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A physiologically relevant role for NO stored in vascular smooth muscle cells: A novel theory of vascular NO signaling

Taiming Liu [a], Hobe Schroeder [b], Gordon G. Power [b], Arlin B. Blood [b,*]

[a] Division of Neonatology, Department of Pediatrics, Loma Linda University School of Medicine, Loma Linda, CA, 92354, USA
[b] Center for Perinatal Biology, Loma Linda University School of Medicine, Loma Linda, CA, 92354, USA

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ABSTRACT

S-nitrosothiols (SNO), dinitrosyl iron complexes (DNIC), and nitroglycerine (NTG) dilate vessels via activation of soluble guanylyl cyclase (sGC) in vascular smooth muscle cells. Although these compounds are often considered to be nitric oxide (NO) donors, attempts to ascribe their vasodilatory activity to NO-donating properties have failed. Even more puzzling, many of these compounds have vasodilatory potency comparable to or even greater than that of NO itself, despite low membrane permeability. This raises the question: How do these NO adducts activate cytosolic sGC when their NO moiety is still outside the cell? In this review, we classify these compounds as 'nitrodilators', defined by their potent NO-mimetic vasoactivities despite not releasing requisite amounts of free NO. We propose that nitrodilators activate sGC via a preformed nitrodilator-activated NO store (NANOS) found within the vascular smooth muscle cell. We reinterpret vascular NO handling in the framework of this NANOS paradigm, and describe the knowledge gaps and perspectives of this novel model.

1. Introduction

Our understanding of the biological role of nitric oxide (NO) has evolved greatly since it was first synthesized in 1772 and later tested in animals [1]. For one-and-a-half centuries NO was deemed merely a toxic gas [2]. Around the 1980s a new era of NO research was brought about by several groundbreaking works: the discovery of endothelial-derived relaxing factor (EDRF) by Furchgott [3], identification of EDRF as NO by Ignarro [4] and Moncada [5], and discovery of the vasodilatory mechanism of NO by Murad [6], leading to a surge of investigations that revealed NO to be a ubiquitous signaling messenger in biology [7].

For many years, the effects of endogenous NO were believed to be only paracrine as it is inactivated in blood within milliseconds which would preclude any endocrine, that is, far reaching and long lasting signaling activity [8–10]. However, early studies of the use of inhaled NO gas to treat pulmonary hypertension surprisingly revealed vasodilation of downstream systemic vascular beds despite the fact that it takes blood more than ten seconds to travel from pulmonary capillaries to the systemic resistance vessels [11,12]. This discovery gave rise to a novel field of research focused on the products of NO metabolism that are stable enough to circulate systemically yet retain NO-like vasoactivity.

NO metabolism in biological matrices is a complex web of reactions leading to a wide array of products. These metabolites of NO can be categorized, based on structural characteristics, into classes that include nitrogen oxoanions such as nitrite (NO_2^-) and nitrate (NO_3^-) [13], S-nitrosothiols (SNOs) [14], iron nitrosyl species (FeNOs) such as dinitrosyl iron complexes (DNICs; non-heme-iron nitrosyl species) [15], nitrated lipids [16], and NO sequestered in the hydrophobic regions of proteins [17]. Importantly, some members of each of these categories of NO metabolite are stable enough to circulate systemically and also have NO-like bioactivity at physiological concentrations. It is widely held that these NO metabolites mediate their effects by protecting the NO from irreversible metabolism during its transit in the circulation and then releasing the same NO moiety at its distant site of action. As such, these compounds are often referred to as "NO storage" forms, or reservoirs of NO vasoactivity [18–21].

Due to their varying chemical structures, the classes of NO metabolites mentioned above comprise a wide range of molecular weights, stabilities, and membrane permeabilities. However, despite these differences, studies in isolated arteries demonstrate that a diverse group of these NO metabolites, which includes many SNOs and DNICs, have remarkable vasodilatory activity at potencies comparable to that of free NO itself. Herein, we will refer to these compounds collectively as 'nitrodilators', a category that we base on five functional criteria

* Corresponding author.
E-mail address: ablood@llu.edu (A.B. Blood).

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(Table 1). First, like NO, all nitrodilators vasodilate via either direct or indirect activation of soluble guanylate cyclase (sGC) and the canonical cGMP vasodilatory pathway. Second, the nitrodilators have an initial vasodilatory potency that is comparable to or even greater than that of free NO itself. Third, different from the transient vasodilatory effect of free NO on isolated arteries, nitrodilators produce a vasodilatory response that is markedly more sustained. Fourth, unlike nitrovasodilators, pharmaceutical agents that relax vessels via donation of NO, nitrodilators are relatively stable and either do not release measureable free NO or release it so slowly that their vasodilatory effects cannot be explained by virtue of free NO release. And finally, nitrodilators have a membrane permeability that is less than that of NO itself. This last characteristic is true of almost any molecule, given NO is a small, non-polar, uncharged molecule that is 3- to 4-fold more soluble in lipids than in aqueous solutions [9]. However, it is important to highlight this characteristic of nitrodilators as it emphasizes the fact that it is not possible for the release of the NO moiety from the nitrodilators to achieve the intracellular free NO concentrations that would be necessary to explain their ability to activate sGC at extent comparable to that of NO itself. Indeed, the latter four characteristics of nitrodilators challenge the commonly held notion that they vasodilate simply by releasing an NO moiety which activates sGC. Herein we propose an alternative explanation for the source of NO involved in nitrodilator-mediated sGC activation.

In the 1950's, it was observed that vascular smooth muscle relaxes upon exposure to both visible and ultraviolet light, a phenomenon that has since been ascribed to the release of NO from a preformed store within the vascular smooth muscle [22–24]. The mechanism activating this light-sensitive NO store remains unknown and its physiological relevance in the absence of light is poorly understood, and its chemical nature is uncertain [24,25]. Nonetheless, these pioneering studies brought forward the notion of an intracellular NO store with a physical basis.

Over the past decade, our attempts to characterize the mechanism of nitrodilator-mediated vasodilation has resulted in a working model that posits that vasodilation by nitrodilators involves the release of an NO-moiety from a store within the vascular smooth muscle cell (Fig. 1), in some respects similar to the activation of NO-mediated vasodilation in response to exposure to light. In this perspective paper, we summarize the existing evidence that nitrodilators cause vasodilation by an uncharacterized pathway, upstream of sGC activation, involving the release of an NO equivalent from a preformed store within the vascular smooth muscle cell, which we refer to as the nitrodilator-activated NO store (NANOS). With focus on the vascular system, we reinterpret existing evidence in the framework of this NANOS paradigm, highlight many of the potential physiological and therapeutic perspectives of this model, and point out important knowledge gaps.

Table 1
Comparison of the chemical and biological properties of NO donor and nitrodilators.

Compounds	sGC-dependence	Dilatory potency	Sustainability of dilation	Membrane permeability	Ability to release NO
NO (authentic NO donor)	+	+	-	++	++
L-cysNO	+	+	+	+	+
D-cysNO	+	+	+	-	+
GSNO	+	+	+	-	-
Glut-MDNIC	+	+	+	-	-
Glut-BDNIC	+	+	+	-	-
NTG	+	++	+	+	-

2. NO storage forms

NO synthetized by NO synthase in the endothelium is thought to diffuse randomly into the neighboring vascular smooth muscle where it can bind to the heme of sGC, resulting in cGMP-dependent vasodilation. It can also react in the blood and tissues to form various stable yet bioactive metabolites, with nitrite, SNOs, and DNICs being three most prominent [26,27]. Fig. 2A summarizes the current commonly held understanding of their production pathways and vasodilatory mechanisms. In this section, we will discuss the prevailing understanding of vasodilatory signaling of nitrite, SNOs, and DNICs, and highlight some remaining gaps in the understanding of their roles in the vasculature wall.

