Nitrite potentiates the vasodilatory signaling of S-nitrosothiols

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Nitrite and S-nitrosothiols (SNOs) are both byproducts of nitric oxide (NO) metabolism and are proposed to cause vasodilation via activation of soluble guanylate cyclase (sGC). We have previously reported that while SNOs are potent vasodilators at physiological concentrations, nitrite itself only produces vasodilation at supraphysiological concentrations. Here, we tested the hypothesis that sub-vasoactive concentrations of nitrite potentiate the vasodilatory effects of SNOs. Multiple exposures of isolated sheep arteries to S-nitroso-glutathione (GSNO) resulted in a tachyphylactic decreased vasodilatory response to GSNO but not to NO, suggesting attenuation of signaling steps upstream from sGC. Exposure of arteries to 1 μM nitrite potentiated the vasodilatory effects of GSNO in naive arteries and abrogated the tachyphylactic response to GSNO in pre-exposed arteries, suggesting that nitrite facilitates GSNO-mediated activation of sGC. In intact anesthetized sheep and rats, inhibition of NO synthases to decrease plasma nitrite levels attenuated vasodilatory responses to exogenous infusions of GSNO, an effect that was reversed by exogenous infusion of nitrite at sub-vasodilating levels. This study suggests nitrite potentiates SNO-mediated vasodilation via a mechanism that lies upstream from activation of sGC.

1. Introduction

Due to its rapid reaction with hemoglobin, nitric oxide (NO) has a very short circulation half-life (merely 2 ms in blood), thus limiting the range of NO itself to paracrine tissues [1,2]. Nonetheless, endocrine effects of NO have been widely reported [3,4], implicating the existence of byproducts of NO that retain its bioactivity and are stable enough to circulate systemically [5–7]. Over the past two decades much research has been focused on determining the chemical identity of these bioactive NO metabolites, with nitrite and S-nitrosothiols (SNOs) receiving the most attention [5–13].

Nitrite, an anion ubiquitous in both blood and vessel walls, can serve as a source of NO bioactivity with vasodilatory effects [14]. Early work put forth the idea that nitrite’s vasoactivity was due to the ability of deoxyhemoglobin to reduce nitrite to NO [5]. Based on the relative abundance of deoxyhemoglobin in hypoxic tissues, this reaction was proposed to promote vasodilation that would serve to restore adequate O₂ delivery [15]. However, more recent work with nitrite suggests that nitrite’s vasodilating properties do not depend solely on the reaction with hemoglobin [16–18]. The vasodilating effects of nitrite may also be derived from its direct action on vascular smooth muscle cells [17,19,20]. The mechanism for these effects remains unclear, although it may involve the conversion of nitrite to NO or some other active NO-adduct within the vascular smooth muscle cell by a number of proposed pathways [9,17,19–21].

S-nitrosothiols are a class of molecules containing NO that has bound to sulfur by replacing the hydrogen of a thiol group. These compounds circulate in blood [22] and cause vasodilation by both NO/cGMP dependent and independent pathways [23]. The NO/cGMP dependent pathway, which appears to be the predominant one [24], involves the activation of soluble guanylate cyclase (sGC) by NO within vascular smooth muscle cells [24–26]. However, the mechanism by which extracellular SNOs, most of which are membrane-impermeable, produce activation of intracellular sGC is still not clear. The following possible mechanisms have been proposed for the cross-membrane vasodilatory signaling of SNOs (Fig. S1): 1) SNOs decompose to release free NO either spontaneously or via catalysis by the cell surface protein disulfide isomerase (csPDI) [27,28], followed by diffusion of the NO...
into the cell to activate sGC; 2) SNOs S-transnitrosylate thiol groups on the surface of the cell membrane [29,30] to initiate cross-membrane signaling events that result in transport of the NO moiety into the cell; or SNOs cross the cell membrane after conversion into either 3) membrane-permeable thionitrous acid (HSNO) formed by S-transnitrosation of H2S [31], 4) S-nitroso-L-cysteine (L-cysNO) which can be taken into the cell via the LAT [32,33], or 5) L-cysNO-glycine formed by hydrolyzation of GSNO via Y-glutamyl transpeptidase (Y-GT) [34] before being taken into the cell through the dipeptidase transporters (PEPT2) [35]. Despite decades of interest, no consensus of support has emerged for any one of these pathways.

