm, inhibit target gene expression by translational repression and/or mRNA degradation. A common characteristic of the different microRNAs involved in hypoxic signalling is their dependence upon HIF (141). HIF may thus be the main modulator of the hypoxic response, either through direct gene induction, or by indirect microRNA-mediated gene repression (140). On the other hand, microRNAs may act as positive and negative feedback regulators of HIF-mediated responses (106). miR-210 is consistently upregulated in hypoxia and may play a central role in hypoxic signalling by modulating factors implicated in various pathways, e.g. downregulating expression of different components of the mitochondrial electron transport chain and the Krebs' cycle, interfering with membrane trafficking, modulating migration and adhesion, differentiation and cell cycle (67).

b) Hemodynamic effects

At the systemic level, adaptation to hypoxemia affects many systems although the most evident changes involve the cardiocirculatory system. This response is composed of essentially unopposed local vasodilation in the heart and brain, and of a balance between the competing effects of locally-induced vasodilation and reflex chemoreceptor-sensed, sympathetic-mediated vasoconstriction in other tissues, e.g. kidney and skeletal muscle (206). This response pattern attempts to maintain an adequate O_2 supply-demand ratio, compensating for any reduction in arterial O_2 content, while preserving arterial perfusion pressure. Over 130 years ago, Roy and Brown recognized that interrupting tissue perfusion

produced a local, non neurally-mediated increase in blood flow, and that blood vessels could vary their diameter independently in response to local metabolic needs (208). Indeed, the main hemodynamic effect of hypoxia is systemic vasodilation with increases in cardiac output and heart rate, and redistribution of regional perfusion with increased coronary, carotid and hepatic and decreased renal blood flow. The sympathetic nervous system is activated with increased catecholamine levels but a reduced response to exogenous vasopressors (55,98,104,156,194,206,207). Increases in forearm blood flow, a reduced vasomotor reflex and a reduced response to exogenous norepinephrine and angiotensin were noted in healthy volunteers made hypoxemic (104).

c) Respiratory

Haldane noted how hypoxia induced a rapid, shallow type of breathing in humans (101). The predominant ventilatory response is an increase in respiratory rate with a rise in bronchiolar tone (127), a response mainly mediated via hypoxemia-responsive peripheral chemoreceptors in the carotid and aortic bodies.

d) Renal and endocrine

Hypoxemia redistributes blood flow away from the kidney which, in response, increases blood volume via an antidiuretic and sodium-sparing effect (20,183). In volunteers breathing 10.5% O_2, blood pressure fell by 10% and urine output by 30% (109), yet vasopressin and cortisol levels significantly increased while urine osmolality more than doubled.

e) Metabolic

Mammalian cells undergo multiple adaptive modifications of metabolism in response to changes in O_2 availability (184,215). Some organs with high metabolic demand (e.g. muscle, liver, brain, heart) cope with the initial energy imbalance through glycogenolysis,

phosphocreatine dephosphorylation and the adenylate kinase reaction (4). Moreover, a switch in fuel selection from lipid to carbohydrate oxidation optimizes the rate of ATP production by taking advantage of the higher ATP yield per mole of O_2 consumed (94,113).

ATP produced from fatty acid oxidation is strictly dependent upon the presence of oxygen. In contrast, glucose-derived ATP originates both from oxygen-dependent glycolysis and on glucose oxidation. During hypoxia, both fatty acid and glucose oxidation decrease, thereby increasing the importance of glycolytic-derived ATP which, in most cell types, only plays a minor role in normoxic conditions (182). However, in order to regenerate NAD^+, pyruvate produced from glycolysis is converted to lactate rather than being utilized within the mitochondria, a process that ultimately depends on the presence of O_2 as the terminal electron acceptor. Moreover, hydrogen ions. generated by the hydrolysis of glycolytic-derived ATP, accumulate as these are not taken up by the mitochondria, and will eventually result in a fall in intracellular pH (210). Hypoxia, besides causing a critical reduction of oxygen availability for oxidative phosphorylation, also affects other mitochondrial processes including a decrease in Complex I-dependent respiration, and reversal of the direction of operation of the F_0F_1-ATPase (Complex V). This latter effect converts mitochondria into major ATP consumers as they attempt to restore and maintain membrane potential to prevent increased mitochondrial permeability transition and cell death (68).

During hypoxia, expression and activity of carbohydrate transporters, and of enzymes involved in glycogenolysis and glycolysis are increased (215), while pyruvate dehydrogenase activity is inhibited (188). The net effect is a shunting of pyruvate away from mitochondria and an increase in glucose availability and glycolytic flux - the ‘Pasteur effect’. Hypoxia also realigns the subunit composition of cytochrome c oxidase (CcO), improving the efficiency of respiration (82). Furthermore, accumulation of the glycolytic intermediate, fructose 1,6-biphosphate, directly inhibits mitochondrial respiration (69), again linking an increase in glycolytic flux to decreases in O_2 consumption.

Further adaptation to hypoxia is achieved through metabolic suppression, measured as a decrease in mitochondrial O_2 consumption during hypoxia. This oxygen conformance (111) is recognized, at least to some extent, in human heart and in hepatocytes. It begins at partial pressures of O_2 above the critical level at which diffusion limitation into the mitochondria affects oxidative phosphorylation (31). In addition, a reallocation of cellular energy between essential and non-essential ATP-demanding processes provides further defense against the energy mismatch. ATP-consuming processes are arranged in a hierarchy, with processes less critical for cell survival being first sacrificed (30). This is mainly achieved at the level of the two principal ATP consumers: ion pumps and protein synthesis. Hypoxia reversibly suppresses Na^+/K^+ ATPase activity and inhibits mRNA translation through multiple mechanisms (244). Indeed, a modified phenotype for adaptive hypoxia tolerance is expressed in indigenous highlander human populations (Quechuas, Sherpas and Tibetans), whereby improved coupling between ATP demand and supply pathways protects against imbalances due to environmental O_2 limitation (112). Similarly, NO was increased in lowlanders acclimatizing to altitude; this was associated with changes in microcirculatory blood flow which increased local tissue oxygen delivery, in agreement with an adaptative role in hypoxia (147).

Nitric oxide synthesis and metabolism

a) Production by nitric oxide synthases

A large proportion of NO synthesis occurs through the L-arginine-NO pathway (177), one of the main metabolic pathways for arginine (246) (as depicted in figure 2). With these reactions, the semi-essential amino acid L-arginine is metabolized to NO and L-citrulline by a family of nitric oxide synthases (NOS) (135). These enzymes are dimers formed by two monomers consisting of a flavin-containing reductase domain and a heme-containing

oxygenase domain. The NO synthesis reaction requires the presence of two oxygen molecules plus NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin as coenzymes/cofactors (29). NOS may be 'uncoupled' in the absence (or reduced availability) of either L-arginine or tetrahydrobiopterin, or in the presence of the endogenous NOS inhibitor, asymmetric dimethylarginine (ADMA) (227,239). Here, electrons flowing from the reductase to the oxygenase domain are diverted to molecular O_2 rather than to L-arginine, eventually resulting in production of superoxide rather than NO (239,247). Three isoforms of NOS have been characterized (135). The endothelial (eNOS) and neuronal (nNOS) isoforms are constitutively expressed. Although initially isolated in vascular endothelium and the nervous system, they have since been found in skeletal muscle, lung and liver and, recently, an eNOS-like NO producing machinery has been described in erythrocytes (134). These constitutive isoforms can rapidly increase NO production. In health, they play important regulatory roles in neurotransmission and the cardiovascular system. Production of the inducible isoform (iNOS) depends on transcription; several hours are needed to reach peak activity. Its gene transcript increases on exposure to pro-inflammatory cytokines and bacterial products. In general, iNOS generates larger (nanomolar vs picomolar) quantities of NO compared to its constitutive isoforms (5). This is related to the amount of protein expressed. The existence of a specific mitochondrial variant of NOS (mtNOS) has been claimed (88,142). However, others challenge its existence as no specific mtNOS sequence has yet been found in mitochondrial DNA, nor has any pathway enabling NOS protein transport into these organelles been identified, nor could it be found when specifically sought (142).

This picture is further complicated by the ability of most cells to express multiple isoforms in different compartments. In the heart, for instance, nNOS is expressed within the sarcoplasmic reticulum and its activation increases contractility, whereas eNOS is confined to the caveolae and inhibits β-adrenoreceptor-mediated increases in contractility (16). Moreover, constitutively expressed NOS can be modulated by post-translational

modifications and phosphorylation (216) or be induced (135), while iNOS may be constitutively expressed at low levels in some tissues (189).

b) Dietary nitrite/nitrate intake

Though traditionally considered as inert oxidative end-products, nitrate (NO_3^-) and nitrite (NO_2^-) may play an important role in NO homeostasis (164). Diet is a major source of NO_3^- with particularly high levels in leafy green vegetables. An average serving of beetroot contains more nitrate than is endogenously generated per day from NO generated by all three NOS isoforms combined. Once absorbed, most nitrate is ultimately excreted in urine. However, up to 25% is taken up by salivary glands and concentrated in the saliva where it reaches levels 10-fold higher than in plasma. Facultative anerobe bacteria within the oral cavity then reduce it to nitrite while using it as an alternative electron acceptor to O_2 during respiration (165). Nitrite-rich saliva is then swallowed where, within the acidic milieu of the gastric lumen, NO_2^- is rapidly protonated to nitrous acid that further decomposes to NO. This is termed the “nitrate-nitrite-nitric oxide pathway” (166) (figure 3).

