New Regulatory, Signaling Pathways, and Sources of Nitric Oxide

Ryszard M. Pluta

Abstract Discovered in 1980 by the late Robert F. Furchgott, endothelium-derived relaxing factor, nitric oxide (NO), has been in the forefront of vascular research for several decades. What was originally a narrow approach, has been significantly widened due to major advances in understanding the chemical and biological properties of NO as well as its signaling pathways and discovering new sources of this notorious free radical gas. In this review, recent discoveries regarding NO and their implications on therapy for delayed cerebral vasospasm are presented.

Keywords Hemoglobin · Neuroglobin · Nitric oxide · SAH · Vasospasm

Nitric oxide (NO), a gas with a half-life of milliseconds in blood is continuously synthesized from L-arginine by NO-synthases (NOS), a family of complex multifactorial enzymes that include neuronal and endothelial (both constitutive) and inducible NOS (Fig. 1). NO released from the endothelium can act locally or can exercise distant effects via activation of soluble guanylyl cyclase after binding to its heme moiety, resulting in increased cGMP, activation of GMP-dependent kinases and different biological effects such as vasodilation, increased blood flow, inhibition of platelet activation, and modulation of inflammatory reaction. Additionally, NO can produce biological effects via cGMP-independent pathways, acting as a neurotransmitter, quenching oxygen free radicals, modulating activity of genes and enzymes, evoking the lipid peroxidation cascade, and modulating apoptosis and angiogenesis [1].

In the blood vessels' lumen NO is oxidated to form nitrite and nitrate, reacts with oxyhemoglobin to form nitrate and methemoglobin, and it nitrosates the thiols and amines to nitrosothiols and nitrosamines, as well as it reacts with metals forming for instance the iron-nitrosyl compounds with heme proteins [1, 2]. It has been a longstanding notion that in the presence of erythrocytes, NO is ultra-rapidly consumed or "inactivated" to nitrate, an inert NO metabolite and removed via the kidneys [3]. Recent experiments revolutionized our understanding of this molecule's biological characteristics and effects.

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New Regulatory, Signaling Pathways

NOSes are a family of complex dimeric enzymes that are multifactorial and contain several co-enzymes. This family consists of neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible (iNOS). nNOS, type I or NOS1 is constitutive and coded by the gene on chromosome 12. iNOS, type II or NOS2 is inducible and its gene is on chromosome 17. eNOS, type III or NOS3 is also constitutive and coded by the gene on chromosome 7.

NOS Synthesis and Regulation

eNOS Single Nucleotide Polymorphism (SNP)

SNP is a change of the DNA sequence that leads to substitution, deletion or insertion of a single nucleotide. Results of the SNP can be silent or produce an inadequate action of the particular gene-product or complete inhibit activity of the coded protein. The eNOS gene promoter T-786C single nucleotide polymorphism (eNOS T-786C SNP) was shown to predict susceptibility to post-subarachnoid hemorrhage (SAH) vasospasm [4]. These authors reported that a single nucleotide polymorphism (T/C) was observed in all patients who developed clinical symptoms of vasospasm. Unfortunately, nitrite levels were not measured in this study. Thus,

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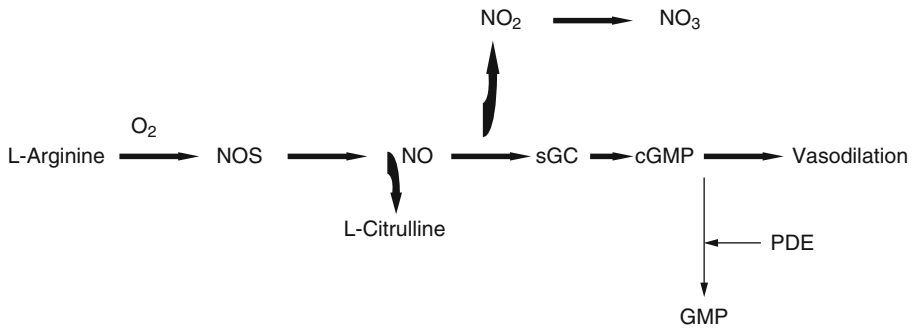