The membrane permeability of, and the rate of release of NO from SNOs varies depending on the chemical nature of the molecule. For instance, L-cysNO and its stereoisomer D-cysNO decompose to release SNOs varies depending on the chemical nature of the molecule. For instance, L-cysNO and its stereoisomer D-cysNO decompose to release SNOs respectively [32]. In contrast, L-cysNO can move across plasma membranes via the L-type amino acid transporter (LAT), whereas D-cysNO and especially GSNO are relatively membrane-impermeable [32]. In this study, we tested the above pre-

2. Materials and methods

2.1. Preparation of SNOs and pharmacological compounds

GSNO, l-cysNO, d-cysNO, and their corresponding blank controls were prepared as reported before [37,38] and described in the Online Supplementary Information (SI). Test compounds were purchased from Sigma Aldrich (St. Louis, MO), and dose information and justification for pharmacological selectivity at those doses are provided in the references shown in Table 1.

2.2. Experimental animals

All animal procedures were conducted in accordance with procedures that were pre-approved by the LLU Institutional Animal Care and Use Committee. In both sheep and rats, blood flow and arterial driving pressure to a hind limb were recorded in response to test compounds so that conductance could be calculated as an index of vasodilation.

2.3. Surgical procedures and intra-arterial infusion protocol in sheep

The surgical procedures have been reported before [37,39] and are described in the SI. The conductance and blood flow of the infused femoral artery, mean arterial blood pressure, and heart rate were recorded. Sheep were randomly divided into three groups: a L-NAME group received L-NAME and GSNO; a L-NAME + nitrite group received L-NAME, nitrite, and GSNO. L-NAME (60 mg kg

l-NAME was administrated intravenously (45 mg/kg bolus) to lower the endogenous nitrite level. Sodium nitrite (1 mM) was infused for 15 min at a rate (1 ml/min) calculated to result in femoral arterial blood nitrite concentrations of ~12.4 μM based on dilution of the infused in the measured femoral flow [17]. GSNO, d-cysNO, or l-cysNO (5 mM; measured value) was infused at rates of 0.2, 0.4, 0.6, and 0.8 ml/min, increasing every 3 min. A baseline period of 30 min was allowed before the nitrite and SNO infusions. Baseline hemodynamic values before infusion of SNOs in sheep that received L-NAME with or without prior nitrite infusion are shown in Table S1.

2.4. Surgical procedures and intra-arterial infusion protocol in rats

The surgical procedures in rats were similar to the sheep experiments mentioned above and described in the SI. Briefly, rats were randomly divided into three groups: a control group received GSNO; a L-NAME group received L-NAME and GSNO; and a L-NAME + nitrite group received L-NAME, nitrite, and GSNO. L-NAME (60 mg kg

In rats, blood flow and femoral blood flow were recorded continuously and used for calculation of femoral artery conductance while GSNO was infused.

2.5. Wire myography

Arterial rings (2 mm diameter and 5 mm long) were dissected from a portion of the mesenteric artery supplying the duodenum or from the lateral circumflex branch of the femoral artery of adult ewes. Arterial rings were denuded of endothelium and mounted in organ bath chambers as described previously [37,38] and described in the SI. Initial wire myography studies of L- and D-cysNO were performed by measuring SNO dose response curves in femoral arteries after con

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical description</th>
<th>Concentration</th>
<th>Action</th>
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<tbody>
<tr>
<td>ODQ</td>
<td>1H-[1,2,4] oxadiazolo [4,3-a] quinoxaline-1-one</td>
<td>10 μM</td>
<td>competitive sGC inhibitor [68]</td>
</tr>
<tr>
<td>CPTIO</td>
<td>2-(4-carboxyphenyl)-4,4,5-tetramethyl imidazoline-1-oxyl-3-oxide</td>
<td>200 μM</td>
<td>membrane-impermeable NO scavenger [43,69]</td>
</tr>
<tr>
<td>BCh</td>
<td>2-Aminobicyclo [2.2.1]heptane-2-carboxylic acid</td>
<td>10 mM</td>
<td>competitive LAT inhibitor [70]</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
<td>10 mM</td>
<td>competitive LAT inhibitor [70]</td>
</tr>
<tr>
<td>Na2S</td>
<td>Na2S</td>
<td>1 μM</td>
<td>H2S donor [71]</td>
</tr>
<tr>
<td>PAG</td>
<td>DL-propargyl glycine</td>
<td>1 mM</td>
<td>H2S synthetase inhibitor [72]</td>
</tr>
<tr>
<td>LNAME</td>
<td>L-N(ω)-nitro arginine methyl ester</td>
<td>1 mM</td>
<td>Y-glutamyl transpeptidase (Y-GT) inhibitor [72]</td>
</tr>
<tr>
<td>HMBA</td>
<td>p-hydroxymercuribenzoic acid</td>
<td>100 μM</td>
<td>nonspecific NOSs inhibitor [73]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 μM</td>
<td>membrane-impermeable thiol modifier [30]</td>
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Details of analytical methods including tri-iodide chemiluminescence...
measurement of nitrogen oxides, ELISA measurement of cGMP, confocal microscopy measurement of intracellular NO, and electron paramagnetic resonance measurement of dinitrosyl iron complexes (DNIC), an intracellular NO-containing molecule [41,42], are given in the SI.