Orally administered nitrate or nitrite can modulate the endogenous NO system in various physiologic and pathophysiologic conditions (41,241,249). Significant increases in plasma NO_3^- and NO_2^- levels were measured in human volunteers after beetroot juice ingestion, with lowering of arterial BP by about 10 mmHg. This was prevented by interrupting the enterosalivary circulation through non-swallowing of saliva (241), or by selective suppression of the oral microflora with an antiseptic mouthwash (193).

c) Generation by other pathways:

(i) Systemic nitrite reduction

Endogenously-formed or dietary nitrite in blood and tissues may be recycled to form NO-like bioactive molecules (Figure 3). In a human forearm blood flow study (90), Gladwin *et al* found that of the various plasma NO-related species, only NO_2^- had a significant arterial-venous gradient, indicating a degree of consumption during circulatory transit, and thus suggestive of possible bioactivity. This gradient markedly increased with exercise and inhibition of regional NO synthesis, suggesting that NO_2^- is a plasma carrier of NO bioequivalents that are peripherally converted into bioactive NO. However, these results could not be replicated by Lauer *et al* (144) who reported no vasodilatory effect following intra-arterial administration of $NaNO_2$ into healthy volunteers. This may relate to a shorter duration of nitrite infusion, or the need for nitrite to undergo metabolic conversion before becoming vasoactive. A careful comparison of the route of administration, the concentration and total dose of nitrite is needed to better understand its role (45).

Various possible *in vivo* pathways by which nitrite is reduced to NO have been investigated (figure 4). Nitrite can form NO non-enzymatically under acidic and/or ischemic conditions (251). Using ^{15}N-labelled nitrite to identify the source of NO, and enzyme inhibitors to exclude other pathways, NO generation was explained by a reaction of spontaneous disproportionation; however, the *in vivo* relevance of this pathway remains uncertain. Dedicated nitrite reductases are present in bacteria, but are lacking in humans. Nonetheless, certain mammalian enzymes show some nitrite reductase activity beyond their normal physiologic function. As an alternative to non-enzymatic reduction, proteins from the heme-globin family (89) or from pterin-based molybdenum enzymes (149) may catalyze the NO_2^--reductase reaction to NO. An *in vitro* reaction of NO_2^- with human deoxyhemoglobin forms NO and methemoglobin while an intra-arterial nitrite infusion produces, after several circulation times, a vasodilatory effect in healthy volunteers (60). Myoglobin, both in the heart and vasculature(105,185), also has significant NO_2^--reductase activity, as do heme protein-containing enzymes such as the mitochondrial electron transport chain cytochromes (17,49,138), the cytochrome P450 family of microsomal heme proteins (151), and aldehyde

dehydrogenase (ALDH2), a mitochondrial enzyme involved in ethanol inactivation that has also been linked to the bioactivation of organic nitrates (13,51). Even eNOS and soluble guanylate cyclase (sGC), being heme-based enzymes, may have possible nitrite reductase activity, thereby offering an important alternative source of NO outside the conventional L-arginine pathway (8,87).

Given that Hb is an effective scavenger of NO, the possibility of a heme-independent pathway of NO synthesis from nitrite merits consideration as the *in vivo* relevance of nitrite reduction by heme-based enzymes may be challenged by the need for the newly synthesized NO to escape from this scavenging. The two most studied NO_2^--reducing molybdenum-based enzymes are xanthine oxidoreductase (XOR) and aldehyde oxidase (149). The former plays a critical role in purine and pyrimidine catabolism, catalyzing oxidation of hypoxanthine to xanthine, and xanthine to uric acid. As it also reduces O_2 to H_2O_2 and $O_2^{\cdot-}$ XOR is a key enzyme in the process of oxidative injury. XOR can catalyze reduction of NO_3^- to NO_2^-, and NO_2^- to NO under anerobic conditions; this can be blocked by the XOR-inhibitor, oxypurinol (152). Similarly, aldehyde oxidase, a cytosolic enzyme involved in biotransformation of drugs and xenobiotics, also has significant *in vitro* NO_2^--reductase activity (150).

(ii) Release from pre-formed storage pools

Attempting to solve the apparent paradox of the NO scavenging process being too rapid and effective to allow this short half-life molecule to exert its physiologic effects, it has been suggested that, depending on the oxygenation state of Hb, NO may either react with oxyHb to be oxidized to NO_3^-, or can bind to the deoxygenated form to generate a nitrosyl-adduct (NO-Hb) that subsequently reacts with thiol groups to produce S-nitrosohemoglobin (SNO-Hb) (121,191,225). SNO-Hb is more stable and has a longer half-life than NO, and its

administration can evoke a hypotensive response suggesting it acts as both carrier and donor of NO bioequivalents (figure 3).

Other reservoirs of potential nitric oxide bioactivity include S-nitrosoalbumin (224), tissue nitrite, S-nitrosothiols (RSNO), N-nitrosamines (RNNO), and dinitrosyl iron complexes (DNIC). Their concentrations vary in different pathophysiologic states, typically showing marked elevation compared to basal levels in acute inflammation and reduction in the more chronic setting. Although little is known about their *in vivo* relevance, they may act as signalling molecules or storage forms of NO (43,73,237). Similar NO storage forms are found in other compartments such as the vascular wall (203).

Regardless of location, all these compounds may be activated to release NO under certain conditions, and to contribute to the body pool of NO-related metabolites. Opinion still remains divided as to the *in vivo* importance of these mechanisms (90,118,198). A mitochondria-targeted S-nitrosothiol was recently shown to selectively induce NO production and S-nitrosylation (addition of an NO^+ group to a protein thiol to form a nitrosothiol) at the mitochondrial level, producing vascular relaxation of pre-contracted aortic rings and protecting against ischemia-reperfusion (197). Molecules able to enhance trans-nitrosation reactions, transferring NO from one cysteine residue to another, constitute an emerging area of research in the field of drug design (86).

d) NO metabolism

The *in vivo* fate of NO is highly complex; several catabolic pathways exist (figure 5), with varying relevance in different body compartments (27,131). *In vitro*, NO rapidly reacts with O_2 to form nitrogen dioxide (NO_2). In the presence of NO at low concentration, the latter reacts with water to form equal amounts of nitrite and nitrate. At higher NO concentrations, NO_2 reacts with another NO molecule to form dinitrogen trioxide (N_2O_3), which hydrolyses to form nitrite. In plasma, in the presence of O_2, the principal reaction is formation of NO_2^-

(115). Whether this is through autoxidation of NO, reaction with the plasma multi-copper oxidase ceruloplasmin (220), or oxidation by the mitochondrial cytochrome c oxidase in vascular cells is currently unclear (192,218,230). The situation differs in whole blood where the relatively high amount of oxyHb favours biotransformation of NO to NO_3^- with concomitant formation of methemoglobin (27). This Hb reaction is considered by some as the primary catabolic process responsible for NO removal (107,124). A similar reaction with oxymyoglobin (to generate metmyoglobin and NO_3^-) has been recently proposed as a crucial regulatory step of NO inactivation in muscle (40,79). However, other studies have shown that this reaction only takes place in conditions of excess NO (192).

NO can also react with superoxide to produce peroxynitrite ($ONOO^-$). The rapidity of this reaction, some 3-4-fold faster than $O_2^{\cdot -}$ dismutation by superoxide dismutase, makes $ONOO^-$ formation a major potential disposal pathway of NO reactivity (92), though this does depend on the rate of tissue superoxide production. $ONOO^-$ itself may trigger oxidation or nitration reactions with various cellular targets modulating their biological activities, and is eventually converted into nitrate or nitrite (228).

Nitrate and nitrite were long considered stable end-products of NO catabolism, however both are now recognized to be subject to further biotransformation (78,131,226). Highly reactive NO by-products (reactive nitrogen species) can react with protein thiol (-SH) groups to form S-nitrosothiols such as S-nitrosoalbumin, S-nitrosoglutathione and S-nitroso-hemoglobin (131), or with amines to generate N-nitrosamines. The physiologic significance of RNNOs is presently unknown, but concentrations change rapidly in response to an acute oxygen shortage (43). NO itself can directly react with metals to generate metal nitrosyls, e.g. NO-Hb. In addition to nitrosation, RSNOs may also be produced by oxidative nitrosylation. This reaction is mediated by generation of thiyl radicals ($RS^{\cdot}$) that may be derived from interaction of thiols with oxidants such as peroxynitrite (43). NO metabolism changes under hypoxic conditions, with greater production of metal nitrosyls, RSNOs and RNNOs (180). Some of

these products share some of the biologic properties of NO (226) and may possess the important biological functions of storing and transporting NO. Moreover, S-nitrosylation of thiol groups is a widespread post-translational redox-based protein modification. Similar to phosphorlyation, this exerts control over many protein classes in various physiologic and pathophysiologic conditions (108,157).

e) Half-life of NO

The process of NO transfer to its target remains incompletely understood. NO has high reactivity and a very short half-life, with measured blood levels being too low to likely exert any physiologically relevant effect (159). From *in vitro* studies, the half-life ranges from as little as 10^{-6} seconds to as much as 11.5 seconds (100). Mathematical modelling estimates an *in vivo* half-life of about 2 ms (158). Such a short half-life plus the rapid intravascular scavenging of NO has to be reconciled with the prominent autocrine and paracrine roles this molecule is believed to play in cellular physiology. Liao *et al* (154) proposed the existence of intravascular erythrocyte-free zones generated by blood flow that may increase the NO half-life by several orders of magnitude due to reduced local NO scavenging, thus allowing the molecule to exert its biologic functions. Another possibility is the concept of stored NO bioactivity (66,221), as outlined above.

