Nitric oxide, vascular function and exercise

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NO (nitric oxide) is a diffusible gas molecule produced intracellularly by three distinct nitric oxide synthase (NOS) enzymes that are highly conserved in mammalian species. The NOS enzymes are haem-containing proteins that catalyse a five-electron oxidation process of the guanidino nitrogen of arginine and in the presence of O_2. NOS activity is, in fact, a tandem of two consecutive reactions: a reductase step catalysed by a moiety of NOS homologous with the cytochrome P450 reductase, including the NADPH and flavin nucleotide cofactor-binding domains, and an oxygenase step coupling the reduction of Fe^{3+} to Fe^{2+} in the haem to the release of NO and citrulline from arginine and molecular oxygen. The reaction requires several cofactors, including tetrahydrobiopterin and calcium/calmodulin.

Two of the NOS enzymes are constitutively expressed and are abundant in specific tissues, therefore they are referred to as neuronal (nNOS or NOS-1) and endothelial NOS (eNOS or NOS-3) according to the cells in which they are normally present. These enzymes are involved in the release of low amounts of NO and require calcium/calmodulin increases to be active. In particular, eNOS is involved in the continuous synthesis of NO in the vascular endothelium and is a main contributor to the regulation of the vascular tone, the blood flow and the inhibition of vascular adhesion and platelet aggregation. The third enzyme is acutely expressed in response to pro-inflammatory cytokines and other cell stress instigators, such as anoxia, hypoxia and bacterial products, or under singular conditions, such as in the placental tissue during pregnancy. This inducible NOS enzyme (iNOS or NOS-2) presents a high-affinity binding to calmodulin and therefore additional calcium inputs are not required for its activity. Indeed, iNOS activity is highly dependent on its transient expression when availability of substrates (arginine, oxygen and cofactors) is not compromised. iNOS reaches high levels of expression in activated cells and is responsible for the high-output NO synthesis observed in macrophages, hepatocytes, chondrocytes, microglia, astrocytes and other cells under pathological circumstances such as inflammation or septicaemia. This high-throughput synthesis of NO exerts profound immune, inflammatory, cytostatic and cytotoxic effects, depending on the target cells (reviewed by Ignarro[1] and Moncada and Higgs[2]); however, in specialized cells such as the placental trophoblasts, iNOS is constitutively expressed and fully functional, without inducing toxic effects or loss of viability and contributing to the transplacental traffic.

In the biological milieu, NO is a mild oxidant molecule with a very short half-life, but its effects are mediated after interaction with different targets that expand its action. Targets of NO include oxygen and superoxide, transition metals and thiol groups, resulting in the formation of nitrite and nitrate, peroxynitrite (the reaction of NO with O_2^-; one of the faster reactions in Nature and a potent oxidant molecule), and metal adducts with NO and S-nitrosothiol derivatives, among others[3,4]. The ability of NO to react with haem groups present in many proteins has proved to be a potent mechanism to exert its biological effects, especially at the vascular level. One of the first identified targets of NO was the soluble form of guanylate cyclase (a haem-containing enzyme). Indeed, the endothelium regulates vascular homoeostasis mainly by adjusting arterial resistance control to the blood flow. The endothelium releases, among others, NO that

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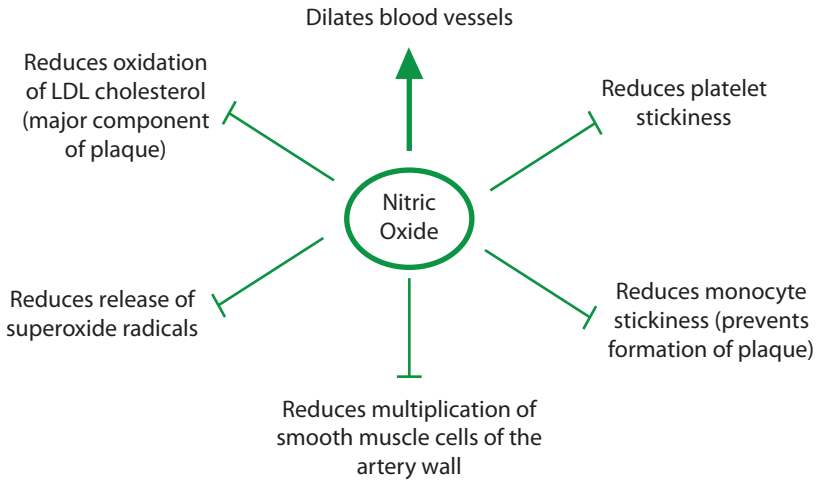
Abbreviations: eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase; ROS, reactive oxygen species.