2.7. Statistics and data analysis

Average values are given as mean ± SEM in the text and figures. Two-way ANOVA was used to compare the infusion dose response curves. One-way ANOVA was used to test significance of changes with infusion rates. One-way ANOVA with Bonferroni post hoc tests or paired t tests were used as appropriate. Statistical analyses were carried out with Prism, v5.0c (Graphpad Software, La Jolla, CA) with significance accepted at p < 0.05. Dose response data was quantified by calculating concentrations at one-half maximal response (EC_{50}), an index of sensitivity, and maximal response (E_{max}), potency of relaxation, by fitting the data to the Gaddum/Schild model in Prism, v5.0c.

3. Results

3.1. Tests of previously proposed mechanisms of cross-membrane vasodilatory signaling by SNOs

In the wire myography studies, removal of the endothelium did not alter dose responses to L- or D-cysNO (Fig. S2). Thus, vasodilatory responses did not depend on an intact endothelium and endothelium-denuded vessels were used in subsequent experiments. The competitive sGC inhibitor ODQ shifted the dose response curves of both SNOs to the right (Fig. S2 and Table S2, p < 0.05) indicating that the vasodilation pathway is dependent on sGC activation.

To test the role of transmembrane movement of SNOs in mediating vasodilation, dose-response curves of L-cysNO and D-cysNO were compared in isolated sheep femoral arteries. Despite the greater membrane permeability of L-cysNO [32] the two SNOs had similar EC_{50} and E_{max} values (Fig. S2 and Table S2).

To test whether L- and D-cysNO-mediated vasodilation was brought about by extracellular release of free NO that would then diffuse across the cell membrane to activate sGC, dose response curves were carried out in the presence of CPTIO, a membrane-impermeable NO scavenger [43]. Although CPTIO completely blocked vasodilation by free NO itself (see Fig. S5), it only partially attenuated the relaxation effects of L- and D-cysNO, suggesting that the vasodilation by these SNOs is not due entirely to extracellularly released NO.

The role of membrane surface thiols was previously proposed based on the observation that HMBA, a membrane-impermeable compound that binds covalently to thiols, attenuated L-cysNO-mediated vasodilation [30]. However, our finding that L-cysNO is rapidly degraded in the presence of HMBA (Fig. S3D) suggests that the attenuation effects might be an artifact in that HMBA actually accelerates the degradation of SNOs and thus should not be used concomitantly. Therefore, we pre-exposed the vessels to HMBA to block extracellular thiols prior to the addition of SNOs for vasodilation. After washing away unreacted HMBA, vasodilatory responses to either L- or D-cysNO were unchanged (Fig. S3A and B). These results do not support the involvement of cell membrane surface thiols in the vasorelaxation. However, it is important to note that although HMBA blocked > 30% of the extracellular surface thiols (Fig. S3C) for a period of time that was longer than required for the wire myography experiments (< 60min), a large portion of extracellular thiols was not affected by the HMBA. Therefore, the HMBA tests cannot exclude the involvement of an extracellular thiol.