To summarize, referring to the half-life of free NO may no longer be relevant given the rapidity of its transformation and interchange between different metabolites, many of which are longer lived than the parent molecule. A more complex system appears to be in place, in which the short half-life of NO itself is important in limiting the action of the molecule to its site of formation and to enable local signalling (autocrine and paracrine levels), while downstream biotransformation reactions make economical use of the NO produced as well as contributing to distant signalling (endocrine level).

Nitric oxide and cardiovascular homeostasis

a) Nitric oxide as a regulator of vascular homeostasis

NO is a core physiologic regulator of many cardiovascular processes, including platelet aggregation and adhesion, myocardial contractility, vascular permeability and tone (187,243). NO is essential for both global regulation and regional distribution of blood flow and pressure. Dysregulation of the NO system thus likely plays a fundamental role in many pathophysiologic conditions ranging from essential hypertension and atherosclerosis to the hypotension seen in acute shock states (243).

Nitric oxide is a key contributor to new vessel formation: in endothelial cells, VEGF induced NO production via eNOS that, in turn, mediated angiogenesis (83). iNOS-derived NO may also have a role in angiogenesis (178). Besides being an effector of VEGF activation, NO also enhances growth factor synthesis in numerous cell types, mimicking the classical hypoxic stimulus (71). Thus, NO appears to act both as an upstream and a downstream mediator of VEGF-dependent angiogenesis.

b) Mechanisms underlying the vasodilatory action of NO

NO regulates blood pressure and flow through its effects on vascular smooth muscle tone. The shear stress generated by flowing blood against the endothelial surface triggers production of NO both basally (114), and in response to mechanostimulation (222). This increase in NO production is nonlinear with respect to shear stress (14). Of note, laminar blood flow (which increases shear stress) increases NO production whereas disturbed flow (causing low and oscillating shear stress) inhibits release of NO and fails to upregulate NOS (58).

Being small and lipophilic, NO rapidly diffuses across membranes to reach vascular smooth muscle cells. Its main mechanism of action is mediated by nitrosylation of the heme-iron within sGC, leading to increased synthesis of cyclic GMP (cGMP) (243). This, in turn,

activates protein kinases that modulate myosin light chain kinase and phosphatase activities, resulting in less phosphorylation of myosin and, eventually, vasorelaxation (143). NO can also cause vasodilation via cGMP-mediated opening of calcium-sensitive (K_{Ca}) (10) and ATP-sensitive (K_{ATP}) (179) potassium channels. When these ion channels open, the outward efflux of potassium hyperpolarizes the plasma membrane, reducing vascular tone. NO also activates K_{ATP} and K_{Ca} channels in a cGMP-independent manner through direct S-nitrosylation (28,129). NO may contribute to the regulation of intracellular free Ca^{2+} levels, either via cGMP-dependent inhibition of calcium influx through L-type Ca^{2+} channels (23), and/or via increased Ca^{2+} removal from the cytoplasm. The latter can occur by accelerating the Na^{+}/Ca^{2+} exchanger (84), or by increasing sequestration into intracellular stores via the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (2,56). This effect may be important as the constitutive isoforms of NOS are calcium-calmodulin-regulated (216). An increase in intracellular Ca^{2+} activates calmodulin leading to NO synthesis. Thus, it could be postulated that reduced intracellular levels of free calcium resulting from increased NO levels could reduce the amount of iNOS-generated NO, contributing to the fine tuning of NO levels. (figure 6)

In health, eNOS mediates most of the hemodynamic actions of NO, contributing to blood flow regulation between different vascular beds according to their varying metabolic needs (177,243). NO derived from iNOS is traditionally considered to be primarily responsible for the vascular hyporeactivity and hypotension seen in inflammatory states (235). However, emerging data indicate an important role for nNOS-derived NO in basal microvascular tone regulation while eNOS-derived NO regulates changes in tone in response to agonists or shear stress (171). This suggests a potentially independent regulation of basal and stimulated blood flow.

Hypoxic vasodilation to improve coupling of oxygen delivery and utilization

(a) Evidence for the involvement of NO

Under hypoxemic conditions, a vasodilatory response occurs, augmenting blood flow in an attempt to maintain O_2 delivery. Given its importance in the regulation of cardiovascular homeostasis, NO likely plays a key role. An association between NO and hypoxic vasodilation was first described in 1989 (196). This response was endothelium-dependent and significantly reduced by administration of oxyHb, acting as a potent NO scavenger. Ten-fold higher levels of circulating NO products were found in residents of the Tibetan plateau compared to sea-level dwellers (76). This was associated with increased resting forearm blood flow suggesting an adaptive role that offsets the O_2 lack caused by high altitude. In volunteers hypoxemia-induced increases in forearm blood flow were blocked by the non-specific NOS inhibitor, L-NMMA (24). In awake sheep, hypoxemia-induced increases in cerebral blood flow and falls in cerebral vascular resistance were reversed by the sGC inhibitor, methylene blue (119).

(b) Alternative theories

Apart from its role as an energy source for cellular metabolic activity, ATP has important signaling characteristics, particularly in situations of reduced energy supply such as hypoxia. ATP release occurs in all major cell types (162), including endothelial cells, vascular smooth muscle cells and circulating red blood cells. In the blood vessel lumen, ATP levels increase during hypoxia (21) or conditions of increased shear stress (26). Precise mechanisms responsible for release are, at present, incompletely understood. Ellsworth et al have postulated a key role for the red blood cell (RBC) in sensing hypoxia (74) and modulating vascular tone via active release of ATP (75). Blood flow is significantly augmented, either through direct purinergic signaling or by inducing synthesis of vasoactive metabolites such as NO. These pathways do appear to intersect and interact with each other; indeed, NO can inhibit ATP release from RBCs (181) while nitrite enhances erythrocyte ATP synthesis and release during hypoxia (46).

(c) NO and control of metabolism

In tandem with its role in hypoxic vasodilation, NO has potent inhibitory effects upon cellular metabolism that are significantly enhanced under hypoxic conditions (53,59). NO potently and reversibly reduces mitochondrial membrane potential (213) by competing with O_2 at CcO (34). As less competition occurs in the presence of hypoxia, after an initial rapid (albeit reversible) inhibition of this enzyme, a potentially irreversible inhibition of Complex I occurs through nitrosylation and nitration that is also accelerated by hypoxia (52,81). For nitrosothiols and peroxynitrite to interact with Complex I, prior transition of the enzyme from its active (A) to its deactive (D) state is necessary, as only the D-form is susceptible to inactivation by these agents. Transition of Complex I from A to D preferentially occurs during hypoxia (85). This process may initially confer some degree of protection, reducing the amount of free radicals produced upon re-oxygenation, but may also initiate pathophysiological modifications of mitochondrial activity. Taken together, under conditions of reduced O_2 availability NO mediates an important compensatory response through both enhancing supply and suppressing metabolic demand (figure 6).

Other important effects of NO on intermediary metabolism are mediated through $ONOO^-$-dependent activation of the AMPK system, or by direct nitrosation of critical thiols of target enzymes (122). The link between NO and the AMPK system involves different levels of regulation; for example, silencing of AMPK caused a decrease in cellular eNOS content (57). In particular, NO can limit energy-consuming anabolic processes such as hepatic gluconeogenesis and glycogen synthesis while inducing energy-producing catabolic pathways via increased expression of transmembrane carbohydrate transporters and a higher glycolytic flux (7,153). Over a longer time-scale, NO stimulates biogenesis of functionally active mitochondria (54). NO may also be an important modulator of the adaptive response to hypoxia; by redistributing O_2 within cellular compartments and between neighbouring cells and interfering with the stabilization process of HIF-1α, it

allows fine-tuning of cellular metabolism (99). Altered levels of NO and ROS likely impinge upon oxygen-sensing pathways. In a variety of cell types, NO stabilized HIF-1α protein and provoked HIF-1 target gene expression under normoxia (36). Whether this is due to NO itself or to a reactive intermediate, and whether or not the mechanism is cGMP-dependent is under active investigation (37). It does appear that the ability of NO to stabilize HIF-1α depends to some extent on the formation of co-signals, such as, for instance, superoxide and the consequent production of peroxynitrite.

Notwithstanding the above findings, some authors reported no impact of NO modulation on whole body (63) or myocardial (136) oxygen consumption. By contrast, others did find a modulating effect on whole body oxygen consumption (217). The negative studies used non-selective NOS inhibition but did not entertain the possibility that NO may be generated by alternate pathways such as nitrite reduction or release from RSNO. Moreover, the lack of effect after administering the NO donor sodium nitroprusside, or authentic NO, may be explained by the potent scavenging properties of haemoglobin and myoglobin. Conceivably, the effect of NO on oxygen delivery and consumption may be tissue-specific. In the brain, NO synergized with hypoxia to induce necrotic death via CcO inhibition in both neurons (120) and glia (170). NO-mediated inhibition of CcO may thus induce an adaptive state of reduced O_2 consumption compensated for by increased glycolytic flux, or it may lead to a critical reduction in ATP production and cell death. The overall impact likely depends on the relative contribution of each process; this is turn depends on the extent and rate of metabolic perturbation and, perhaps, the cell type affected.