Fig. 1 NO production and metabolism. NO released from the endothelium acts locally or exercises a distant effect by activating soluble guanylyl cyclase after binding to its heme moiety, resulting in increased cGMP, activation of GMP-dependent kinases and different biological effects such as vasodilation, increased blood flow, inhibition of platelet activation, and modulation of inflammatory reaction. But, NO can also produce biological effects via cGMP-independent pathways, acting as a neurotransmitter, quenching oxygen free radicals, regulating gene and enzyme activity, evoking lipid peroxidation cascade, and modulating apoptosis and angiogenesis [1]. When NO is released into the vessel's lumen, several biochemical reactions occur, including NO oxidation to form nitrite and nitrate, reaction with oxyhemoglobin to form nitrate and methemoglobin, nitrosation of thiols and amines to form nitrosothiols and nitrosamines, and formation of iron-nitrosyl compounds with heme proteins [1, 2]. It has been a longstanding idea that, in the presence of erythrocytes, NO is ultra-rapidly consumed or "inactivated" to nitrate, an inert NO metabolite of NO and removed via the kidneys [3]. Recently, our understanding of this molecule's biological characteristics and effects has changed significantly

one can only hypothesize that this nucleotide substitution resulted in decreased production of NO and subsequent development of delayed cerebral vasospasm.

eNOS Phosphorylation

Phosphorylation by kinases and de-phosphorylation by phosphatases are two processes that activate or deactivate different enzymes. Activation of eNOS and increased production of NO have been attributed to its phosphorylation in response to physiological (shear stress, ischemia) or pharmacological (lipopolysaccharide, bradykinin, statins, sildenafil) stimuli that activate kinases Akt, AMPK, CaMK-2, PK A and PK G. However, eNOS is unique among other enzymes since the effect of phosphorylation depends on its locus. Phosphorylation of Ser 615, Ser633, or Ser1177 activates the enzyme but phosphorylation of Thr495 inhibits its activity. Nevertheless, dephosphorylation of the enzyme always decreases NO production [5]. Preventing and reversing delayed cerebral vasospasm by stimulating eNOS phosphorylation has been intensively investigated.

NO Synthesis

Asymmetric Dimethylarginine (ADMA)

L-arginine is a substrate not only for NOS but also for several other enzymes and metabolic pathways. In 1992, P. Valance and colleagues [6] discovered an endogenous NOS competitive inhibitor, double methylated L-arginine by protein arginine *N*-methyltransferase (PRMT I) ADMA; this recently received significant attention because it has been linked to development of vasospasm [7]. Despite the negative results of an experimental study to inhibit ADMA's effect [8], the recently confirmed prevention of vasospasm by statins has led to increased interest in this endogenous NOS inhibitor.

Arginase

Arginine is a semi-essential or conditionally essential amino acid that is used for NO production, protein synthesis, creatine, agmantine and the urea cycle [9]. When arginase is activated and produces ornithine and urea, such up-regulation may deplete eNOS of its substrate and indirectly decrease NO production that in turn may produce or aggravate the vasospasm after SAH. This hypothesis is currently being investigated.

Substrate and Coenzyme Deficiency

Each member of the NOS family is a complex (Fig. 2) enzyme that consists of several co-enzymes including flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and co-factors, tetrahydrobiopterin (BH4), calmodulin (CAM), heme, and calcium. NOS produces NO by cleaving terminal nitrogen from arginine in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen. But, eNOS not only produces NO. When it is deprived of L-arginine, oxygen [10] or co-factor tetrahydrobiopterin, it produces hydrogen peroxide H_2O_2 [11], a potent vasoconstrictive and neurotoxic agent. Furthermore, in the presence

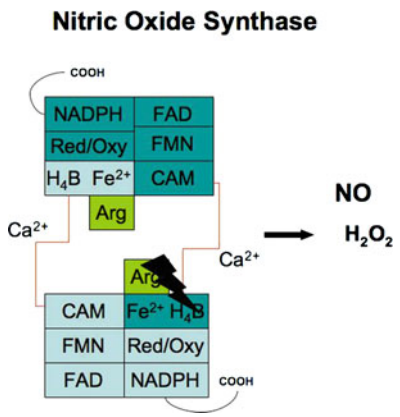

Fig. 2 Nitric oxide synthase (NOS) structure. Each member of the NOS family is a complex enzyme that consists of several co-enzymes including: flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and co-factors, tetrahydrobiopterin (BH4), calmodulin (CAM), heme, and calcium. NOS produces NO by cleaving terminal nitrogen from arginine in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen. eNOS not only produces NO. When it is deprived of L-arginine, oxygen [10] or the co-factor tetrahydrobiopterin, it produces H_2O_2 [11], a potent vasoconstrictive and neurotoxic agent. Furthermore, in the presence of equal volumes of NO, H_2O_2 reacts with the NO to yield the extremely cytotoxic peroxynitrite (ONOO). This pathway as a source of vasospasm has been investigated only in relation to the unlikely depletion L-arginine [12]

of equal volumes of NO, H_2O_2 reacts with it, yielding the extremely cytotoxic peroxynitrite (ONOO). This pathway, as a source of vasospasm, has been investigated only in relation to the unlikely depletion of L-arginine [12]. However, it might be interesting because of the neuronal death that was reported both in the cortex and adventitia of cerebral vessels after SAH [13].