2.1. Nitrite

Until the mid 1990s, nitrite was considered to be inert at physiological concentrations, and simply a byproduct of NO metabolism that is excreted in the urine. However, it is increasingly apparent that although nitrite is orders of magnitude less vasoactive than NO [19], it can serve as a source of NO bioactivity [28]. A growing body of literature reports that increasing circulating nitrite concentrations by dietary intake results in decreased systemic vascular resistance and improved exercise performance [13,29]. One proposed mechanism of nitrite's vasoactivity is based on the ability of deoxyhemoglobin to reduce nitrite to NO. By this reaction, the abundance of deoxyhemoglobin in hypoxic tissues would facilitate the conversion of nitrite into NO, leading to vasodilation that would serve to restore adequate O_2 delivery to hypoxic tissues [20,30]. Consistent with this idea, it has been demonstrated that the vasodilatory effects of nitrite are potentiated under hypoxic conditions [31–35]. However, since erythrocyte hemoglobin scavenges free NO at rates roughly one million-fold faster than the rate at which deoxyhemoglobin produces NO from nitrite, how NO produced by this reaction escapes the red blood cells to reach the vessel wall remains unclear [36]. Alternatively, a number of experiments present evidence that the vasodilatory effects of nitrite are not facilitated by deoxyhemoglobin. For instance, infusion of nitrite into either the relatively deoxygenated blood of the pulmonary artery, or of the femoral artery during systemic hypoxia, does not result in a decrease in pulmonary or femoral vascular resistance to flow until intravascular nitrite concentrations are orders of magnitude above the physiologic range [37,38]. Likewise, although fetal hemoglobin reduces nitrite to NO twice as fast as adult hemoglobin does, and normal fetal oxyhemoglobin saturations are at a level that favors the reduction of nitrite to NO, infusion of nitrite into chronically instrumented fetal sheep does not result in cerebral vasodilation until plasma nitrite concentrations reach >100-fold greater than the physiological range [39,40].

As an alternative to the hemoglobin reductase hypothesis, multiple lines of evidence indicate that the vasodilating effects of nitrite are derived from its direct action in the vascular wall [38,41]. In line with this idea, the vasodilatory effects of treatment with nitrite are found to persist even after plasma nitrite levels have returned to baseline [42,43]. The mechanism for these effects remains unclear, although it may involve the reduction of nitrite to NO, or some NO-adduct, within the vascular smooth muscle cell by a number of different proposed enzymes [38,41,44–48]. For example, a central role of xanthine oxidoreductase (XOR) has been proposed based on the fact that isolated vessels show poor response to nitrite as they have low levels of XOR, whereas animals or humans with high circulating and hepatic XOR levels demonstrate a more pronounced lowering of blood pressure in response to nitrite [46,49].

Intriguingly, measurements of nitrite concentrations also suggest it may play a special role in vascular smooth muscle [50]. For example, while concentrations measured in most tissues are well below 1 μM, including the blood, concentrations in the walls of the rat aorta range from 5 to 25 μM [51,52]. The physiological role of such high

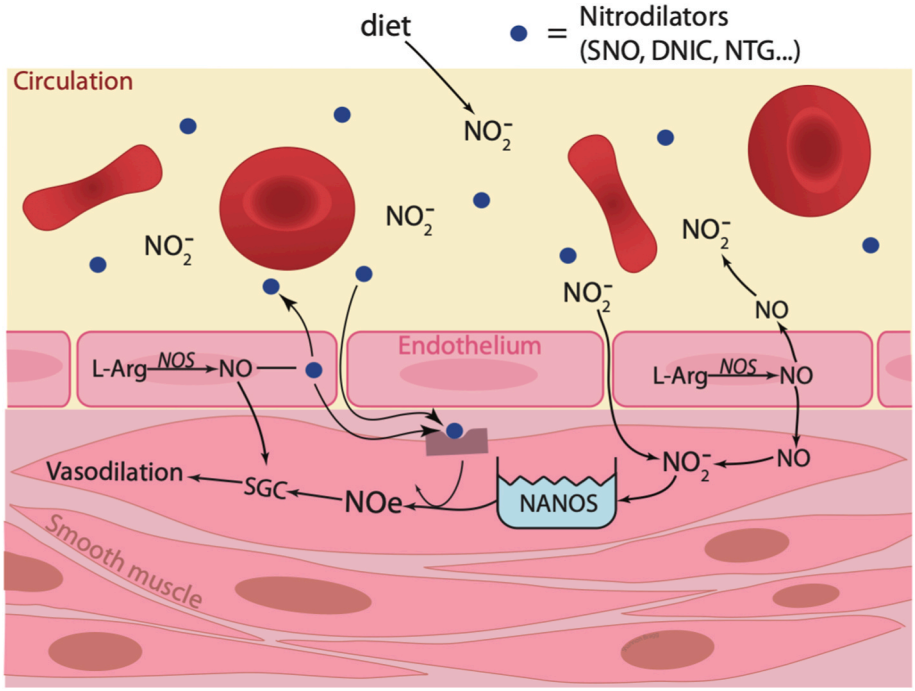

Fig. 1. Working model of the nitrodilator-activatable intracellular NO store (NANOS). Nitrite (NO_2^-) and nitrodilators (NTG, SNOs, and DNICs; the latter two can be endogenous NO metabolites) circulate systemically. NO_2^- contributes to a store of NO bioactivity (NANOS) within the vascular smooth muscle cell. Extracellular nitrodilators cause sGC-dependent vasodilation by stimulating release of an NO equivalent (NOe) from the NANOS. The means by which extracellular nitrodilators mobilize the intracellular NO store, and the chemical nature of the NANOS, remain unknown.