(d) NO and microRNAs

The interplay between NO and microRNA signalling is intriguing. Such cross-talk may connect a very fast and ubiquitous signalling pathway for the acute response to hypoxia with the master regulator of chronic hypoxia. This was recently demonstrated in studies of the mechanisms of ischemic or hypoxic myocardial preconditioning (209). miR-21, a

microRNA induced in vascular tissue by shear stress, increased NO availability through phosphorylation of eNOS (242). Conversely, a NO donor modulated production of miR-21 and other microRNAs, thereby regulating smooth muscle cell contraction (137). Brief bursts of myocardial ischemia induced miR-1, miR-21 and miR-24; this, in turn, induced eNOS mRNA and upregulated eNOS protein, whereas no effect was seen on iNOS. This miR-induced, eNOS-derived NO had cardioprotective effects against ischemia-reperfusion injury, possibly by restoring the O_2 supply/demand balance (248).

Very recently, another aspect of miR-mediated regulation of the NO pathway was discovered: a well-known paradox of iNOS-derived NO is that cytokine stimulation can upregulate iNOS gene expression >2000-fold but, in some tissues, the increase in NO levels was far less (167). miR-939 decreased cytokine-induced iNOS protein expression but with no effect on iNOS mRNA levels or stability, thereby contributing to post-translational silencing through direct binding to the iNOS gene (93). Similarly, increases in miR-146a activity inhibited of LPS-induced iNOS expression and NO production (64). These findings have been interpreted as an endogenous protective mechanism against the untoward consequences of prolonged iNOS overexpression.

(e) NO and the renin-angiotensin system

The renin-angiotensin system (RAS) is an important regulator of blood flow and pressure through renal, vascular and central mechanisms (97). The classical pathway involves binding of angiotensin II to the angiotensin II type 1 receptor (AT1), to exert inotropic and vasocontrictor actions through increasing intracellular free calcium. The type 2 receptor (AT2) serves to counterbalance activation of the AT1 pathway; one of the main pathways associated with AT2 activation is stimulation of NO production (47). AT2 activation significantly attenuated mitochondrial respiration, and this was reversed by the NOS inhibitor L-NAME (1).

(f) NO levels in hypoxia: balance of synthesis and metabolism

The NO concentration at any given location represents the balance between local synthesis and metabolism/elimination. During hypoxic vasodilation, the rise in NO levels may derive from increased production via NOS isoforms, and/or increased NO synthesis from alternate pathways (e.g. nitrite reduction or RSNO release), and/or reduced elimination, e.g. by conversion to NO_2^- or NO_3^- (figure 7). An alternative (or perhaps concurrent) mechanism is of vasodilation due to other factors such as ATP release by RBCs; the resulting increase in shear stress increases NO production which then further enhances the vasodilatory response.

(i) Does hypoxia increase NO production by NOS?

NOS-related NO production increases during hypoxia. In dogs, NOS inhibition reversed hypoxemia-induced tachycardia, hypotension and increases in cardiac output (12), findings subsequently replicated in human volunteer studies (25,48,236). However, NOS inhibition also accentuated the hypoxia-induced rise in pulmonary vascular resistance (25).

Given the rapidity (seconds to minutes) of hypoxic vasodilation, the initial increase in NO synthesis is likely to be primarily mediated by a constitutively expressed NOS isoform. O_2 regulates transcription of eNOS (11) and possibly nNOS (19). With prolonged hypoxia, NO levels progressively rise; in an *ex vivo* macrophage model iNOS mRNA was detected after 1.5 hours of hypoxia (9). HIF-1 may influence with iNOS expression under hypoxic conditions (125). In some cell types such as macrophages, hypoxia cannot by itself induce iNOS expression, whereas the synergistic combination of hypoxia and interferon-γ was a potent inducer (172). In other cell types (e.g. cardiomyocytes), hypoxic activation of the HIF-1 pathway could upregulate iNOS expression by itself, though this was significantly amplified with interleukin-1β (125). In pulmonary artery endothelial cells, hypoxia alone did not induce iNOS expression, but it significantly modulated cytokine induction of the gene, prolonging the half-life of cytokine-induced iNOS mRNA from 6 to 17 hours (250).

However, hypoxia itself can induce expression of inflammatory cytokines (133) which then can activate iNOS. Thus, a more complex scenario probably exists *in vivo* with co-participation of all three isoforms.

The oxygen atom in NO and citrulline is derived from molecular oxygen, regardless of synthesis by constitutive or inducible isoforms of NOS (146). Hypoxia could attenuate the NO component of endothelium-dependent vascular relaxation, likely due to decreased NO production secondary to oxygen depletion (123,200). The apparent Km values for oxygen were 17, 6 and 5 mmHg for neuronal, endothelial and inducible isoforms of NOS respectively(201). These values are close to the Km values of other enzymes that utilize O_2 as a substrate, e.g. CcO. As the neuronal isoform shows a higher Km value for O_2, it is thus more sensitive to the prevailing oxygen concentration.

Despite its apparent simplicity, the likelihood that hypoxic vasodilatation can be predominantly explained by *de novo* NOS synthesis presents at least three major contradictions. Given that NOS-derived NO synthesis requires molecular O_2, it seems counter-intuitive in situations of O_2 lack to record an increase in NO production. Indeed, some *in vitro* experiments report reductions in eNOS mRNA expression and decreased NO production during hypoxia (155,200,245). These results conflict with those cited above and may relate to the degree of hypoxia (or anoxia) experienced, the varying O_2 sensitivity of the different cells/tissues studied, and/or a possible biphasic nature of events relating to the time course of the overall response (e.g. feedback inhibition of NOS expression secondary to an acute initial increase in NO availability, followed by a gradual increase in expression as hypoxia persists). Nevertheless, these studies raise important concerns regarding the true significance of NOS-derived NO in hypoxia. Secondly, increased arginase activity during hypoxia is a well-known process (139,163), and this reduces the amount of substrate available for the reaction by NOS.

A more complex regulation of NO synthesis from NOS may exist during hypoxia. In a rodent model of chronic hypoxia, exposure to 10% O_2 significantly increased pulmonary eNOS expression, in addition to an increase in ADMA concentration, reduced DDAH, (the enzyme responsible for ADMA disposal) and reduced tissue nitrate/nitrite (NOx) concentrations (175). They postulated that hypoxia reduces DDAH activity that, in turn, increases ADMA concentrations that leads to eNOS inhibition and reduced synthesis of NO. Finally, in several studies NOS inhibition could only partially reverse hypoxic vasodilation, thus other mechanisms must be implicated (35,148).

(ii) Does hypoxia increase NO release from SNO-Hb and other NO storage forms?

Stamler's theory for hypoxic vasodilation demands a central role for erythrocytes in matching blood flow to local metabolic demands. The affinity of hemoglobin for NO is similar to that for O_2, i.e. high in the relaxed deoxygenated state, and low in the tense oxygenated state (225). A cysteine residue on the hemoglobin β-chain reacts with NO to form a nitroso-adduct (SNO-Hb) that acts as a carrier of NO bioactivity. Erythrocytes thus act as O_2 sensors to control regional blood flow. Erythrocytes could rapidly relax thoracic aortic rings from both rabbits and mice under hypoxic but not normoxic conditions, though this relaxation could be inhibited by either depletion of SNO-Hb or sGC blockade (70).

(iii) Does hypoxia increase reduction of nitrite to NO?

The findings of an increased concentration of NO during hypoxic vasodilation, full reversal of these hemodynamic effects by sGC inhibition but not by NOS blockade, a strict O_2 dependence of the NOS reaction, and the possibility that NO_2^- can be converted to NO, particularly under acidic conditions, suggest that nitrite itself may act as a NO-equivalent donor during hypoxia (3,176). cGMP-dependent nitrite vasodilation and the rise in measured NO have been interpreted as evidence for the involvement of a NO-mediated relaxation, rather than a direct NO_2^- effect. Intravenous infusion of sodium nitrite (1

μmol/min) into healthy volunteers increased forearm blood flow and reduced pulmonary artery pressure only under hypoxemic (12% O_2) conditions, but was not related simply to an increase in plasma nitrite concentration (116). These data are consistent with a direct extravascular metabolism of NO_2^- to NO to exert hypoxia-associated bioactivity. Whether endogenous nitrite concentrations are sufficient to cause similar hemodynamic effects remains uncertain at present.

Of the many different mammalian molecules with nitrite reductase activity (figure 4), the most extensively studied is hemoglobin. Addition of erythrocytes induced a left shift in the vasodilatory dose-response curve and cGMP accumulation in response to nitrite but only under hypoxic conditions (61). This could be inhibited by the NO scavenger, C-PTIO. Thus, NO_2^- exerted a higher vasodilatory effect when deoxygenated Hb was present, again emphasizing the role of NO-dependent mechanisms in hypoxic vasodilation.