Erythropoietin (EPO)

Influence of EPO on NO production by eNOS remains unclear. There are at least three mechanisms that have been investigated. A. Desai and colleagues showed that in vitro EPO at low and high concentrations does not affect eNOS formation, but at a middle concentration (5 U/ml, it down-regulated expression of eNOS protein [14]. This confirmed the authors' hypothesis that administration of EPO accelerates atherosclerosis via decreased NO availability. Decreased availability of NO in the arterial wall was proposed as a mechanism for developing delayed cerebral vasospasm [13]. Thus, EPO could contribute to the increased severity of vasospasm after SAH. On the other hand, A–L Siren and her colleagues reported that EPO provides neuroprotection against ischemic insults inhibiting apoptosis [15], one of the purported pathomechanisms of vasospasm. Furthermore, in the in vitro study, 4 U/ml EPO (almost the same dose that was used in the Desai et al. experiment) induced eNOS activity in several human cells lines [16] by EPO-related NOS phosphorylation [5]. There have been several experimental and clinical trials investigating the possible therapeutic effect of EPO on vasospasm after SAH.

Soluble Guanylyl Cyclase (sGC) and cGMP Regulation

J. Isenberg and colleagues [17] discovered that transpondin-1, a multi-domain glycoprotein, inhibits neovascularization and tumorigenesis. Reacting with multiple receptors including the integrin-associated protein (IAP), AKA CD47, transpondin-1 inhibits sGC. This binding makes it impossible for NO to exercise its vasodilatory effect via the cGMP pathway. Recently, this pathway was examined experimentally (Fathi et al. in preparation) but did not confirm a direct relationship with vasospasm in a primate model of SAH.

Up-regulation of phopshodiesterases, enzymes responsible for cGMP metabolism, has been known for several years as being a good experimental target to treat vasospasm [18]. This was of interest because cGMP levels are decreased in the artery in spasm [19, 20] but the presence and activity of eNOS remained unaffected despite the decrease of cGMP [21]. Different enzymes have been targeted (PDEV by Sildenafil, PDEIII by Cilostazol, and PDE IV by multiple agents) [22, 23] in experimental and clinical trials but the results remain questionable.

New Sources of Nitric Oxide Biological Activity

Since the discovery that NO is cleaved from L-arginine by NOS, studies assessing NO activity focused on protein synthesis and regulation of the enzyme. However, as reported by Malinski et al. [24] release of NO from the tissue could not be explained adequately by increased NO production by NOS. This effect occurred too quickly, almost immediately (within milliseconds) after ischemic injury to the brain. Thus, there was a lot of speculation about other sources of NO activity that included the presence of S-NO thiols, Fe–NO hemoglobin or S–NO hemoglobin, nitrite or even nitrate. Recently, such speculation has ended because it has been experimentally [25, 26] and clinically proven [27] (Pluta et al. in preparation) that deoxygenated hemoglobin in an acidic environment reduces nitrite to NO.

Nitrite an On-Demand NO Donor

When NO is released into the vessel's lumen, several biochemical reactions occur, including NO oxidation to form

nitrite, reaction with oxyhemoglobin to form nitrate, nitrosation of thiols and amines to form nitrosothiols and nitrosamines, and formation of iron-nitrosyl compounds with heme proteins [1, 2]. It has been a longstanding notion that in the presence of erythrocytes, NO is ultra-rapidly consumed or "inactivated" to nitrate, the inert NO metabolite of NO [3]. However, this hemoglobin "sink effect" [28] has been questioned. A recent report showed that during inhalation of NO there was a subtle increase in forearm blood flow despite regional inhibition of NO synthase. This blood flow increase was associated with a concomitant rise in both heme-bound NO and nitrite [29] evoking artery-to-vein gradients and suggesting that nitrite produces vasodilation [2, 26, 30, 31]. Thus, nitrite represents a major bioavailable pool of NO and deoxygenated hemoglobin acts as nitrite reductase in vivo contributing to hypoxic vasodilation. Reversing and preventing cerebral vasospasm by NO/NO donors strongly suggest that decreased availability of NO is at least contributing to delayed vasospasm after SAH [32, 33]. Thus, we hypothesized that nitrite should act as the "on-demand" NO donor in the presence of deoxyhemoglobin [34] and lower pH [35, 36] in the subarachnoid space after SAH. We tested this hypothesis and demonstrated that intravenous, continuous long-lasting infusion of sodium nitrite prevented development of vasospasm [26]. This encouraging result was followed by a Phase I toxicity and safety study of prolonged intravenous sodium nitrite infusion in healthy volunteers (Pluta et al. in preparation) and a Phase II efficacy study in patients following an aneurismal SAH.