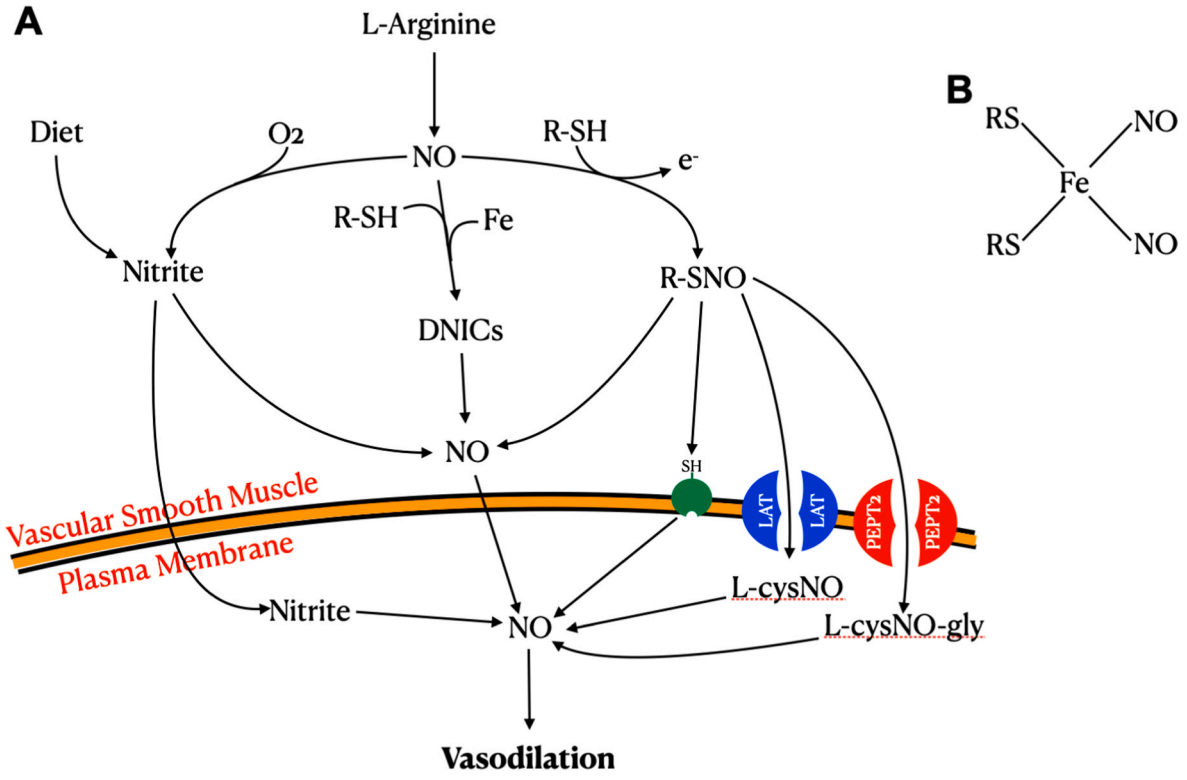

Fig. 2. A. A summary of prevailing understanding of the production pathways and vasodilatory mechanisms of three prominent NO storage forms: nitrite, DNICs, and SNOs. All three have been proposed to mediate vasodilation via various routes of conversion to free NO. DNICs and SNOs, most of which are membrane impermeable, have been proposed to release free NO outside the vascular smooth muscle cell, with the exception of possible selective transport of SNOs such as S-nitrosocysteine (L-cysNO) or L-cysNO-glycine (L-cysNO-gly) across the plasma membrane via the L-type amino acid transporter (LAT) or dipeptide transporters (PEPT2), respectively. Handoff of NO from extracellular SNOs to membrane thiols via transnitrosylation with subsequent transport of the NO to the cytoplasm has also been proposed. **B.** Structure of a mononuclear DNIC.

concentrations remains largely unknown.

2.2. SNO

2.2.1. SNO as circulatory endocrine NO adduct

S-nitrosothiols, a category of NO adduct with potent vasodilatory effects, are formed by the addition of NO to the thiols of molecules ranging in size from small molecules such as thionitrous acid (HSNO) and nitrosoglutathione (GSNO) to high molecular weight compounds such as the nitrosylated cysteine residues of large proteins, including hemoglobin and albumin [53,54]. Since the direct reaction of NO and thiols are spin forbidden and therefore kinetically hindered, the addition of NO to thiols involves a process termed oxidative nitrosylation, i.e. one-electron oxidation of NO, thiol, or S–NO radical intermediate [54–57]. In addition, SNOs can be produced by the 'handoff' of an NO moiety from one SNO to another thiol, a reaction called transnitrosylation (R_1-SNO + R_2-SH ⇔ R_1-SH + R_2-SNO, where R_1 and R_2 are different molecules) [58]. As a result of transnitrosylation, the various SNOs in plasma exist at equilibrium with each other, with the more stable high molecular weight (HMW) forms such as albumin-SNO predominating [59,60]. For example, intravascularly-infused low molecular weight (LMW) SNOs such as S-nitroso-cysteine (cysNO) or S-nitroso-glutathione (GSNO) are converted to HMW plasma SNOs within one systemic circulatory transit time [60]. Advancing the idea that SNOs may be a circulating form of NO bioactivity, Stamler *et al* proposed that a cysteine thiol on the beta subunits (β-93) of hemoglobin constitutes an important carrier of NO in the blood [18,61,62]. Albeit not without challenge [63,64], there is evidence that the NO moiety of hemoglobin-SNO can be exported out of erythrocytes in the form GSNO via a process that is facilitated by the R-to-T shift in conformation that occurs upon deoxygenation [18,61,62]. As discussed in the next section, GSNO and other membrane-impermeable SNOs are potent vasodilators. What is still not understood is how the exported GSNO, which is membrane impermeable and does not release free NO, can cause vasodilation [60,65,66].

2.2.2. Possible mechanisms for SNO-mediated vasodilation

The mechanisms underlying SNO-mediated vasodilation have puzzled investigators in the field for over four decades [67]. Like NO, SNOs cause vasodilation primarily via activation of sGC [68], although they are also capable of signaling via transnitrosylation of proteins, resulting in posttranslational modification of protein function. However, the mechanism by which extracellular SNOs activate cytosolic sGC is still not clear. Due to their different thiol ligands, SNOs can have widely-varying stabilities and membrane permeabilities (Table 1). For instance, L-cysNO and its stereoisomer D-cysNO are 10-fold less stable than GSNO and thus more likely to release NO, although L-cysNO merely releases <10% of its NO moiety over a duration of 1 h in the presence of metal chelator in PBS [14,69,70]. On the other hand, Hogg *et al* demonstrated that L-cysNO can be taken up into cells via the L-type amino acid transporter (LAT), whereas D-cysNO and GSNO (200-fold less membrane permeable than L-cysNO) are largely membrane-impermeable [71]. Based on these properties, one might expect the vasodilatory potency of these three compounds to differ greatly, with the L-cysNO being the most potent and GSNO being the least potent. However, despite these different rates of decomposition, stereo conformation, and membrane permeability, all three of these SNOs have vasodilatory potencies comparable to NO itself [72]. In other words, although a majority of the NO moiety of GSNO remains outside the cell, GSNO is able to activate sGC with potency equal to that of L-cysNO and even free NO itself, both of which enter the cell much more rapidly. Although multiple mechanisms have been proposed to explain this phenomenon (Fig. 2A), no consensus of support has emerged for any one of these pathways [72].

2.3. DNICs

Dinitrosyl iron complexes are a class of FeNOs that contain complexes of NO with ferrous non-heme-iron (Fig. 2B) and usually thiol ligands, although other ligands have also been described. These compounds contain one (mononuclear) or two (binuclear) $Fe(NO)_2$ nuclei (MDNICs or BDNICs, respectively) [15]. Similar to SNOs, DNICs are capable of transferring their $Fe(NO)_2$ nuclei to thiols on other molecules, resulting in a wide variety of DNICs with varying molecular weights, structures and stabilities. DNICs have been found to vasodilate isolated pre-constricted arteries at submicromolar concentrations– an effect that is blocked by inhibition of sGC [73]. For example, glutathione-liganded DNICs have a vasodilatory EC_{50} on the order of 1 μM, comparable to that of free NO itself [74]. As with SNOs, the comparable vasodilatory potencies of glutathione-liganded DNIC and free NO raises a mechanistic conundrum: how can a DNIC that is membrane-impermeable and stable [73,75] activate intracellular sGC just as potently as free NO itself? Furthermore, how do DNICs and SNOs produce a vasodilatory response that is markedly more sustained than that of free NO [15,72]?

While DNICs are clearly potent vasodilators with pharmacologic potential [74], their physiologic relevance remains unclear due to a lack of assay methodologies with adequate selectivity and sensitivity to measure DNICs in blood and tissues. The gold standard method, electron paramagnetic spectroscopy (EPR), is capable of distinguishing DNICs from other NO metabolites. However, the lower limit of detection of EPR is at or above the vasoactive concentrations of DNICs, and thus EPR cannot definitively determine whether DNICs are present at vasoactive concentrations. Ozone-based chemiluminescence offers the ability to detect DNICs at concentrations that are within or below the vasoactive range. However, this method requires sample handling and pre-processing that may result in the degradation of DNICs to form other NO metabolites before they reach the assay instrument [73,76–78]. In spite of these interchanges, cell culture experiments with both exogenous and endogenous NO sources suggest DNICs are the most abundant intracellular NO adduct [79]. In line with this, Vanin *et al* proposed that DNICs are the "working form" of NO in cells and tissues [80], with high molecular weight DNICs representing a predominant reservoir of intracellular NO bioactivity while the less stable LMW DNICs transfer the NO bioactivity to heme- or thiol-containing targets [76,81].