diffuses to the smooth muscle and interacts with the haem group of soluble guanylate cyclase, making it active. This enzyme synthesizes cGMP from GTP, activating intracellular signalling pathways that decrease the vascular smooth muscle contraction, leading to vessel relaxation. Moreover, vasodilation events associated with NO-dependent cGMP activity in the vasculature are a hallmark of all three NOS mechanisms of action[5].

The dual role of nitrite as end-product and source of NO in the vascular system

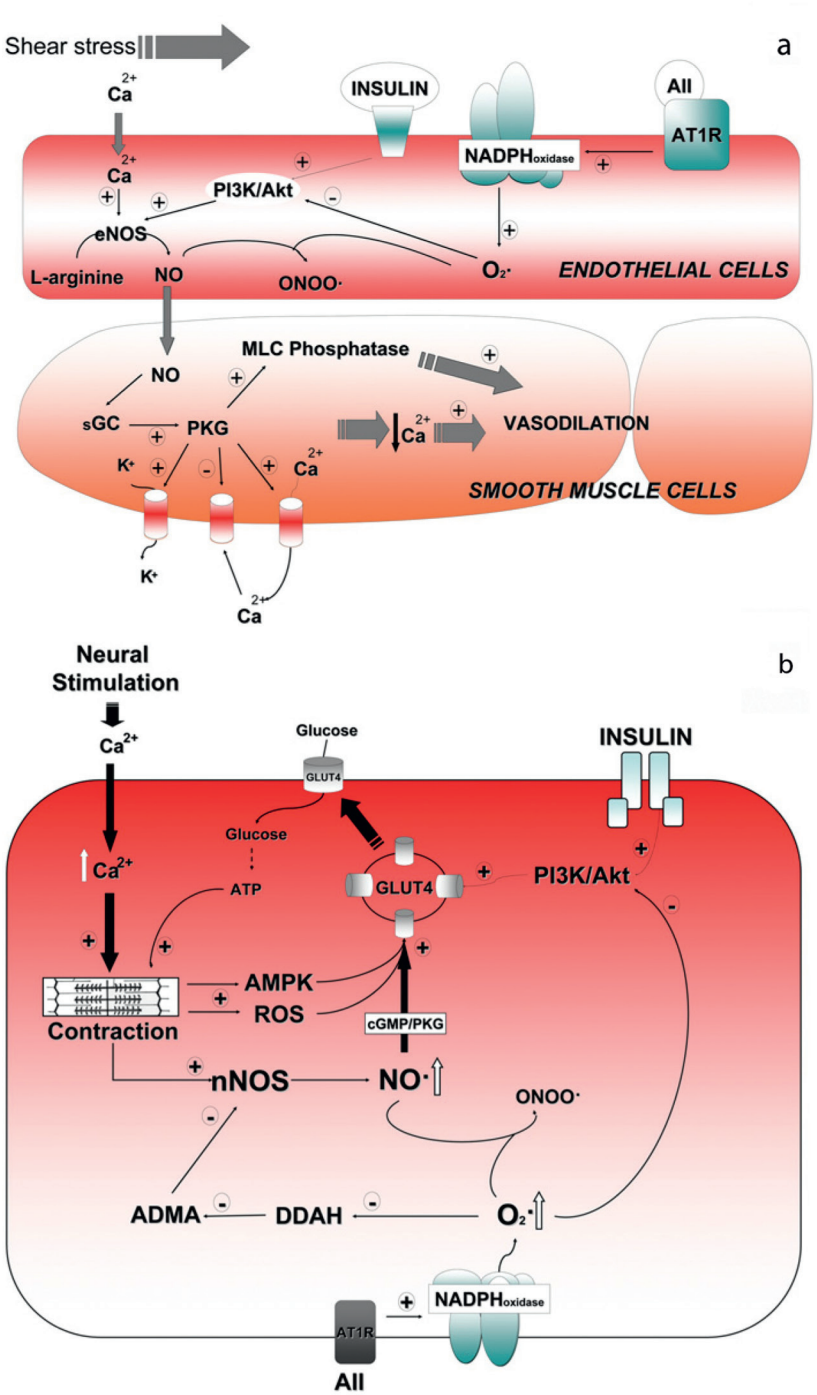
Studies in animal models and from the area of human vascular physiology indicate that the plasma nitrite pool results mainly from the oxidation of NO. In fact, cumulative experimental evidence indicates that plasma nitrite levels rely primarily in eNOS activity that accounts for approximately 60–80% of the nitrite pool. Interestingly, in addition to the enzymatic synthesis of NO from NOS, there is solid support in favour of NO or biologically active NO derivatives coming from plasma nitrite. The first evidence was provided in the early 1980s by the observation that nitrite reacts with deoxyhaemoglobin (nitrite reductase activity of deoxyhaemoglobin?) under acidic conditions to release NO and forming methaemoglobin. This reaction requires low pO_2 levels (that also impair the activity of eNOS) and acidic environments, typical of hypoxia or transient anoxia. According to this mechanism, nitrite may account for a significant contribution of NO under circumstances of poor oxygen availability[6,7]. Evidence that support this mode of action came from pathological situations, such as vascular occlusion and cardiac infarction[8–10]. The chemistry of the release of NO from nitrite is complex and involves several alternative pathways in addition to the deoxyhaemoglobin pathway: for example, xanthine oxidoreductase activity may reduce nitrite, although the conditions of pO_2 and pH required are perhaps too extreme to be relevant under normal conditions, including exercise. Perhaps a definitive proof that verifies the generation of NO from nitrite is the observation of significant vasodilation in animal models of systemic administration/perfusion with nitrite and in human forearm artery perfusion with nitrite. In these cases, increased modification of vascular thiols (nitrosylation) is a common event, suggesting that this nitrite-derived NO synthesis contributes to vasodilation with a profile of NO modifications (i.e. in haemoglobin) similar to that observed by the activation of the classic eNOS activity. This dual role of nitrite as a sink of NO under normoxia and a source of NO intermediates upon hypoxia is important in vascular biology in view of the anti-apoptotic and pro-survival activity of the low concentrations of NO attained under these conditions[11].