Hemoglobin may be an important physiologic O_2 sensor that can modulate vascular tone by (i) scavenging excess NO and (ii) increasing local blood flow through NO generation from nitrite when O_2 content is reduced. This theory remains controversial because of the avid NO-scavenging properties of hemoglobin to the point that it has been argued that this phenomenon has minimal, if any, *in vivo* relevance (6). The Gladwin group countered with a recent *ex vivo* study using rat vascular rings (117) wherein the balance between the NO-scavenging and generating properties of hemoglobin was specifically targeted during both normoxia and hypoxia. They found that nitrite displays a particular interaction with deoxyhemoglobin that promotes vasodilation despite its scavenging properties.

Myoglobin can also act as a nitrite reductase, with NO being produced *in vitro* by reaction between NO_2^- and myoglobin (199). This conversion was significantly reduced in cardiac tissue taken from a myoglobin knockout mouse model but restored by adding exogenous myoglobin. Under hypoxic conditions *in vitro* (238) and *ex vivo* (240) eNOS also displays

nitrite reductase activity. As for the pterin-based enzymes, both xanthine oxidoreductase (174,240) and aldehyde oxidase (195) are sources of NO_2^- reduction, particularly during co-existing hypoxia and acidosis.

The apparently conflicting theories of NO_2^- reduction and S-nitrosothiols acting as non-NOS dependent sources of NO, and as regulators of local blood flow under both physiologic and hypoxic/ischemic conditions, may be reconciled (42,77). Under physiologic conditions, nitrite is not directly reduced to NO but rather modulates many signalling pathways, including sGC activation. It also induces post-translational modifications normally associated with NO, such as the formation of nitroso- and nitrosyl species (42). NO_2^- may therefore exert its signalling functions directly, without the need for intermediary formation of free NO. Hypoxia markedly potentiates tissue NO production from NO_2^- in a dose-dependent manner (77). This occurs particularly in heart, liver and vascular tissue, with multiple heme, iron-sulfur cluster and molybdenum-based reductases distributed among distinct subcellular compartments acting in a multifactorial and cooperative manner to catalyze the reaction. Acute hypoxia also reduces NO_2^- concentrations yet enhances formation of NO metabolites such as RSNOs and RNNOs in an NO-independent manner. This suggests a pathway that generates bioactive NO metabolites directly from NO_2^-. In this paradigm, conversion of NO_2^- to NO and the storage of NO bioactivity as RSNOs may both be constituents of a more complex regulatory mechanism of interaction of multiple NO-related species. The differences in oxygen dependence of nitrite reductase activity in tissues (exponential) versus red blood cells (optimum around the p50) suggest that the regulatory range of the latter may operate at intermediate levels of hypoxia whereas the former predominates as PO_2 drops further.

(iv) Does hypoxia reduce NO metabolism by cytochrome c oxidase?

Moncada's group suggested that the hypoxia-induced increase in NO is due to reduced elimination rather than increased production (186,234). Plausibility has been tested in a

computational model of brain O_2 transport and metabolism (205), with description of the kinetic parameters that link decreased NO metabolism by CcO with low O_2 concentrations. They showed in L-arginine-supplemented iNOS-overexpressing cells that the capacity of CcO to metabolize NO was diminished at low O_2 tensions, and that this correlated with both the enzyme's redox state and consequent sGC activation. The same effect was seen when the redox state was altered by cyanide rather than hypoxia. Whereas NOS inhibition was effective at reducing NO levels, NO_2^- administration had no effect on NO release at any O_2 tension (186,234). NO production was also similar at all O_2 tensions, regardless of whether CcO was oxidized or reduced (234). They thus concluded that CcO (in its oxidized state) constantly inactivates NO, thus regulating its intracellular concentration. However, in the reduced state seen in hypoxia, impaired inactivation would account for the increase in NO. This leads to local vasodilatation with improvements in oxygen delivery and a concurrent reduction in oxygen consumption due to direct inhibition of CcO, thereby facilitating the matching of delivery to needs under hypoxic conditions. Further studies are needed to demonstrate the *in vivo* relevance of these findings.

Conclusions

Hypoxic vasodilatation is an adaptive response that involves elevations in local NO concentrations in response to an acute reduction in arterial PO_2. This both increases blood flow to restore O_2 delivery and also modulates local metabolic requirements, thus attempting to re-balance an acute oxygen supply-demand mismatch. Several mechanisms are implicated (Figure 7) including increased NO synthesis from NOS, increased reduction of nitrite to NO by heme- or pterin-based enzymes, increased release of NO from NO storage forms, and reduced deactivation by mitochondrial cytochrome c oxidase. Many of these mechanisms have been shown either in *in vitro/ex vivo* conditions or by utilization of pharmacologic dosing regimens, so the question remains as to their *in vivo* (patho)physiologic relevance. While tissue hypoxia can result from decreases in arterial PO_2,

blood flow or hemoglobin concentration (15), the adaptive mechanism for each form of hypoxia need not be identical. A recent animal study (62) demonstrated that NOS inhibition did not blunt the increase in myocardial blood flow during acute normovolemic hemodilution, and suggested, at least in this form of tissue hypoxia, that NO did not mediate vasodilation. Further work is needed to fully elucidate the multiple and varied roles of NO under hypoxic conditions, and to integrate these into an overall picture.

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Glossary of non-standard Abbreviations and Acronyms

ADMA: asymmetric dimethylarginine; ALDH2: aldehyde dehydrogenase; AMPK: AMP-activated protein kinases; AO: aldehyde oxidase; CcO: cytochrome c oxidase; cGMP: cyclic GMP; DO2: oxygen delivery; Hb: haemoglobin; HIF-1: hypoxia inducible factor-1; N2O3: dinitrogen tetroxide; NO: nitric oxide; NO2-: nitrate; NO2: nitrogen dioxide; NO3-: nitrate; NOS: nitric oxide synthase; O2.-: superoxide radical; ONOO-: peroxynitrite; PO2: partial pressure of oxygen; RNNO: N-nitrosamines; ROS: reactive oxygen species; RSNO: S-nitrosothiols; sGC: soluble guanylate cyclase; SNO-Hb: S-nitrosohemoglobin; VO2: oxygen consumption; XOR: xanthine oxidoreductase

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Figures and figure legends

Figure 1. Oxygen supply-demand relationship.

The relationship between oxygen delivery to cells and oxygen consumption is non-linear. Initial reduction in the amount of oxygen delivered are compensated by a series of adaptive mechanisms. If delivery is reduced further, a critical point is reached ($DO_{2\ crit}$) below which tissue extraction cannot increase any further, leading to a fall in consumption. Several complementary macro- and microcirculatory mechanisms act to prevent the onset of tissue hypoxia in the face of a reduced delivery, and many of them are mediated by nitric oxide.

Figure 2. Metabolic pathways for arginine.

Four major metabolic pathways for arginine exist: first, arginine is degraded to urea and ornithine by isoforms of the enzyme arginase. A large part of arginine is used for protein synthesis, and arginine is also involved in the biosynthesis of creatine. Another metabolic pathway of arginine is the synthesis of nitric oxide (NO) by endothelial, neuronal or inducible isoforms of the enzyme NO synthase (respectively, eNOS, nNOS and iNOS) with concomitant formation of citrulline. The NO synthesis reaction requires the presence of two oxygen molecules plus NADPH as coenzymes/cofactors, and proceeds with two steps, a first one in which the intermediate N(G)-hydroxy-L-arginine (N-OH-L-Arg) is formed, and a second one in which NO and citrulline are formed as products.

Figure 3. Multiple pathways of NO synthesis.

Nitric oxide (NO) synthesis occurs in blood and tissues through the classical L-arginine-NO pathway, where the semi-essential amino acid L-arginine is metabolized to NO and L-citrulline by a family of nitric oxide synthases (NOS) that use oxygen as co-factor.

Endogenously-formed or dietary-derived nitrite in blood and tissues may be reduced to NO. Dedicated nitrite reductases are present in bacteria, but are lacking in humans. Nonetheless, certain mammalian enzymes under hypoxic conditions may show some nitrite reductase activity beyond their normal physiologic function, such as proteins from the heme-globin family or from pterin-based molybdenum enzymes. S-nitrosothiols (RSNO) are other circulating reservoirs and carriers of potential nitric oxide bioactivity. Their concentrations vary in different pathophysiologic states, and under hypoxic conditions they may be activated to release NO from their thiol (-SH) group. The most studied of such storage pool is hemoglobin (Hb): a cysteine residue on the β-chain may react with NO to form a nitroso-adduct (SNO-Hb) that, under hypoxic conditions releases NO bioactivity. Traditionally considered as inert oxidative end-products, dietary nitrate (NO_3^-) and nitrite (NO_2^-) contribute to NO homeostasis through the gastroenteric "nitrate-nitrite-nitric oxide pathway". Once absorbed from the diet, a significant part of plasma NO_3^- is concentrated in the saliva. Bacteria within the oral cavity then reduce it to nitrite. Nitrite-rich saliva is swallowed, and within the acidic milieu of the gastric lumen NO_2^- is rapidly converted to NO.

Figure 4. Enzymes with putative nitrite-reductase activity.