3. Light-sensitive NO store

As outlined earlier, the existence of a light-sensitive NO store in the vascular muscle is well established. For example, precontracted endothelium-denuded arteries are relaxed by exposure to light [82–84], particularly UV light [22,23], and this photo-relaxation is due to NO-mediated activation of sGC, despite the absence of endothelial NO production [85,86]. Moncada *et al* demonstrated that the photoactivated NO store can be depleted by repeated exposure of the arteries to light (Fig. 3), and that it can also be gradually repleted when endothelium-derived NO production is preserved or when endothelium-denuded arteries are incubated with an NO donor [24,87].

3.1. Activation mechanism

The activation mechanism for the light-sensitive NO store is not well understood. Earlier reports favored UV light irradiation as the activator of the NO store [88]. However, more recent work found far red, near infrared, and visible light are also capable of activating photo relaxation [82–84,89]. In fact, the nature of the vasodilatory response varies with respect to the wavelength of light used for stimulation. For example, visible light causes a transient and reversible relaxation that is endothelium-dependent, whereas the relaxation caused by UV light includes an initial transient response superimposed on a sustained one that is endothelium-independent (Fig. 4). This evidence has led to proposals

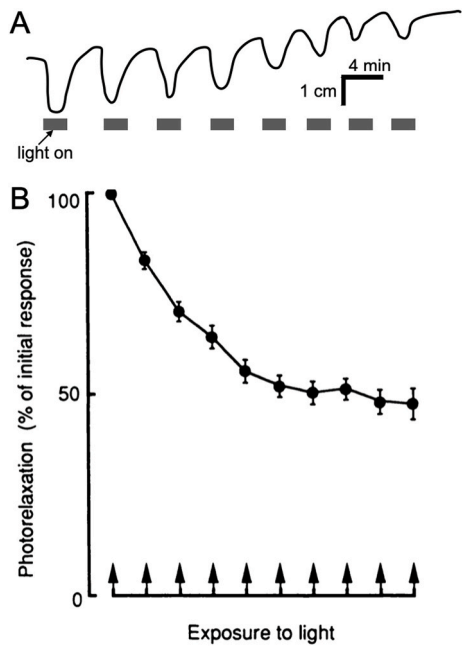

Fig. 3. A. Illustration of the vasodilatory effects of intermittent exposure of a preconstricted rabbit aorta to light from a tungsten filament lamp. **B.** The magnitude of the vasodilatory response diminishes significantly with repeated light exposures. Figures adapted from Moncada et al. [87].

of two or more light-sensitive NO stores that are distinct in chemical nature, one in the endothelium and the other in the vascular smooth muscle cells [82–84,89].

In addition to light, LMW thiols at low millimolar levels, far in excess of physiological levels, can also activate an NO store and mediate endothelium-independent vasodilation, although it remains to be determined whether this thiol-sensitive NO store is identical to the light-sensitive one [82]. Muller et al. demonstrated that membrane-permeable N-acetyl-L-cysteine (NAC) relaxes rat aortas that were pretreated with LPS and L-arginine to form a putative intracellular NO store, but not those pretreated with LPS or L-arginine alone, or those with LPS, L-arginine, and NOS inhibitor L-NAME [82]. It has been proposed that LMW thiols facilitate the dissociation of NO from intracellular NO storage forms to cause vasodilation [25,82].

3.2. Chemical nature

The chemical nature of the light-sensitive NO store has only been partially characterized [51,90]. SNOs, heme-bound NO, and DNICs are all capable of photolytic release of NO under irradiation by UV, red and infrared light [51,91,92]. In contrast, the photolytic liability of nitrite is relatively low, such that unphysiologically high intracellular concentrations (>1 mM) of nitrite would be required for photo relaxation [88,91].

Several lines of evidence suggest a role for SNOs as an intracellular NO store. For example, exposure of isolated arteries to compounds that deplete SNOs attenuates both light- and thiol-mediated relaxation [86,93–95]. Conversely, incubating vessels with membrane-permeable SNOs to increase concentrations of NO adducts in the vessel wall augments thiol-mediated relaxation [96]. In addition, exposure of arteries to red light stimulates the release of a vasodilator with characteristics of SNOs from the vessel [84], highlighting the close relation of SNOs to the intracellular NO store.

There is also evidence supporting a role for DNICs as an intracellular NO store [97]. Pre-incubation of arteries with LPS and L-arginine in combination [82], or with an NO donor [81] leads to an increased intracellular store of DNICs with protein ligands that can be mobilized by membrane-permeable LMW thiols to release NO in the form of LMW thiol-liganded DNICs, leading to vasodilation. However, because SNOs, DNICs, and other NO adducts are interconvertible and are likely to exist in equilibrium, it is possible that they all play some role in the function of the intracellular NO store.

3.3. Physiological relevance of a photoactive NO store

Although a vast majority of the vascular smooth muscle in the body is shielded from the amount of light that would be necessary to activate NO release from the photosensitive NO stores, both light- and thiol-sensitive NO stores in the vasculature have been associated indirectly with cardiovascular function and pathology [25,89], suggesting they participate in vascular smooth muscle function even in the absence of light. For example, in young, spontaneously hypertensive rats, the function of the thiol-sensitive NO store decreases in parallel with the development of endothelial dysfunction and hypertension. Conditioning with mild intermediate hypoxia, on the other hand, enhances the function of the store and also prevents endothelial dysfunction and hypertension [25]. In humans, Feelisch et al. suggested that UV light

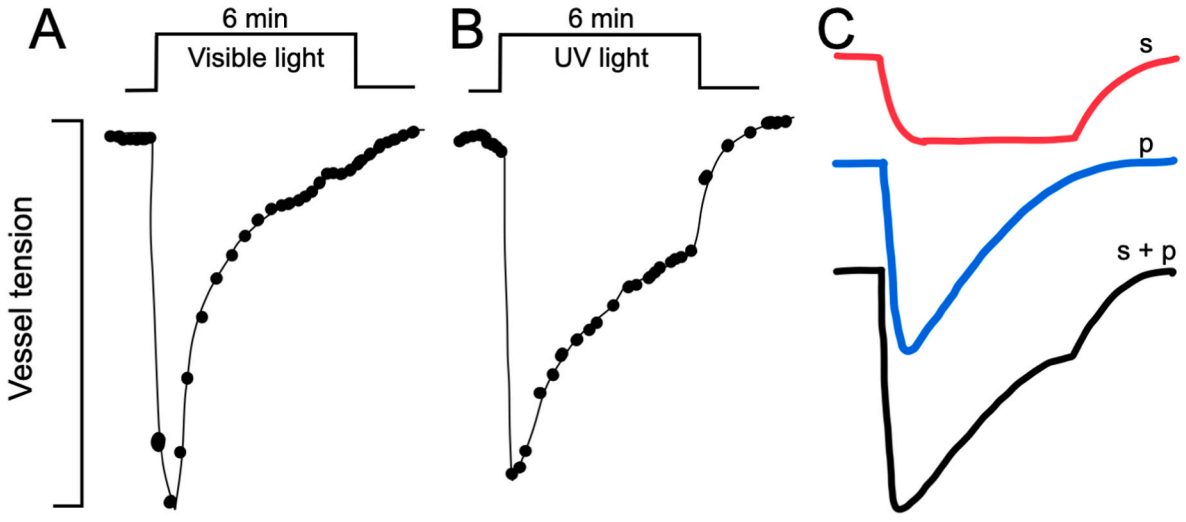

Fig. 4. Comparison of relaxation caused by visible and UV light. **A.** Visible light leads to a transient and reversible relaxation that is endothelium-dependent. **B.** Relaxation caused by UV light includes an initial phasic (p) response similar to that of the response to visible light, superimposed on a sustained (s) response that is endothelium-independent **(C)**. Figures adapted from Ref. [89].

modulates systemic NO bioavailability by mobilizing the NO store in the skin, and that this might contribute to the latitudinal and seasonal variation of blood pressure and cardiovascular diseases [90,98,99].