To test the role of free NO outside the cell, because the impermeable NO-scavenger CPTIO had no effect, Q) S-nitrosothiol to extracellular thiols, as the thiol blocker HMBA did not affect, D) conversion to membrane permeable thionitrosative compound H_{2}S donor Na_{2}S did not potentiate vasodilation and also the H_{2}S synapse inhibitor PAG even significantly left-shifted the GSNO dose response curve (p = 0.043), E) conversion to L-cysNO which can cross the membrane via L-type amino acid transporters (LAT), because the LAT blocker threonine (Thr) had no effect, or F) hydrolyzation by Y-glutamyl transpeptidase (Y-GT) to L-cysNO-gly which can cross the membrane via dipeptide transporters (PEPT2), as the Y-GT blocker GSM had no effect. N = arterial rings from ≥5 animals.
The role of the LAT was tested by performing dose response curves in the presence of the competitive LAT inhibitors BCH and Thr. Rather than desensitize vasodilation as would be expected if uptake of L-cysNO as an intact molecule was required, these inhibitors both sensitized the vasodilatory effects of L- and D-cysNO (Fig. S4 and Table S2), indicating that uptake of SNOs may actually desensitize vasodilation instead.

We then performed wire myography experiments with mesenteric arteries using GSNO, which is impermeable to vascular smooth muscle cell membranes but a potent vasodilator nonetheless [38]. Again, as in femoral arteries, ODQ right-shifted the GSNO dose response curve indicating a critical role for the NO/cGMP pathway in GSNO-mediated vasodilation (Fig. 1 and Table S3). The membrane-impermeable NO scavenger CPTIO, which abolishes NO-mediated vasodilation (Fig. S5), did not alter the responses to GSNO, indicating that the GSNO-mediated vasodilation was not dependent on NO released outside the cell. Likewise, HMBA (impermeable thiol blocker), Thr (competitive LAT inhibitor) did not alter the responses to GSNO, indicating that the GSNO-mediated vasodilation was not dependent on NO released outside the cell. Likewise, HMBA (impermeable thiol blocker), Thr (competitive LAT inhibitor), and GSM (Y-GT inhibitor) did not have any measurable effects suggesting that the relaxation might be independent of membrane surface thiols, LAT, and Y-GT, respectively (Fig. 1, and Table S3). The H_{2}S source Na_{2}S did not potentiate vasodilation, and H_{2}S synthetase inhibitor PAG did not attenuate vasodilation but actually left-shifted the GSNO dose response curves (p = 0.0429), indicating that HSNO formed by transnitrosation of H_{2}S by GSNO does not potentiate but may actually attenuate vasodilation.

To test the possibility that GSNO-mediated vasodilation might be occurring by real-time activation of L-arginine-dependent NOS within the vascular smooth muscle, wire myography experiments were conducted in the presence of L-NAME, a non-selective NOS inhibitor. L-NAME added 15 min before contraction did not alter GSNO-mediated vasodilation (Fig. S6), indicating no detectable role for NOS in GSNO-mediated vasodilation.

3.2. Pre-exposure of arteries to GSNO attenuates vasodilatory responses to GSNO but not NO

The above evidence against the various means by which SNOs might trigger vasodilation led us to experiments designed to further characterize the nature of GSNO-mediated vasodilation. We hypothesized that the mechanism involved signaling steps upstream from sGC activation, and that these steps would be attenuated by repeated exposure of the vessel to SNOs. Consistent with this idea, pretreatment of isolated arteries with GSNO resulted in attenuation of the maximal vasodilatory response to subsequent exposures to GSNO, with no significant effect on the EC_{50} (Fig. 2B, F, and G) or the basal contractility (Fig. S7). In contrast, pretreatment with GSNO did not alter dose responses to NO itself, indicating the attenuating effects of GSNO pretreatment were upstream from sGC activation. In addition, pretreatment of the vessels with NO (Fig. 2B), GSH, or blank control of GSNO (Fig. S8) instead of GSNO did not alter the GSNO dose-response curve, suggesting that the attenuation response is unique to pretreatment with SNOs as opposed to NO or other species derived from the SNOs. Together with the finding that GSNO mediates vasodilation by the NO/cGMP pathway (Fig. 1A and [24]), these results are all consistent with the idea that GSNO-mediated vasodilation involves signaling steps which are attenuated by repeated exposure to GSNO, and that these steps lie upstream from sGC and thus are not involved in vasodilation by NO itself (Fig. 2C).

We next tested the effects of nitrite on SNO-mediated vasodilation. We observed that the presence of 1 μM nitrite to the bath markedly increased the maximum vasodilation caused by GSNO (Fig. 2D and 2F). In addition, the attenuation of vasodilation caused by pretreatment of the vessels with GSNO was reversed by exposure of the vessels to 1 μM nitrite (Fig. 2E and F), a concentration more than two orders of magnitude below that required for nitrite itself to cause vasodilation [17].