Dedicated nitrite reductases are present in bacteria, but are lacking in humans. Nonetheless, certain mammalian enzymes show some nitrite reductase activity beyond their normal physiologic function. As an alternative to non-enzymatic reduction, proteins from the heme-globin family (such as deoxygenated haemoglobin – HHb, and myoglobin – HMb, the mitochondrial cytochrome bc1 complex – CIII, cytochrome c oxidase – CcO, and cytochrome c – CytC, as well as aldehyde dehydrogenase – ALDH2, the endothelial isoform of NOS – eNOS, and soluble guanylate cyclase – sGC) or from pterin-based molybdenum enzymes (such as xanthyne oxidoreductase – XOR, sulphide oxidase – SO and aldehyde oxidase –

AO) may all catalyze the NO_2^--reductase reaction to NO, expecially under hypoxic conditions.

Figure 5. Pathways of NO metabolism in normoxic conditions.

NO rapidly reacts with molecular oxygen (O_2) to form nitrogen dioxide (NO_2). If NO is present at low concentration, the NO_2 reacts with water to form equal amounts of nitrite and nitrate. At higher NO concentrations, NO_2 reacts with another NO molecule to form dinitrogen trioxide (N_2O_3), which hydrolyses to form nitrite (NO_2^-), or may dimerize to form dinitrogen tetraoxide (N_2O_4). In plasma, the principal fate of NO is formation of NO_2^-, although the exact reaction is unclear (autoxidation, reaction with ceruloplasmin, or oxidation by the mitochondrial cytochrome c oxidase – CcO). In whole blood NO reacts with oxygenated hemoglobin (oxyHb) to form nitrate (NO_3^-) with concomitant formation of methemoglobin (MetHb). A similar reaction with oxymyoglobin (oxyMb) happens in tissues. NO can also react with superoxide ($O_2^{\cdot-}$) to produce peroxynitrite ($ONOO^-$); the latter may directly trigger oxidation or nitration reactions with various cellular targets, and is eventually converted into nitrate or NO_2. Highly reactive NO by-products ("reactive nitrogen oxide species", i.e. $ONOO^-$, NO_2, N_2O_3, N_2O_4), possibly through the formation of nitrosonium equivalents (NO^+), can react with protein thiol (-SH) groups to form S-nitrosothiols (RSNOs), or with amines to generate N-nitrosamines (RNNOs). NO can also directly react with metals (Me^x) to give metal nitrosyls (Me^xNO). In addition to nitrosation, RSNOs may also be produced by oxidative nitrosylation.

Figure 6. Effects of increased NO levels during hypoxia in the balancing of oxygen supply and demand.

Left panel (A). Metabolic and contractile state of an ideal cell during normoxic conditions. ATP supply is maintained through mitochondrial and cytoplasmic reactions. Glucose (Gluc)

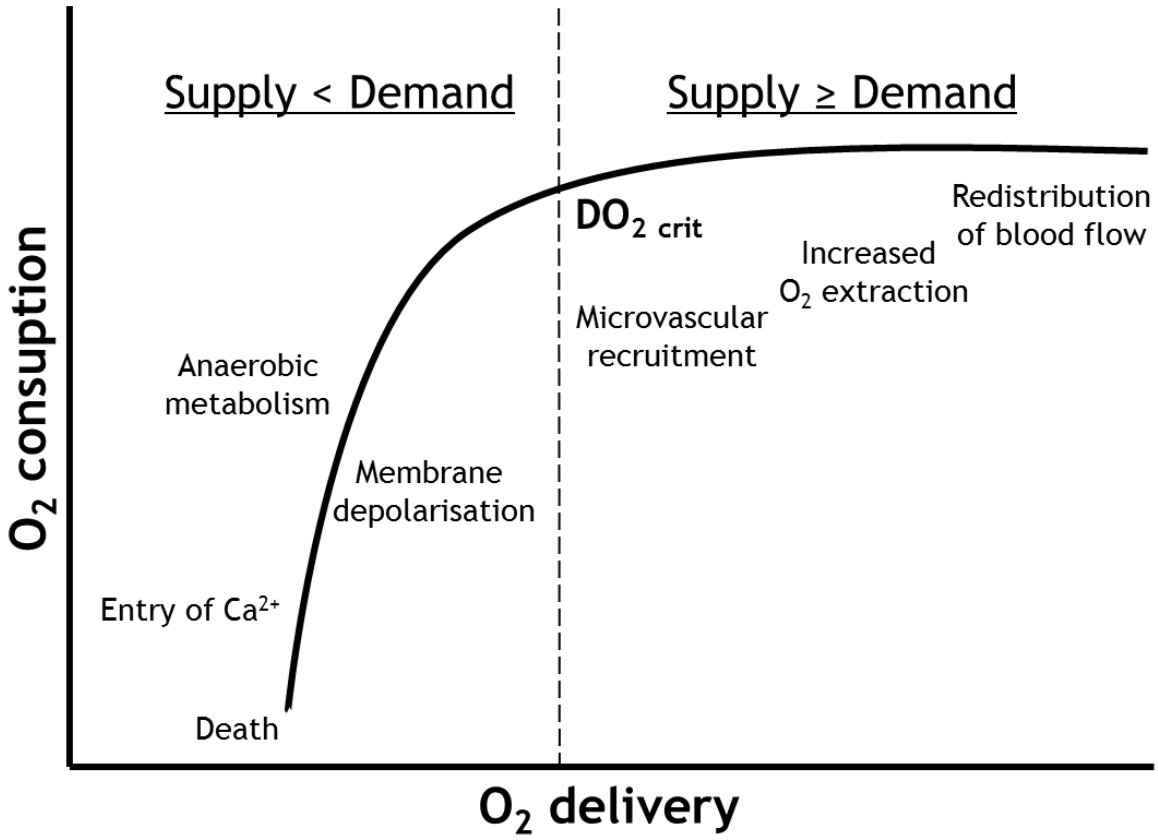
is transported from the interstitium via GLUT carriers. The vast majority of pyruvate, the end-product of glucose breakdown, enters the mitochondria after oxidation by the enzyme pyruvate dehydrogenase (PDH). Here, it is completely oxidized with reactions coupled to the synthesis of ATP. Oxygen (O_2) is crucially required as the terminal electron acceptor of the respiratory chain, Cytochrome c Oxidase (CcO). A small part of the pyruvate is reduced to lactate by lactate dehydrogenase (LDH). In conditions of excess of substrate availability, glucose is stored as glycogen. The contractile state of the cell is maintained through cycling of myosin from a phosphorylated to a dephosphorylated state, via the enzymes myosin light chain kinase and phosphatase, respectively (MLCK and MLKP).

Right panel (B). Adaptive modifications of metabolism and contractile state during hypoxia. The increased levels of exogenous or endogenously formed nitric oxide (NO) during hypoxia contribute to the adaptation to the energy supply-demand mismatch. NO reduces mitochondrial metabolic demand by competing with O_2 at CcO. It also limits energy-consuming anabolic processes such as glycogen synthesis and induces energy-producing catabolic pathways via the increased expression and activity of GLUT transporters, and of enzymes involved in glycogenolysis and glycolysis, while PDH activity is inhibited. The net effect is a shunting of pyruvate away from mitochondria and an increase in glucose availability and glycolytic flux. NO also activates the enzyme soluble guanylate cyclase (sGC), leading to increased synthesis of cyclic GMP (cGMP). This, in turn, modulates MLCK and MLCP activities, resulting in less phosphorylation of myosin and, eventually, vasorelaxation. NO can also cause vasodilation via cGMP-mediated opening of calcium-sensitive (K_{Ca}) and ATP-sensitive (K_{ATP}) potassium channels. When these ion channels open, the outward efflux of K^+ hyperpolarizes the plasma membrane, reducing vascular tone. NO may also activate those ion channels in a cGMP-independent manner through direct S-nitrosylation of cysteine (SH) residues. Eventually, NO regulates the intracellular free Ca^{2+} concentration, either via a cGMP-dependent inhibition of calcium influx through L-type Ca^{2+} channels (LTC), and/or via an increased Ca^{2+} removal from the cytoplasm. The latter may

occur by accelerating the Na^+/Ca^{2+} exchanger (SCE), or increasing the sequestration into intracellular stores via the sarcoplasmic/endoplasmic reticulum (SER). The net effects of an increase in NO levels are reduced consumption of oxygen, increased glycolytic ATP production, and vasodilation that increases the supply of oxygen and nutrients. Solid arrows indicate pathways induced by NO; dashed arrows indicate inhibition.