4. The nitrodilator-activated intracellular NO store (NANOS)

4.1. Rationale

The idea that nitrodilators utilize the intracellular NO store, herein the "NANOS", to effect vasodilation was borne out of experiments intended to determine the mechanism underlying SNO-mediated vasodilation. Previous hypotheses were based on the assumption that these NO adducts release their NO moiety, which then directly activates sGC (Fig. 2A). Many pathways by which the NO of the extracellular SNO might reach the intracellular sGC have been proposed [71,100–107]. However, in our own hands, experiments indicate none of these pathways play a key role [72]. Instead, these results raised the alternative possibility that SNO causes vasodilation via mobilization of an NO moiety from a source other than the extracellular SNO itself [72], essentially a preexisting intracellular NO store reminiscent of the light-sensitive one.

4.2. Core proposal

As shown schematically in Fig. 1, the NANOS model holds that a preformed intracellular NO store is mobilized by extracellular nitrodilators to mediate vasodilation. We find that either inhibition of endogenous NOS or repeated stimulation by these nitrodilators will attenuate the vasodilatory response to subsequent nitrodilator exposure via depletion of the NANOS. Furthermore, incubation of arteries with compounds capable of contributing to the store potentiates nitrodilator-mediated vasodilation.

4.3. Evidence that the NANOS can be depleted and replenished

One expectation consistent with the concept of a NANOS would be that its contents can be depleted, and that its function would diminish accordingly, as has been demonstrated repeatedly for the light-sensitive NO store. Indeed, consistent with depletion, we found that isolated vessels lose ~50% of their initial vasodilatory response to GSNO after repeated exposures to GSNO, despite retaining a full response to NO itself [72]. This finding is consistent with a use-dependent loss of GSNO-mediated vasodilation that results from attenuation of a pathway component that lies upstream of sGC activation. We propose that the initial exposure of arteries to GSNO triggers mobilization of the NANOS, which becomes depleted, leading to an attenuated response to subsequent GSNO exposures.

Based on the hypothesis that endogenous eNOS activity is necessary to constitutively maintain the NANOS, we tested whether treatment with L-NAME would attenuate vasodilatory responses to GSNO in intact rats and sheep. In both cases, animals treated with L-NAME had a greatly diminished or even absent femoral vasodilation in response to GSNO, D-cysNO, and L-cysNO [72]. Interpreted in the context of the NANOS model, these results suggest that inhibition of endogenous NO production by NOS leads to depletion of the NANOS-dependent vasodilation caused by SNOs.

In further accordance with a NANOS model, fortifying the store should potentiate vasodilatory responses to nitrodilators. To increase the store experimentally, isolated sheep femoral arteries were pre-exposed to 1 μM nitrite, a concentration two-to-three orders of magnitude below which nitrite itself causes vasodilation [38]. Such treatment was found to potentiate vasodilatory responses to GSNO [72]. In addition, the attenuation of vasodilatory response to GSNO that occurs in isolated arteries after repeated exposure to GSNO to deplete the NANOS can be reversed by subsequent incubation of the arteries with 1 μM nitrite for 30 min. Likewise, intravenous infusion of sub-vasodilatory concentrations of nitrite to intact rats and sheep pre-treated with L-NAME led to potentiated vasodilatory responses to subsequent infusions of SNOs in the femoral arteries that are otherwise not responsive to SNOs [72]. Interpreted in the context of the NANOS model, these findings suggest that nitrite can replenish the NO store after it has been depleted by inhibition of endogenous NOS activity.

A natural example of a reduced NO store is found in newborn humans and lambs. In both species their plasma nitrite concentration decreases by more than 50% within minutes after birth to levels even lower than those observed following NOS inhibition [108–110]. During this postnatal period, treatment of newborn lambs with low micromolar concentrations of intravenous nitrite is found to potentiate GSNO-mediated vasodilation [111]. This finding is again consistent with the idea that nitrite serves to replenish the NANOS.

In recent work we find, somewhat unexpectedly, that under some conditions L-NAME itself can contribute to the NANOS. Pretreatment of rats with L-NAME for four days potentiated the mesenteric vasodilatory response to nitrodilators but not to NO itself [74], a finding that suggests upregulation of a pathway component that lies upstream of sGC activation, reminiscent of the enlargement of the NO store. Thus we postulated that L-NAME itself contributed directly to the NANOS in mesenteric arteries and in support found that L-NAME had increased the concentration of NO-containing compounds in the mesenteric arterial wall [74]. In addition, in vitro and in vivo application of L-NAME that had an isotopically labeled nitro group ($R\text{-}^{15}NO_2$) demonstrated that metabolic conversion of L-NAME releases NO from its nitro group [74].

L-NAME is known to compromise endothelium-dependent vasodilation, and has been applied in vivo to create a model of experimental hypertension [112,113]. As further evidence that the potentiating effects of L-NAME are due to its release of NO but not to NOS inhibition, we found that its stereoisomer D-NAME, which does not inhibit NOS but does release NO from its nitro group, also potentiated nitrodilators-mediated vasodilation. Furthermore, the potentiation was not observed after treatment with L-NMMA, a NOS inhibitor that does not contain a nitro group [74]. In addition, the potentiating effects of L-NAME were augmented in the presence of oxidative stress, which was found to facilitate the release of NO from the nitro group [74]. Although the detailed mechanism underlying the release of NO from nitro group of L-NAME by oxidative stress remains unclear, oxidative stress has also been proposed to chemically reduce the nitro group of nitrotyrosine in mitochondria [114]. Thus, there is evidence that chronic treatment with L-NAME can potentiate vasodilation of the mesenteric vasculature by nitrodilators via contribution of its nitro group to the NANOS.

4.4. The contribution of nitrite to the NANOS

Nitrite likely contributes to the NO store via conversion into NO, but the underlying mechanism remains unclear. A number of different metalloproteins such as hemoglobin (Hb), myoglobin, xanthine oxidase, cytochrome C oxidase, and eNOS have been proposed to be involved in the reduction of nitrite to NO [20,38,41,44,47,48,115–117]. In many cases, nitrite reduction is favored by hypoxia, in that O_2 competes with nitrite for metal active sites on these proteins so effectively that their ability to reduce nitrite to NO is largely inhibited under physiological O_2 tensions. Furthermore, the oxygenated forms of many of these metalloproteins avidly scavenge NO, converting it to nitrate. Thus, the importance of these pathways of conversion of nitrite to NO under physiological conditions, particularly in the wall of resistance vessels where PO_2s are near arterial levels, is questionable. Nevertheless, nitrite effects are also observed in the absence of hypoxia [42,118–121]. In line with this, nitrite efficiently contributed to the NANOS in isolated vessels under well-oxygenated conditions [72]. Furthermore, xanthine dehydrogenase, a form of XOR with a K_m for O_2 of 260 μM, may be capable of nitrite reduction at O_2 levels in and above the physiologic range [122,123]. Therefore, there is much evidence suggesting that ischemia/hypoxia conditions may not be a prerequisite for nitrite bioactivation. It is

also possible that nitrite is activated via disproportionation at low pH, which converts nitrite to NO [28]. The pKa for nitrite is 3.3, thus the contribution of this pathway to nitrite reduction is expected to be very limited at physiological pH. However, it is worth noting that in mitochondrial intermembrane space the pH can be significantly lower, and that nitrite-mediated nitrosation appears at pH values up to 5 [124]. Further work is needed to determine the mechanism of nitrite bioactivation.