Nitrate as a Source of NO

Nitrate has been known as a metabolically inert end-product of NO oxidation that is removed by the kidneys. However, this opinion has dramatically changed when a panel of researchers "advocate(d) consumption of a diet high in nitrate(s) to protect individuals at risk of adverse vascular events" [37]. This revolutionary change was spearheaded by the studies of Jon Lundeberg and Eddie Weitzberg [38]. As with the earlier discovery that nitrite can become an NO donor, this time it was nitrate that became a source of nitrite and NO. The authors proposed a "recycling" process (Fig. 3) in which both nitrate and nitrite from food are reduced by the nitrate reductase present in bacteria residing in the mouth. After the saliva is swallowed, the nitrite is reduced to NO by chemical disproportionation in the acidic milieu of the stomach. The residual nitrate/nitrite is absorbed in the intestines and part of it is excreted as nitrate by the kidneys. The remaining in plasma exogenous nitrate combined with the nitrite and nitrate produced from the endogenous NO is "recycled" by being absorbed into the salivary glands and then excreted back into the saliva [38]. This very efficient cycle protects nitrite and facilitates its delivery to the plasma to be stored for "on-demand" NO delivery.

However, the mechanism of nitrite reduction to NO is not limited to the red blood cells and the presence of deoxygenated hemoglobin. Recently, it was shown that xanthine oxydo-reductase in plasma also can also act as a nitrite reductase [39]. In addition, neuroglobin [40], the newly discovered member of the globin family, specific for neurons,

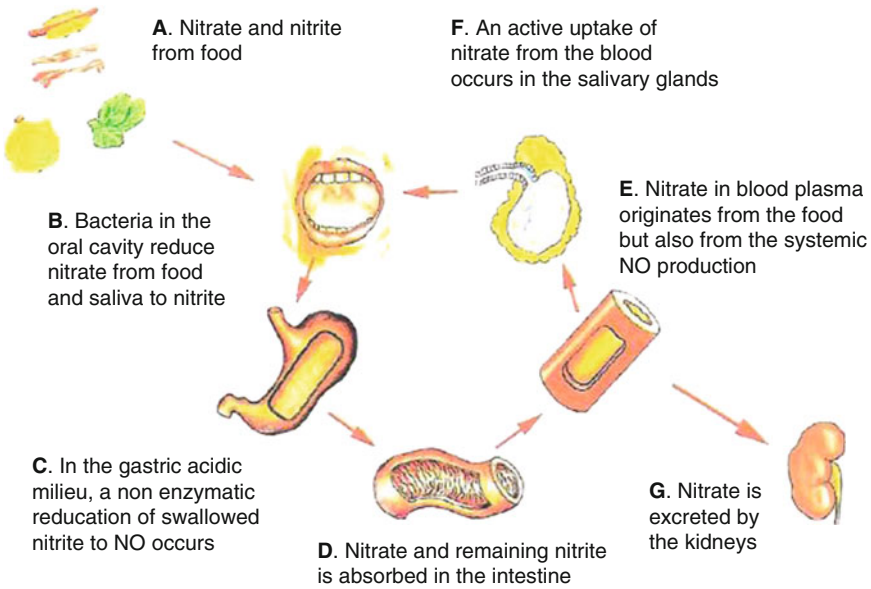

Fig. 3 Nitrite/nitrate "recycling" from [38]

may also reduce nitrite [41]. The latter provides additional support for neuroglobin's protective mechanisms against ischemia proposed several years ago; it also explains the reported high levels of NO-heme in the brain [42].

Conclusion

New pathways and sources of NO activity as well as a deeper understanding of the biological effects of NO have enabled us to develop a new group of therapeutic agents that, by regulating the presence of NO can provide therapeutic effects against vasospasm but may also be useful against the injuries from ischemia/reperfusion, high blood pressure, pulmonary hypertension, cancer development and metastasis, as well as organ transplants.

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Conflict of interest statement The author is one of holders of the international patent on sodium nitrite use in cerebrovascular diseases.

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