To test whether the potentiating effects of nitrite were specific to SNO-mediated vasodilation we studied the effect of 1 μM nitrite on dose response curves to fasudil, an NO- and sGC-independent vasodilator. Nitrite did not potentiate vasodilation by fasudil, or by NO itself (Fig. S10), suggesting that the potentiating effects of nitrite are specific to SNO-mediated vasodilation, and lie upstream from the activation of sGC by intracellular NO.

3.3. Effect of nitrite on SNO-mediated vasodilation in sheep in vivo

Contrary to the potent vasorelaxation caused by SNO in isolated arteries, we observed no vasodilation in response to GSNO and D-cysNO, and only weak vasodilation in response to L-cysNO infused into the femoral artery of L-NAME-treated sheep [37]. Thus, because NOS inhibition is known to lower circulating nitrite concentrations [44,45], we hypothesized that the lack of vasodilatory response to SNOs in our previous in vivo experiments [37] was due to decreased nitrite available to facilitate vasodilation by SNOs. We measured basal plasma nitrite concentrations before infusion of L-NAME (0.22 ± 0.02 μM), after infusion of L-NAME (0.12 ± 0.05 μM), and following intravascular infusion of nitrite (0.40 ± 0.17 μM, a level not vasoactive per se in sheep [17]). We observed that the vasodilatory effects of L-cysNO were markedly increased under baseline conditions compared to responses following L-NAME administration. Furthermore, we found that infusions of nitrite to L-NAME-treated animals resulted in markedly increased vasodilatory responses of the femoral artery to infusions of GSNO, D-cysNO, and L-cysNO (Fig. 3B, C, and 3D). These results in the sheep hind limb are consistent with our myography experiments in that SNO-mediated vasodilation is augmented by nitrite.

3.4. Role of nitrite on GSNO-mediated vasodilation in anesthetized rats

Our previous observation that the sensitivity of sheep arteries to nitrite-mediated vasodilation is nearly 2 orders of magnitude less than that of rat arteries [17] raises the possibility of species-specific pathways of nitrite and SNO-signaling. We therefore further tested the interaction between SNO and nitrite in adult rats. Intraperitoneal injections of L-NAME for four days to lower nitrite levels resulted in a decrease in nitrite concentrations in the wall of the femoral arteries from 0.11 ± 0.01 to 0.07 ± 0.01 μM/mg protein (p = 0.0017). The plasma nitrite concentrations were also decreased by L-NAME (from 0.27 ± 0.09 μM to 0.09 ± 0.02 μM) but were restored (to 0.37 ± 0.06 μM) by infusions of nitrite (Fig. 4E). In contrast to sheep that were unresponsive to nitrite itself, femoral arterial conductance of the anesthetized rats increased significantly from baseline in response to nitrite infusion (p = 0.01), although no significant difference was observed between saline controls and nitrite (p = 0.22; Fig. 4B). Similar to sheep, the vasodilatory effects of GSNO were absent in rats pretreated with L-NAME, but were restored by infusions of nitrite (Fig. 4D). The increased vasodilatory response to GSNO following pretreatment with L-NAME + nitrite could not be explained by a greater baseline vascular tone against which the GSNO could act because the baseline arterial conductances in these animals were already greater than those of controls and greater than those animals treated only with L-NAME (Fig. 4C). These results are all consistent with the idea that GSNO-mediated vasodilation involves signaling pathways that are at least partially facilitated by the presence of nitrite.

3.5. GSNO stimulates higher cGMP levels in the presence of nitrite

To further test the hypothesis that nitrite enhances signaling of GSNO through the sGC pathway, we measured the effects of nitrite on cGMP levels in sheep femoral arteries after exposure to GSNO (Fig. 5A). GSNO stimulated greater increases in intracellular cGMP concentrations when nitrite was present (Fig. 5B), consistent with the idea that the synergistic effects of GSNO and nitrite involve cGMP-mediated signaling.
3.6. No detectable effect of nitrite on intracellular NO production from GSNO

The observation that GSNO mediates vasodilation by activation of sGC led to the hypothesis that GSNO produces increase in intracellular free NO by mechanisms that are augmented in the presence of nitrite. To test this possibility, we performed experiments to measure the effect of GSNO and nitrite on concentrations of free NO or NO metabolites. First, we found that although low levels of NO are released from GSNO added to PBS (pH 7.4; ~1% NO), there was no increase in the release of free NO by mechanisms that are augmented in the presence of nitrite.