Figure 7. Effects of hypoxia on NO metabolic pathways

Possible mechanisms of increased NO levels under hypoxic conditions include: 1) increased production by NOS; 2) increased release from storage forms such as RSNO; 3) increased reduction from nitrite; 4) reduced metabolism by CcO.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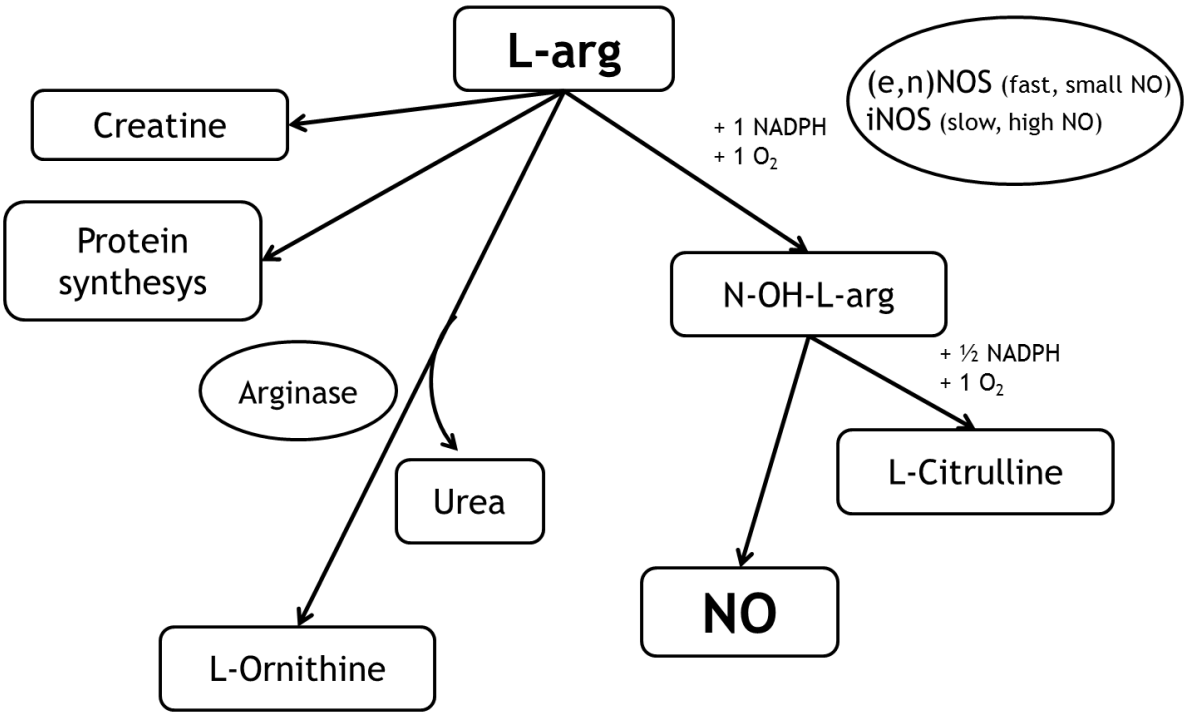
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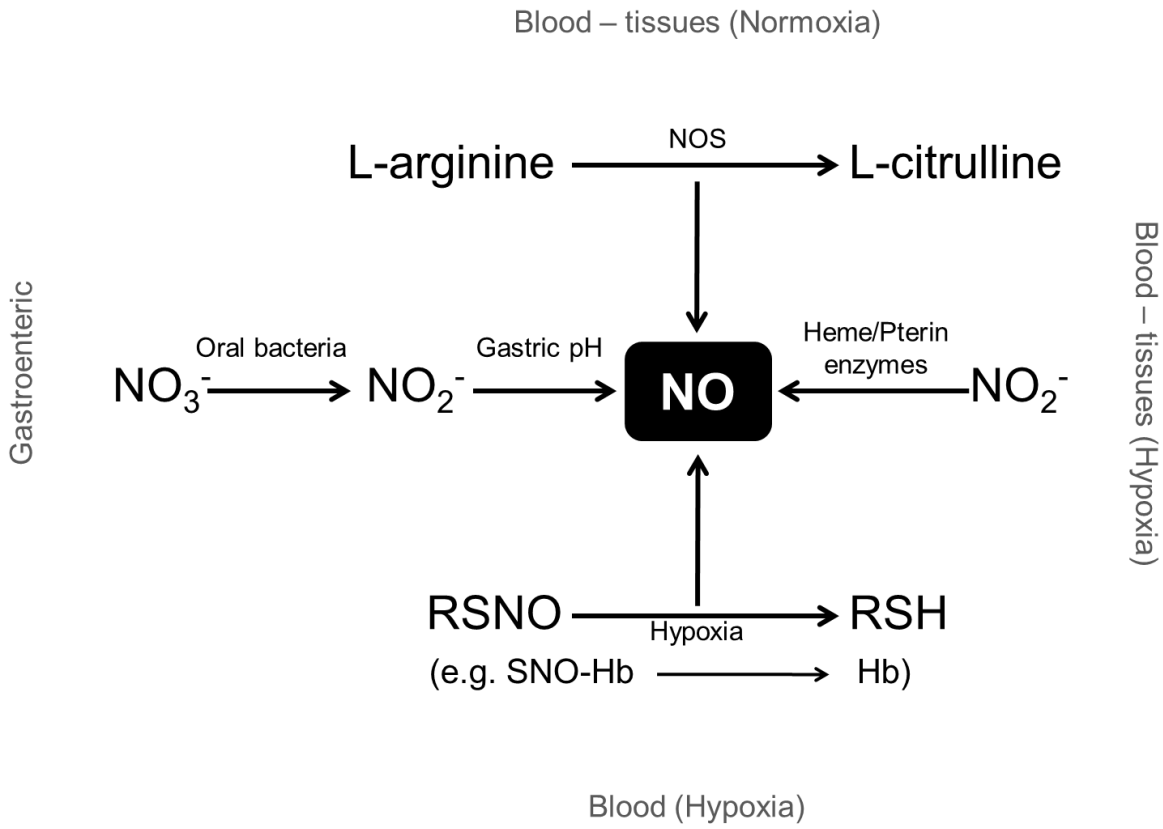