4.5. Nitrodilators as stimulators of the NANOS

Despite representing a wide range of chemical reactivities, molecular sizes, and stoichiometries, DNICs and nitroglycerin (NTG) appear to cause vasodilation in a manner similar to SNOs. These NO-moiety containing compounds cause vasodilation with sensitivities comparable to that of NO itself, and by activation of sGC, but the mechanisms by which they activate sGC remain unclear [68,69,72,73]. Although these compounds are often considered to be NO donors, most SNOs and DNICs differ from authentic NO donors, such as NONOates, in that they do not readily release free NO and also because they cause a sustained relaxation in isolated vessels [15,72] unlike the more transient vasodilation elicited by NO donors. Likewise, NTG has been repeatedly propagated as mediating vasodilation by activation of sGC via releasing the NO moiety from its nitrate group via the bioactivation action of enzymes such as aldehyde dehydrogenase-2 (ALDH-2) [4,125–127]. However, this conversion action produces nitrite rather than NO, especially at therapeutic NTG concentrations (<1 μM), and there is apparent dissociation between NTG-mediated vasodilation and NO generation [128–132]. With an EC_{50} of <0.1 μM, the vasodilatory sensitivity of NTG is approximately one order of magnitude greater than that of NO per se when tested in isolated arteries [74]. These similarities led us to reclassify these NO-moiety-containing vasodilators as nitrodilators [74], a designation that does not include authentic NO donors such as NONOates. This term also serves to categorize these compounds based on their common role as stimulators of the NANOS, as suggested above for SNOs. Consistent with this classification, vasodilation of the mesenteric vasculature of rats by GSNO, glut-BDNIC and NTG are all potentiated by chronic pretreatment with L-NAME and D-NAME, which have a nitro group that may contribute to the NO store, but not by pretreatment with L-NMMA or L-arginine, both of which lack a nitro group [74]. In addition, the potentiation effects are all augmented under oxidative stress, which may facilitate the contribution of nitro group to the NO store via the release of NO [74]. This evidence further justifies the reclassification of SNOs, DNICs, and NTG as nitrodilators, and supports their role as stimulators of the intracellular NO store.

It is important to note that NTG causes tolerance, a loss of responsiveness after repeated use. Multiple mechanisms, including the bioactivation of NTG by ALDH-2, have been proposed for this tolerance effect (see reviews [125,131–134]). However, the phenomenon of NTG tolerance is not yet fully understood [135,136], and the NANOS paradigm raises interesting new hypotheses on this issue. NTG tolerance is often accompanied with a cross-tolerance to other organic nitrates and even endothelium-dependent vasodilators [137]. Although cross-tolerance also occurs with some so-called NO donors such as sodium nitroprusside [138,139], these chemicals actually fall into the category of nitrodilators in that they do not release significant amounts of free NO [69,140]. For authentic donors of NO such as DEA-NONOate [141], cross-tolerance is not observed. In addition, the NTG tolerance can be gradually reversed by a drug-free interval [135]. These features of NTG tolerance are consistent with the involvement of a depleted intravascular NANOS.

4.6. Role of the endothelium in NANOS-mediated vasodilation

Under the NANOS working model, the endothelium provides a constitutive source of NO and nitrite for integration into the NO store of the neighboring vascular smooth muscle. Accordingly, prolonged inhibition of endothelial NOS results in depletion of the NANOS and attenuation of nitrodilator-mediated vasodilation. There is also evidence that the endothelium itself may release SNOs and DNICs in addition to free NO [95,142,143]. Significant aspects of work in this area are in keeping with the NANOS model. For example, vasodilation in response to bradykinin decreases upon repeated use, and this decrease occurs more rapidly in the presence of NOS inhibition [144]. The use-dependent loss of vasodilation is also observed for acetylcholine-mediated vasodilation in the presence of NOS inhibition, despite no change in the vasodilatory response to NO donors [145]. Furthermore, recent work demonstrates that endothelium-dependent flow-mediated vasodilation in humans is improved by dietary nitrite [146] and nitrate [147], which may contribute to the intracellular NO store. Together, these facts raise the possibility that endothelium-dependent vasodilation may be at least partly dependent upon the NANOS.

4.7. Knowledge gaps and hypothesis

The hypothetical NANOS involves contributors, nitrodilators, *trans*-membrane signaling to activate the store, the constituents of the store itself, NO equivalent and mechanisms for the uptake, retention, and release of NO bioactivity by the store. While some of its features are reasonably well established above, others are uncertain as will be discussed below.

As shown in Fig. 1, the direct contributors to the store include NO produced by endothelial NOS, nitrite, and nitro-group-containing compounds. Nitrodilators, including SNOs, DNICs, and NTG, are shown as well-established vasodilators that would stimulate the NO store. Moreover, the endothelium is shown to act as a source of nitrodilators, in addition to release of free NO. A *trans*-membrane signaling mechanism is included, as it would seem necessary to account for cross-membrane vasodilatory signaling of nitrodilators which are membrane-impermeable and stable. Such a membrane receptor/transporter would seem likely to depend on recognition of the NO moiety because it is common to all the nitrodilators. Although seemingly necessary, such a membrane receptor/transporter has not been identified.

4.7.1. Molecular candidates for the NANOS

The NANOS model is reminiscent of the intracellular calcium store, where actual calcium cations constitute the store and mechanisms exist for their uptake, retention, and intracellular release to facilitate vasoconstriction. However, the molecular components of NANOS are unknown. Nor is the identity of the components in the light- and thiol-sensitive NO stores known. Because NO is a highly reactive and freely diffusive gas, it is less likely to be stored and regulated as such. Rather, the NO store is likely to be a more stable NO adduct such as SNOs and DNICs. Taking advantage of a cell-based model that is amenable to surrogate genetic approaches regulating cellular GSNO level via overexpression or knockdown of GSNO reductase, it has been suggested that intracellular GSNO plays an important role in mediating the cGMP-dependent pathway [148]. To test if the intracellular NO store is related to GSNO, we used GSNO encapsulated within nano-liposomes to preload GSNO into the arterial wall while avoiding potential mobilization of the NO store through extracellular stimulation. Preloading the arteries with liposomal GSNO did not reverse the use-dependent loss of vasodilatory response to GSNO, suggesting that GSNO is an unlikely candidate for the NO store. On the other hand, parallel experiments with glutathione-liganded MDNIC encapsulated in nano-liposomes raised DNIC levels in the artery and reversed the use-dependent tolerance to GSNO-mediated vasodilation without altering NO-mediated vasodilation (Fig. 5). These findings support intracellular DNICs as a candidate for the NO store.

Several reports are in line with our nano-liposome experiments and support DNICs as the intracellular NO store. NO can be stored as DNICs in ferritin, a cytosolic protein that stores and releases iron in controlled