The role of nitric oxide

Fine-tuning of NO synthesis in the vascular system: iNOS expression and eNOS genetic polymorphisms

Although NO synthesis at the vascular level mainly involves eNOS activity, under certain pathophysiological conditions iNOS might also contribute to vascular relaxation. In fact, this is one of the reasons for the vasodilation characteristics of sepsis, which is mainly due to a high expression and activity of iNOS. Smooth muscle vascular cells or infiltrating monocyte/macrophages in the vasculature are potential candidates to express iNOS and to release high amounts of NO, with the special characteristics that they are not regulated by the classic calcium-mobilizing drugs. As previously above, the three NOS enzymes are significantly conserved among mammals. However, one characteristic of the iNOS isoform is the attenuated expression in primates in response to the most common cell challenges. Indeed, the biological activity of many genes involved in adaptive responses is regulated mainly at the transcriptional level, whereas fine-tuning is usually regulated at the post-transcriptional level. In this regard, the iNOS gene constitutes an exception. The iNOS coding region is highly conserved not only in mammals, but also in many vertebrates, although its transcriptional regulation differs significantly. The inducibility observed in primate species is more restricted



General mechanisms of NO· actions on vessel relaxation (a) and glucose uptake by the skeletal muscle (b). (a) Endothelial shear stress or other stimuli results in increased intracellular Ca^{2+} and, hence, eNOS activation. NO· is released from the endothelial cells and diffuses to the smooth muscle cells located nearby. NO· binds and activates soluble guanylate cyclase (sGC) in the vascular smooth muscle underlying the endothelium, producing increased concentrations of cGMP. The increase in cGMP thus activates cGMP-dependent kinases that promote relaxation by altering the activity of K^+ and Ca^{2+} channels, leading to cell hyperpolarization and decreased intracellular Ca^{2+} respectively. PKG (protein kinase G) can also activate MLC phosphatases that promote myosin dephosphorylation and then vessel relaxation. Insulin induces vasodilation via a PI3K/Akt-dependent mechanism. AngII (AII) activates the NADPH oxidase in the endothelium cells, resulting in $O_2^{\cdot-}$ production, which, in turn, decreases NO· availability by reacting with NO·, causing ONOO• formation. Skeletal muscle activation results in increased intracellular Ca^{2+}, leading to contraction of muscle fibres and nNOS activation. (B) Skeletal muscle contraction also results in AMPK activation and ROS formation by different mechanisms, and both are likely to induce the translocation of GLUT4 (glucose transporter 4) to the plasma membrane. NO· also regulates GLUT4 translocation by a cGMP/PKG activation mechanism. Increased $O_2^{\cdot-}$ production from NADPH oxidase reduces the NO· availability due to reactivity with the gas and by the inhibition of a key enzyme, DDAH, leading to increased levels of ADMA, a known NOS inhibitor, causing reduced glucose uptake and insulin resistance. (Reproduced from Newsholme, P., De Bittencourt, P.I.H., O'Hagan, C., De Vito, G., Murphy, C. and Krause, M.S. (2010) Exercise and possible molecular mechanisms of protection from vascular disease and diabetes: the central role of ROS and nitric oxide. Clinical Science **118**, 341–349).

than that seen in rodents and other mammals, suggesting a divergent evolution of the iNOS promoter sequence. Extensive studies of the mouse iNOS promoter have shown that only the proximal 1 kb sequence of the 5′-flanking region is necessary for complete inducibility by bacterial lipopolysaccharide (LPS) and cytokine treatment. To confer full promoter activity in the rat, 2 kb of additional 5′-flanking region are required. In contrast, the proximal region of the human iNOS promoter shows no inducibility: the proximal 3.7 kb sequence does not respond to LPS or cytokines in several human cells and, although the 4.7 kb upstream region has basal promoter activity, it does not show any cytokine-inducible activity. These differences between human and rodent iNOS promoters correlate with differences in iNOS expression and NO synthesis, which is markedly less inducible in human cells. In fact, functional and bioinformatic analysis of the promoter region of iNOS shows a higher level of conservation between non-rodent mammals than in rats and mice, perhaps due to the specific evolutionary pressure of pathogens[12].

Regarding the fine-tuning of human eNOS, activation of the enzyme located in the periplasmic domain of the endothelial cell via receptors (i.e. acetylcholine, bradykinin or shear stress) releases NO that rapidly diffuses reaching the vascular smooth muscle cell and directly activates guanylate cyclase activity. This in turn increases calcium pump activity, decreasing the concentration of calcium in vascular smooth muscle cells. In lowering calcium levels, the vascular smooth muscle contraction process is interrupted and vasodilation occurs. The opposite is also true; low NO-releasing activity has been strongly associated with hypertension. In view of the relevance of NO in the regulation of vasodilation, studies to understand the fine-tuning of eNOS in humans have been conducted. The eNOS gene (22 kb) has a complex genetic structure containing 26 exons and 25 introns, and multiple polymorphisms at the promoter and coding and non-coding domains, among them single nucleotide polymorphisms (SNPs) and variable numbers of tandem repeats (VNTRs).