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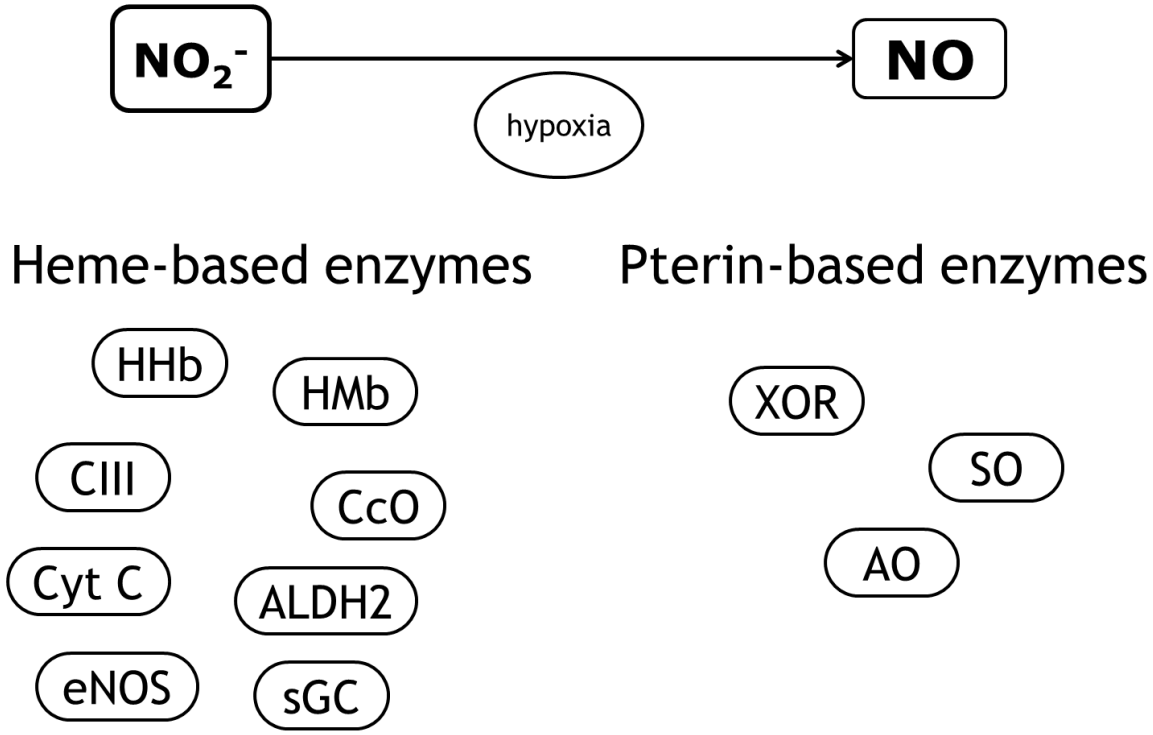
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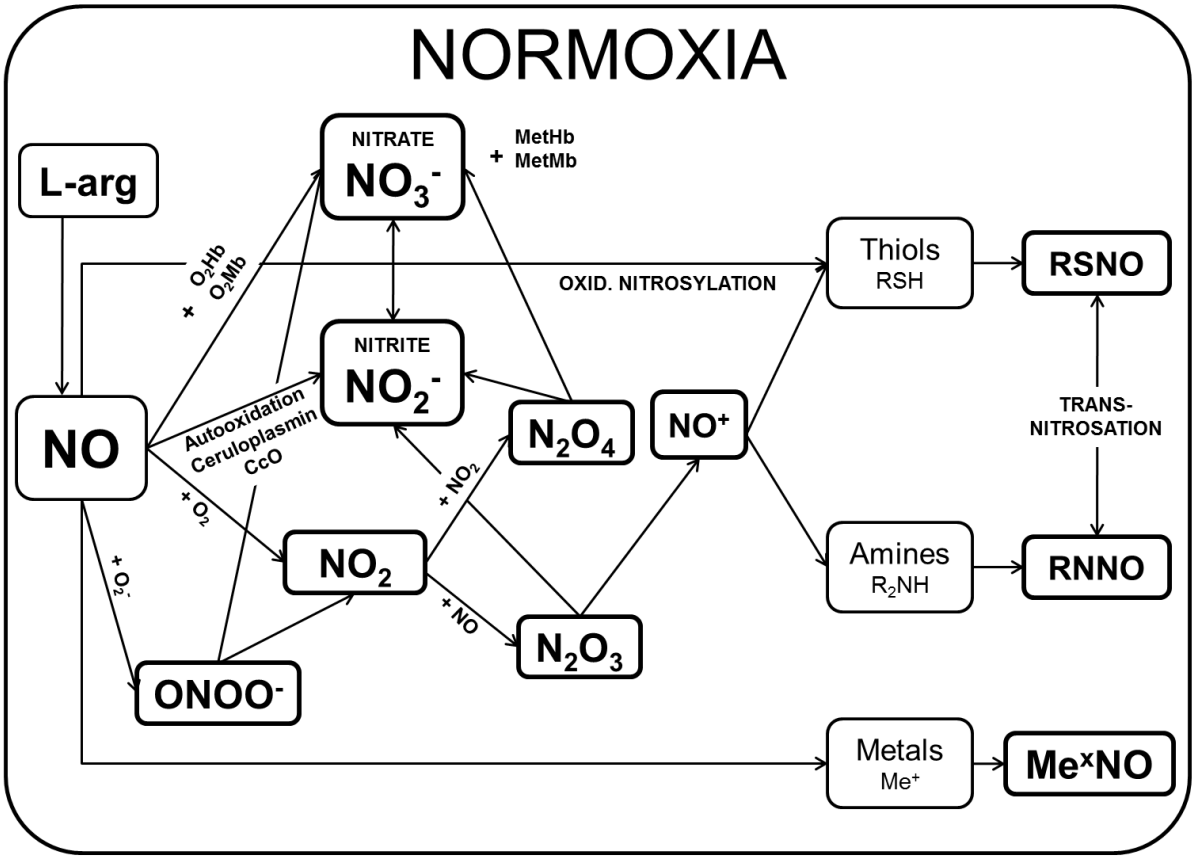
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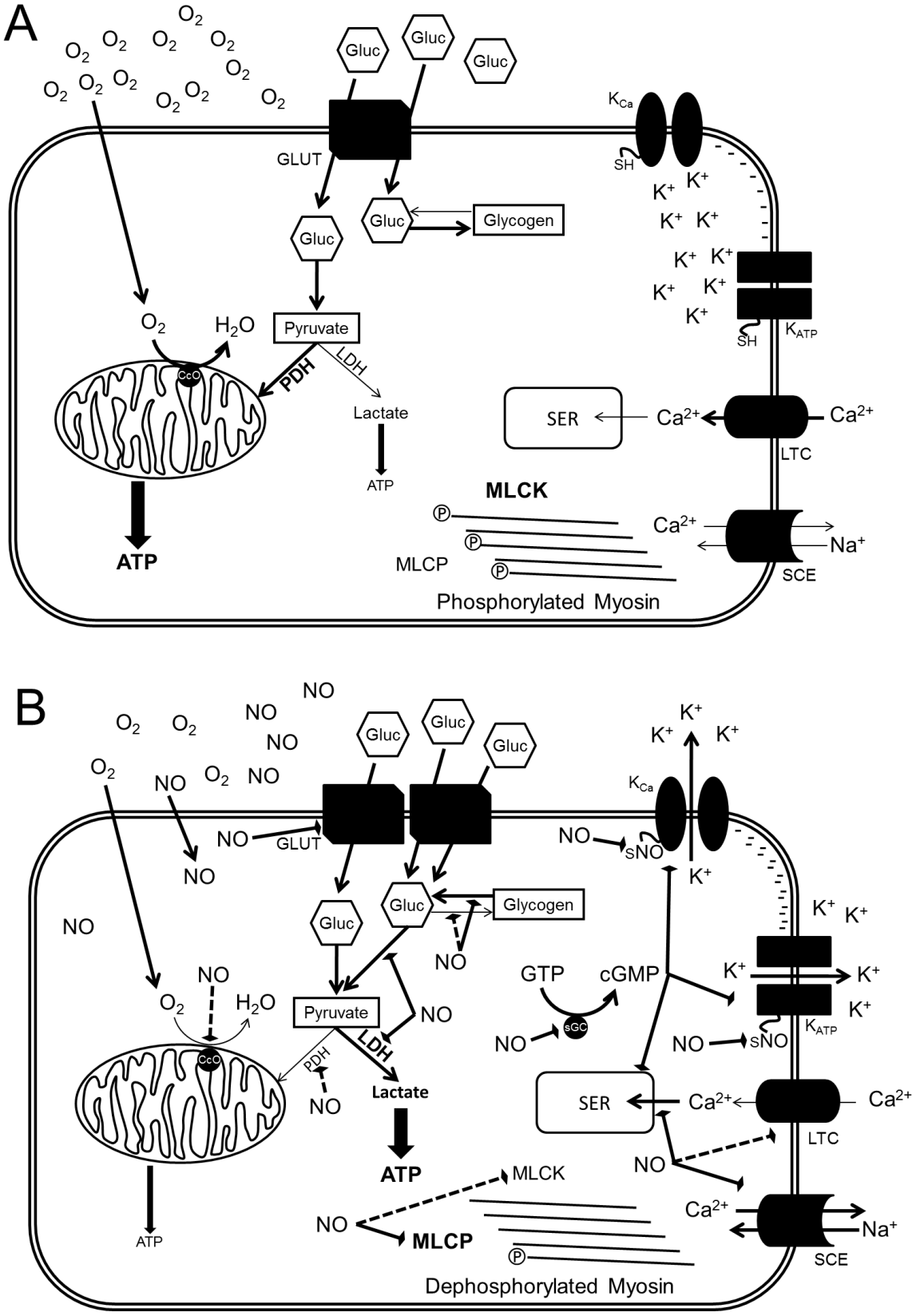
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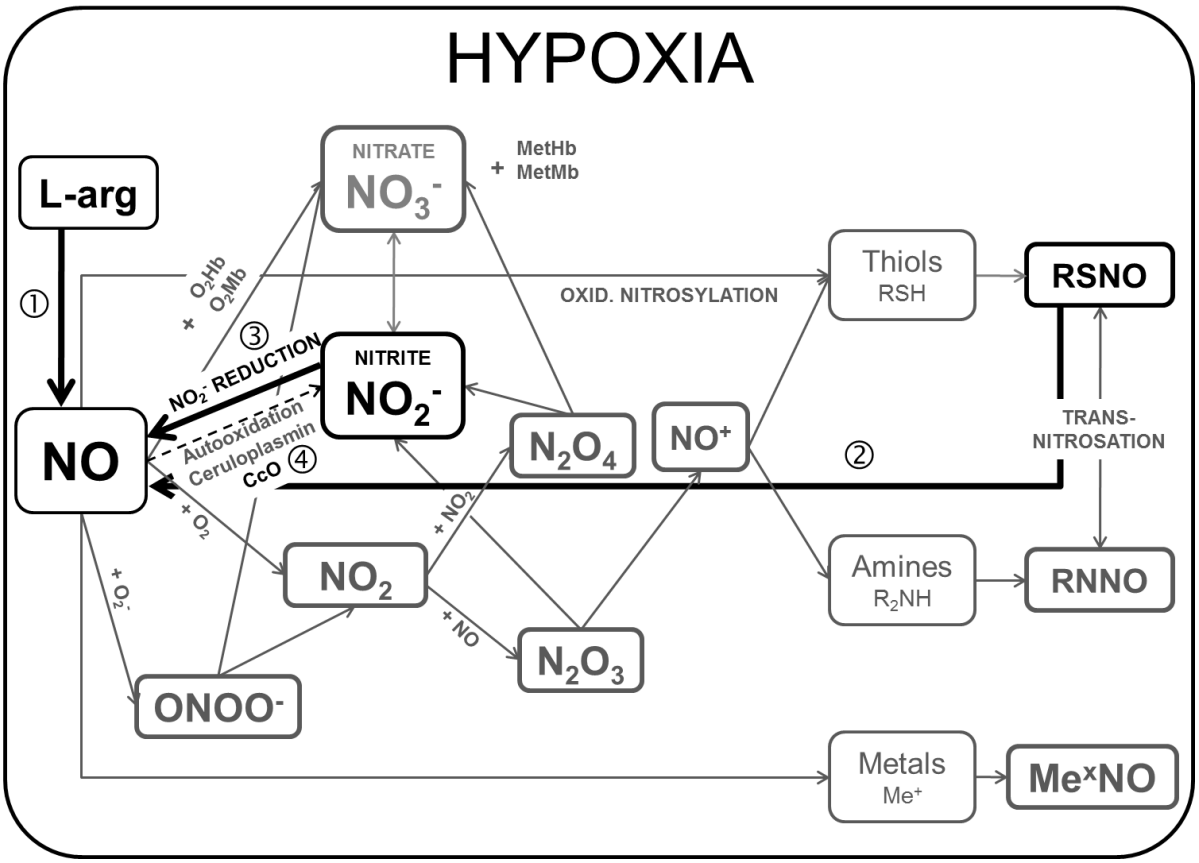
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CcO ④
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