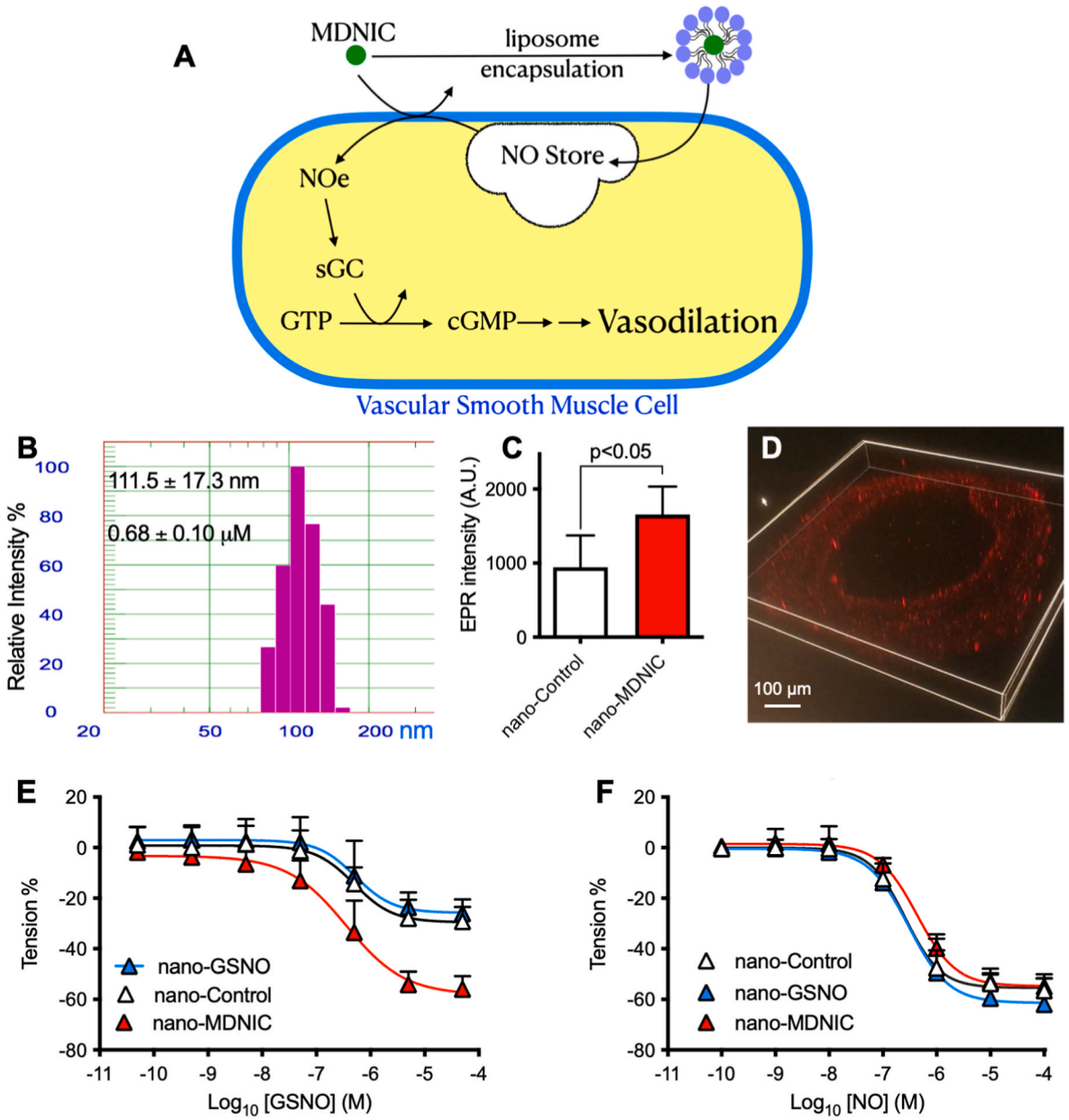

Fig. 5. Evidence favoring MDNICs as a candidate of the NANOS (n = 5). **A)** Proposed model for contribution of the NANOS by nanoliposomes containing MDNIC. **B)** Diameter of nano-liposomes that enclose glut-MDNIC, with a mean concentration of 0.68 μM. **C)** Incubation with MDNIC-containing nanoliposomes introduces MDNIC into the arteries as measured by electron paramagnetic resonance spectroscopy. **D)** Uptake of nanoliposomes tagged with Texas Red into the arterial wall as evidenced by fluorescence image. **E)** Incubation of arteries with nanoliposomes containing MDNIC reverses loss of vasodilatory response that occurs following repeated exposure to GSNO, while nanoliposomes encapsulated with GSNO or buffer (Control) do not. **F)** The incubation does not alter NO-mediated vasodilation. The details of the methods are given in supplemental materials. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

fashion [149]. Nitrite, a NO store contributor, results in considerable cytosolic DNICs in experiments with isolated tissues and in whole animals [78,150]. Incubation of cells with GSNO, a putative NANOS activator, stimulates mobilization of intracellular DNICs, a complicated process that involves glucose metabolism, iron release, glutathione efflux, and the cooperation of carrier and transporter of DNICs [151, 152]. In accordance with this, the general mobilization of arterial intracellular NO-containing species (NOx) upon extracellular stimulation of nitrodilator has recently been observed. As shown in Fig. 6, we demonstrate that incubation with GSNO results in an efflux of NOx from the arterial wall, providing physical evidence in agreement with the NANOS model. Further investigation is needed to determine the correlation between the level of a specific intracellular NO storage molecule and nitrodilator-mediated vasodilation.

4.7.2. *HNO-like NO equivalent*

As discussed above, there are reasons to doubt whether NO itself is the mediator of sGC activation in the NANOS model. Rather, an equivalent of NO may be released by activation of the NANOS. Despite persisting controversy, HNO, the redox cousin of NO, has been suggested to be an alternative activator of sGC [153,154]. Several of our observations suggest the NO equivalent has HNO-like characteristics. We find (Fig. 7) that CPTIO, a membrane-impermeable NO scavenger, does not affect NTG- or GSNO-mediated relaxation, whereas SOD1, which converts HNO into NO, enables inhibition of vasodilation by CPTIO [73,154, 155]. In addition, similar effects of SOD1 on CPTIO were also observed

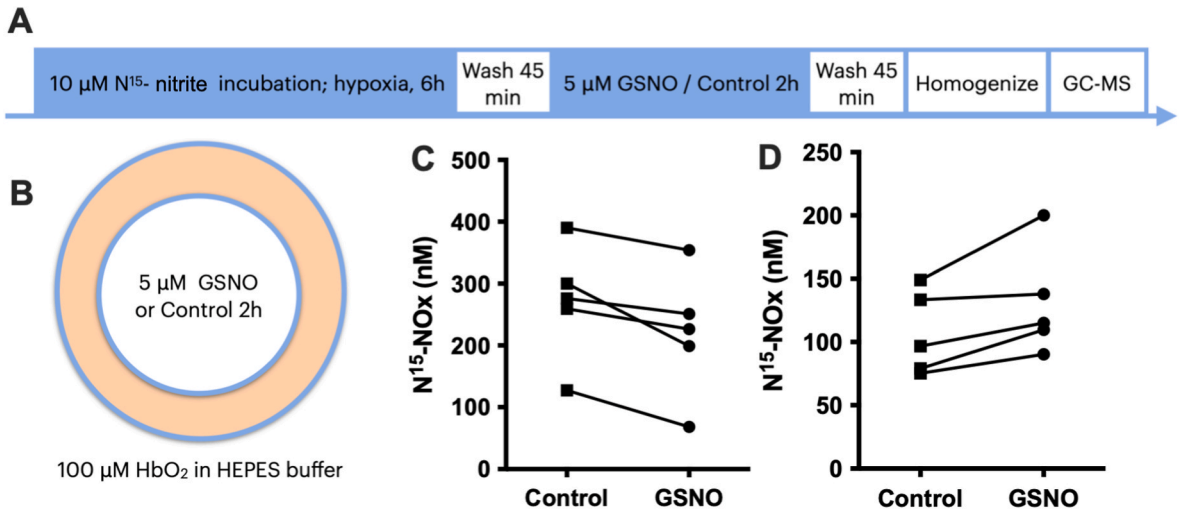

Fig. 6. Physical evidence showing mobilization of the NANOS. **A)** Protocol. A sheep carotid artery (length 8 cm) was incubated with 10 μM N^{15}-nitrite in DMEM under 10.5% O_2 for 6 h to load the vessel with N^{15}-NOx (NO metabolites). After extensive washes, **B)** the arteries were well sealed and incubated for 2 h with 3 ml 100 μM oxyhemoglobin in HEPES buffer on the extraluminal side and 5 μM GSNO or control (0.7 ml HEPES buffer containing 0.1 mM DTPA) on the luminal side. **C)** N^{15}-NOx levels were lower ($p = 0.0206$) in the homogenate of arteries exposed to GSNO. **D)** N^{15}-NOx in extraluminal buffer was higher ($p = 0.0400$) in the arteries exposed to intraluminal GSNO. N^{15}-NOx was measured by GC-MS. The details of the methods are given in supplemental materials. The results are consistent with intraluminal GSNO stimulating an efflux of NOx from the vascular smooth muscle as manifested by a decrease in intracellular NOx and an increase in extracellular NOx.