Fig. 2. Tachyphylaxis to repeated GSNO exposure, and potentiation by nitrite. A) Experimental protocol used to study changes in the GSNO dose-response curves of sheep mesenteric artery. Isolated arterial rings were exposed to three 15-min incubations with 5 μM GSNO (GSNOpreTx) followed by constriction with KCl and then GSNO dose response curves. Comparisons were made to vessels that were not pre-exposed to GSNO (controls) or vessels pre-exposed to 5 μM NO (NOpreTx) instead of GSNO. To test for the effects of nitrite, some GSNOpreTx vessels were exposed to 1 μM nitrite for 30 min (GSNOpreTx + nitrite) prior to the constriction. B) Vasodilatory responses to GSNO were diminished after pretreatment with GSNO but not pretreatment with NO. C) NO dose response curves are not affected by pretreatment with GSNO or NO, suggesting that the tachyphylaxis to GSNO-mediated vasodilation does not involve signaling downstream from sGC activation. D) Addition of 1 μM nitrite to control vessels potentiated GSNO-mediated vasodilation in vessels that were not pretreated with GSNO. E) Addition of 1 μM nitrite to GSNO pretreated vessels reversed attenuated vasodilatory responses to GSNO. F) The decrease of Emax (an index of overall vasodilation) by GSNO pretreatment was restored by nitrite. G) EC50 (an index of sensitivity to GSNO) was not significantly changed. N = arterial rings from ≥5 animals. *P < 0.05, **P < 0.001 for changes relative to Control (no GSNO pretreatment).
NO upon addition of up to 50 μM nitrite (Fig. S9), suggesting nitrite does not directly stimulate the release of NO from GSNO. In addition, we observed no difference in free NO release (limit of detection < 25 nM) between isolated arteries with and without nitrite pretreatment (Fig. S11). Based on recent evidence that a major portion of intracellular NO may be stored in the form of dinitrosyl iron complexes (DNICs) [41,42], we tested whether DNIC concentrations varied in homogenates of arteries following exposure to GSNO, nitrite + GSNO, or buffer controls. In all cases, DNIC levels were below the lower limit of quantification (1 μM) by EPR (Fig. S12D). We also tested for evidence of changes in concentrations of other NO metabolites following exposure of isolated arteries to GSNO, nitrite + GSNO, or buffer controls. In all cases, DNIC levels were below the lower limit of quantification (1 μM) by EPR (Fig. S12D). We also tested for evidence of changes in concentrations of other NO metabolites following exposure of isolated arteries to GSNO with or without nitrite. We did not detect any significant difference in NO metabolites in homogenates of vessels subjected to the above treatments, as measured by triiodide chemiluminescence, which detects NO, nitrite, SNOs, DNICs, and iron nitrosyls, but not nitrate (Fig. S12).

Finally, the fluorescent NO probe DAF-FM did not detect a measurable increase of intracellular free NO or its oxidation products (lower limit of detection > 16.5 μM) in isolated smooth muscle cells following GSNO stimulation (Fig. S13 and [37]). Thus if GSNO stimulated any increases in intracellular NO, they were below current limits of detection with our methodologies.

4. Discussion

The current study reveals several important aspects of SNO-mediated vasodilation. Consistent with previous reports [24], we find that the vasodilation occurs primarily via activation of sGC. However, the current experiments fail to support previously proposed pathways (Fig. S1) [27–35]. In addition, our results demonstrate that vasodilation by GSNO involves signaling upstream of sGC that is attenuated by repeated exposure of the vessel to GSNO. We also present the novel observation that sub-vasoactive amounts of nitrite markedly potentiate the vasodilatory effects of SNOs.

It is worth noting that the overall effect of SNOs on vascular tone is likely a complex result of several concomitant processes. For example, vasodilation caused by the decomposition of L-cysNO into NO under simplified wire myography conditions (Fig. 2E) may be significantly hindered in vivo due to scavenging of free NO by hemoglobin. Also, uptake of L-cysNO into the cell via the LAT may be followed by the activation of sGC due to NO release [33]. At the same time, the L-cysNO taken into the cell (Fig. S4) may cause desensitization to vasodilation via S-transnitrosation of sGC and/or increases in oxidative stress [33,46,47]. The relative importance of each of these processes will likely differ between different experimental conditions, animal species, and SNOs. One example of such multiplicity of SNO activities is our observation that all three SNOs exhibited potent vasoactivity in vitro (Fig. S4), but only L-cysNO caused vasodilation in vivo in our L-NAME treated sheep (Fig. 3). Further study of this multiplicity is needed.