Clinical studies have identified the pathological relevance of some of these polymorphic sites in hypertension, cardiovascular risk and other vascular pathologies. Although studies are still in progress, polymorphisms in the coding and promoter region of eNOS have been associated with impaired NO levels. For example, a significant association of a common variant in exon 7 (G894T) with hypertension has been described and an impaired expression has been observed in individuals with a T786C polymorphism in the promoter region of eNOS. The cellular location of eNOS and its binding to caveolin-1 play a fundamental role in enzyme activity and, indeed, eNOS phosphorylation by Akt plays an important regulatory role (for a review, see [13]). For example, endothelial cells carrying the polymorphism G298D exhibit a lesser presence of eNOS in the caveolae and the same holds true for other polymorphisms affecting the eNOS–caveolin-1 interaction. Even though controversy exists regarding the real contribution of these genetic polymorphisms to NO synthesis by eNOS and their impact on cardiovascular diseases. This is because the relevant factor is the dynamics between NO synthesis and degradation and therefore not only a reduced eNOS activity, but also enhanced NO-scavenging mechanisms influence NO biodisponibility at the vascular level. Also keep in mind that NO can be rapidly scavenged by an increase in the release of reactive oxygen species (ROS), leading to the formation of the vascular harmful and potent oxidant peroxynitrite. In addition to this, current view in the field suggests that perhaps the main relevance of the genetic studies on eNOS polymorphisms relies on the fact that they confer different susceptibilities to drugs used in the treatment of cardiovascular diseases. In particular, recent evidence suggests a modulation of statin effects by genetic polymorphisms of eNOS, regulating the transcription of the gene, the response to angiotensin 2 antagonists or the response to several diuretics. All of these aspects are relevant in the area of the regulation of eNOS activity during and by exercise.

Exercise

Evidence for vascular remodelling in humans, adapting physical activity to blood flow under different sport regimes are quite solid. Indeed, both physical (dynamics of flow, arterial dilation, etc.) and molecular markers for this adaptation have been identified in support of this view (i.e. serum nitrite, and nitrosylated proteins, ROS, reactive nitrogen intermediates (RNI) and thiobarbituric acid reactive substances (TBARS) are the main indicators)[14,15]. One key component of these adapting mechanisms is shear stress. In this regard, aerobic exercise constitutes the main stimulus that promotes eNOS changes to accommodate blood flow to peripheral tissue demands during exercise. Studies in humans attending the various polymorphisms in eNOS promoter and protein suggest that the contribution in terms of changes in eNOS transcription are less relevant than those involving changes in the enzymatic activity because of the phosphorylation of the enzyme by Akt at Ser^{1177} and better interaction with caveolin-1. Remarkably, exercise also involves a decrease in insulin resistance, a rise in factors that activate nuclear receptors, decreasing inflammation and an improvement in glucose availability and use. In addition to this, reduction in scavenging vascular NO appears to be a relevant mechanism activated during exercise, among other factors, due to the release of extracellular superoxide dismutase and a reduced release of ROS as result of the impaired low-grade inflammation. Another consistent observation in the improvement of blood flow in the course of exercise is that the time required to observe such a benefit is rather short; this is probably due to the fact that the main contributor to these changes is integrated through the changes in shear stress and that both transcriptional and post-translational mechanisms appear to act in a quite efficient way[16]. Studies in patients with vascular dysfunction, such as coronary artery disease clearly show notable improvements after 4 weeks of moderate aerobic exercise.

Finally, it is reasonable to propose that chronic inflammation, even at low levels, might play a dual role; on one hand, the possibility exists of a moderate expression of iNOS in different types of cells, including macrophages and smooth muscle cells, but on the other hand, inflammation markedly enhances ROS production decreasing the bioavailability of NO from both eNOS and iNOS. Whether this scenario applies to pathophysiological situations requires further analysis, looking not only to the validation of end-points such as nitrite, but also to the molecular signatures associated with specific regional and tissular area in which this fine balance of NO dynamics occurs[14,17]. ■

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