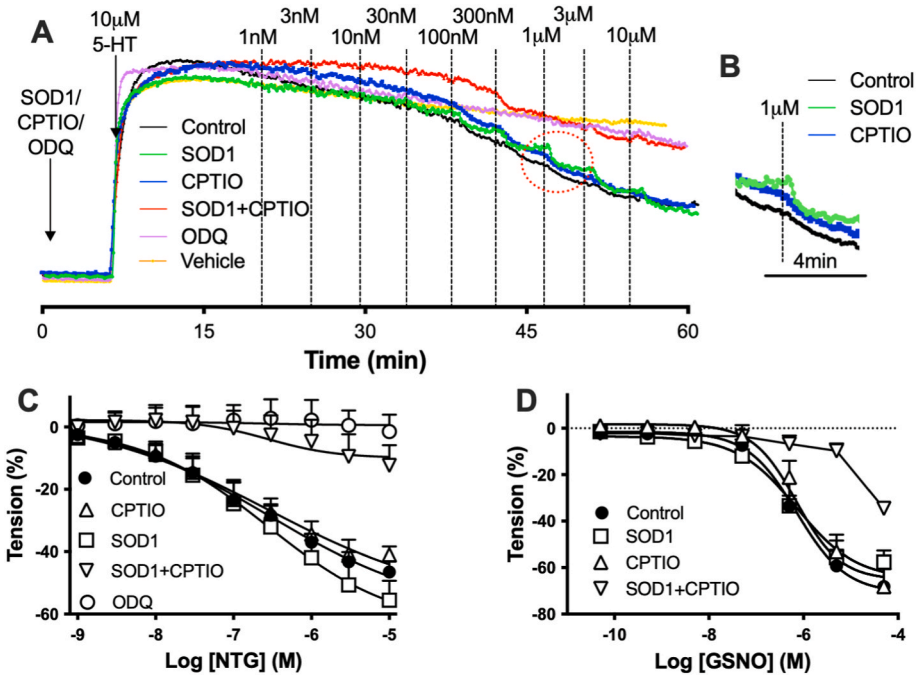

Fig. 7. Evidence showing HNO-like properties of the NO equivalent released from the NANOS. Isolated sheep mesenteric arteries were denuded of endothelium and contracted with 10 μM 5-HT (n = 5). **A)** Example of relaxation traces (one animal, six arterial strips) of NTG in the absence and presence of a sGC inhibitor (ODQ) (10 μM) or superoxide dismutase (SOD1) (1000 U/ml, converts HNO into NO [154]), or the extracellular NO scavenger CPTIO (200 μM), or SOD1 and CPTIO together. All traces were normalized for comparison of extent of relaxation. **B)** Amplification of the area in the red circle in 'A'. **C)** Effects of SOD1, CPTIO, SOD1+CPTIO, and ODQ on relaxation in response to nitroglycerine (NTG). **D)** Effects of SOD1, CPTIO, and SOD1+CPTIO on relaxation in response to nitrosoglutathione (GSNO). SOD1, via putative conversion of HNO to NO, enables the inhibitory effects of CPTIO on relaxation caused by NTG and GSNO. The details of the methods are given in supplemental materials. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

for glut-BDNIC and the HNO donor Angeli's salt [73]. Finally, like SOD1, TEMPOL [156] and CuCl [157], chemicals that convert HNO into NO but do not affect vasodilation per se, also enable the inhibitory effects of CPTIO (not shown due to limited space). However, it remains to be determined whether the NO equivalent is HNO, or whether it is ultimately converted to NO to activate sGC.

4.7.3. Limitations and alternative possibilities

It is important to note again that although the existence of a depletable and repletable NANOS is well supported by multiple points of functional evidence, chemical evidence is still largely missing. While this model may explain some puzzles that otherwise have not been resolved so far, its components are still mostly conceptual. The relationship between light-, thiol-, and nitrodilator-sensitive stores also remains unclear. Many questions, such as those raised above, call for future investigations. Key gaps include the unknown mechanism for transmission of the nitrodilator signal across the plasma membrane and the chemical identity of the NANOS.

Although there is much evidence consistent with the NANOS model, other alternative possibilities are worth consideration. One such possibility is that the observed alterations in the vasodilatory effects of nitrodilators during NANOS depletion and replenishment may actually result from the changes in apo- or inactive-sGC. However, given that the response of NANOS-manipulated arteries to NO itself remains intact, it is

more likely that the alteration lies in a signaling component upstream of sGC, and unlikely that the dilatory signaling cascade from sGC onward is altered. Alternatively, the nitrodilators may act on some receptors or channels that result in alterations in the sensitivity of the vessels toward endocrine vasodilators (e.g. PGI2, bradykinin, natriuretic peptides) or vasoconstrictors (e.g. noradrenalin, ET-1, AT-II, vasopressin) or even ions (e.g. K^+ or Ca^{2+}) in the buffer solution and thereby change the nitrodilator-mediated relaxation. Nevertheless, the synergistic effects of nitrodilators and nitro-group-containing compounds such as nitrite suggest that the mechanism is most likely related to NO.

5. Perspectives and implications

5.1. Physiological and therapeutic perspectives

As discussed above, the endothelium may play a role in the NANOS via the production of nitrodilators as stimulators and the production of NO and nitrite as contributors. This correlation adds another dimension to the connotation of endothelial function, one of the most valuable predictors of future cardiovascular events [158]. Conversely, it also has significant physiological and pathological implications for the NANOS model. Consistent with this rationale, plasma levels of nitrite, which correlate to the NO store function [72,111], have been suggested to reflect the degree of endothelial function [159]. Extrapolating from endothelial NOS, NO sources such as inducible and neuronal NOS and dietary nitrite and nitrate may also play a role in the NANOS model. Through their impacts on these NO sources, various physiological and pathological conditions could be related to the NANOS. It is worth noting that physiological and pathological conditions may not merely affect the size of the NANOS. As has been suggested for the thiol-sensitive NO store [25], the size, capacity, and efficiency of the NO store might all be altered depending on the condition.

The presence of a NANOS also provides opportunities for advancing medical treatments. Examples in which modulation of the NANOS might have therapeutic potential include treatment of a wide variety of hypertensive disorders, erectile dysfunction, hypercoagulation, and improved exercise performance, where dietary nitrite has already proven effective. A specific example where increasing the NANOS could be of marked benefit is in avoiding tolerance to nitroglycerin, a century-old problem in treatment of angina.

5.2. Theoretical implications of NANOS

The proposal of a NANOS participating in the regulation of vascular tone is a novel notion that challenges the fundamental paradigm of NO signaling. In contrast to the conventional thought of NO as a freely diffusive gas that signals without need for a membrane receptor, conceptually the NANOS mediates a controlled mobilization of NO bioactivity upon extracellular stimulation. In addition, the NANOS model may also shed new light on understanding the signaling of different NO storage forms. As described above, the vasodilatory mechanisms of nitrite, SNOs, and DNICs have long puzzled scientists [21]. The NANOS model provides a framework for the crosstalk between different NO species, such as that between nitrite and SNOs in vasodilation [72,111]. This model not only provides potential explanations for the mechanisms by which nitrite and SNOs cause vasodilation, but also builds a conceptual bridge between evidence for the endocrine vasodilatory functions of nitrite and SNOs. Moreover, the proposed synergistic actions of intracellular and extracellular NO species in vasodilation, that is the NANOS in the vessel wall and the circulating nitrodilators, adds new temporal and spatial aspects to the signaling of NO and its storage forms.

Declaration of competing interest

Drs. Liu and Blood disclose they are named on a provisional patent for the use of nitrite to prevent the tachyphylaxis in response to repeated use of nitrodilators.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102327.

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