4.1. Cross-membrane vasodilatory signaling by SNOs

While a majority of evidence indicates that activation of sGC is a primary factor in SNO-mediated vasodilation, how SNOs signal across
the plasma membrane to activate sGC has been unclear. Perhaps the most obvious possibility would be that it is the NO moiety on the SNO that enters the cell to activate sGC. However, this possibility is not supported by a number of our results. First, despite GSNO and D-cysNO being less membrane permeable than L-cysNO [32], there is no difference between the dose response curves of these SNOs (Fig. 1, Fig. S2 and [38]). Second, NO is released from GSNO at a rate several-fold slower than from L- and D-cysNO [48], yet, the dose response curves of these three SNOs are similar [38], indicating that the release of free NO from the SNOs outside the cell is not a rate-limiting factor. This argument is further supported by the observation that CPTIO, an extracellular NO scavenger, did not modify the dose response curve of GSNO (Fig. 1B), providing strong evidence that the release of free extracellular NO is not a requisite factor in SNO-mediated vasodilation. Besides, free NO-mediated vasodilation is of short duration whereas SNO-mediated vasodilation is long lasting (Fig. S14). This temporal difference suggests that SNO may activate sGC via direct means or at least via intermediate steps that do not involve the presence of free NO outside the cell. In addition, in contrast to free NO, which diffuses freely and rapidly across cell membranes [2], the membrane-impermeable GSNO is a poor NO donor [36] and thus intracellular NO concentrations are expected to be markedly lower following application of GSNO compared to NO (Fig. S13). However, the sensitivity of arteries to these two compounds was similar (Fig. 2B, C), suggesting that vasodilation by GSNO occurs either

Fig. 4. Effect of L-NAME and nitrite on femoral conductance responses to GSNO in rats. A) Rats were given L-NAME for 4 days (60 mg kg^{-1} day^{-1}, i.p.) to block endogenous NOSs activity and thereby lower plasma nitrite levels. Animals that received nitrite infusions were then compared to those that received no nitrite. Femoral conductance was then recorded while increasing doses of GSNO were infused into the lower abdominal aorta. B) Saline infusion did not increase the femoral arterial conductance (p = 0.12), whereas nitrite did (p = 0.01), although no significant difference was observed between saline and nitrite (p = 0.22). C) L-NAME pretreatment decreased baseline femoral arterial conductance compared to controls (no L-NAME or nitrite), an effect that was reversed by treatment with nitrite. D) Control animals responded to GSNO infusions with increases in femoral vascular conductance. This effect was lost in animals treated with L-NAME to lower nitrite levels, and then restored in L-NAME-treated animals that were also given exogenous nitrite to replenish plasma levels. E) L-NAME pretreatment decreased baseline plasma nitrite concentrations, whereas nitrite infusions returned it to control levels. Average results from 5 or more animals; *P < 0.05, **P < 0.01, ***P < 0.001.
at lower intracellular concentrations of free NO, or by utilization of NO from a source other than the applied GSNO per se. Together with the negative results from the tests of several previously proposed mechanisms (Fig. S1) by which the NO moeity from the extracellular GSNO enters the cell, and with the novel evidence of a role for nitrite, these results call for the consideration of novel hypotheses for the mechanism of SNO-mediated vasodilation, as discussed below.

4.2. Tachyphylactic response to repeated GSNO exposure

In vitro, we find evidence that the vasodilatory response to GSNO is decreased following repeated exposure of the vessel to GSNO (Fig. 2B). One possible explanation would be that GSNO exposure has resulted in attenuation of one or more of the components of the sGC-mediated vasodilatory pathway. However, this possibility is not supported by the observation that vasodilatory responses to free NO itself remain unchanged (Fig. 2C), indicating the attenuating effects of GSNO pre-treatment are upstream of sGC activation, or that GSNO activates sGC by a mechanism that differs from that of free NO. It is also possible that pre-exposure to GSNO results in attenuation of one or more mechanisms that may be involved in the uptake or handling of GSNO in a way that facilitates its activation of sGC. Although the current experiments do not support commonly proposed mechanisms for GSNO-mediated activation of sGC (Fig. 1 and Fig. S1), the possibility of undiscovered pathways, such as those discussed below, cannot be discounted.

4.3. Synergistic vasodilation by nitrite and SNOs

S-nitrosothiols [7,49] and nitrite [5] have each been argued to be endocrine carriers of NO bioactivity [10]. Intriguingly, although much early emphasis was placed on the conversion of nitrite to NO in blood, evidence from others and us suggests that the vascular wall plays an important role in the bioactivation of nitrite to produce vasodilation [17,19]. This compartment provides a potential site for cross talk between SNOs and nitrite. Our evidence that the circulating SNOs in blood cross talk with nitrite in the vascular smooth muscle cells to cause vasodilation suggests that SNOs and nitrite may work synergistically rather than independently as endocrine NO adducts. However, it is important to note that the present work does not rule out possible roles for blood in the bioactivity of nitrite or SNOs [5,7].

Several viable hypotheses can be proposed to explain the novel interaction observed between SNOs and nitrite. For example, endocytotic activity is known to be particularly high in the plasma membrane of vascular smooth muscle cells [50], and thus it is possible that GSNO (and other SNOs) are taken into the vascular smooth muscle cell via endocytosis. A targeted delivery of GSNO to cytosolic sGC via endocytotic vesicles would be consistent with our evidence of no involvement of extracellular free NO in GSNO-mediated vasodilation, and with our observation of similar potencies of SNOs with varying degrees of membrane permeability. In addition, binding of endocytotic vesicles is facilitated by dynamin, a GTPase that is activated by S-nitrosation [51]. Depletion of active dynamin could explain the tachyphylactic response to repeated GSNO exposure, and the ability of nitrite to play a role in S-nitrosation of membrane-associated proteins via nitrated lipid intermediates [52,53] would constitute a model consistent with the results of this study.

Another possibility involves a role for oxidative stress in response to exposure of cells to SNOs. Our observation of a diminished vasodilatory response following repeated exposure of arteries to GSNO is reminiscent of the more widely known tolerance effect that develops as a result of repeated exposure to organic nitrates such as nitroglycerin. Although the mechanism for such tolerance is still not fully understood, stimulation of oxidative stress that would serve to dampen vasodilation is proposed to play a role [54,55]. Depletion of intracellular anti-oxidative reserves could explain the tachyphylactic response to repeated GSNO exposure, and the known ability of nitrite to attenuate reactive oxygen species production [56–58], would also be consistent with the current results.

A third possible hypothesis would propose that SNOs stimulate the release of NO or an NO equivalent from a preformed intracellular store in vascular smooth muscle cells (Fig. S15). The existence of preformed intracellular NO stores has been appreciated for over three decades, beginning with the observation that endothelium-denuded vessels relax in response to light [59,60], and that this phenomenon involves activation of sGC [61,62]. Similar to our findings for SNO-mediated vasodilation, a tachyphylactic phenomenon is observed upon repeated exposure of the arteries to light [63,64]. Nitrite itself is unlikely to be more than a precursor for the production of more bioactive NO adducts, as supraphysiological concentrations of nitrite are required in order to stimulate vasodilation in isolated sheep arteries [17]. However, by reacting with a number of metal-containing enzymes found in vascular smooth muscle cells [65], nitrite can serve as a source of NO adducts, such as SNOs and DNICs, that may serve as a depot form of stored NO bioactivity. SNOs have been implicated as the source of intracellular NO that mediates photorelaxation [66]. However, our in vitro results offer little support for this possibility since inhibition of cellular uptake of SNOs did not attenuate but instead facilitated the vasodilation (Fig. 1D and Fig. S4 C-F). DNICs, which have recently been proposed to serve as a major intracellular NO adduct [42], are capable of activating sGC [67]. However, as demonstrated by our attempts to measure DNICs in arteries (Fig. S12D), more sensitive assay methods are needed to explore this possibility further. It is also not known how extracellular SNOs might interact with the cell to promote activation of an intracellular NO store.

These and other possible explanations for the synergistic effects of nitrite and SNOs warrant further studies that may lead to a deeper understanding of how endothelium- and sGC-mediated vasodilation is regulated.

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Disclosure

Dr. Power is listed as a co-inventor on a NIH patent for the use of inhaled nitrite for cardiovascular conditions. The remaining authors report no conflicts.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.niox.2018.01.011.

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