L-NAME releases nitric oxide and potentiates subsequent nitroglycerin-mediated vasodilation

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ABSTRACT

L-N^G-Nitro arginine methyl ester (L-NAME) has been widely applied for several decades in both basic and clinical research as an antagonist of nitric oxide synthase (NOS). Herein, we show that L-NAME slowly releases NO from its guanidino nitro group. Daily pretreatment of rats with L-NAME potentiated mesenteric vasodilation induced by nitrodilators such as nitroglycerin, but not by NO. Release of NO also occurred with the NOS-inactive enantiomer D-NAME, but not with L-arginine or another NOS inhibitor L-NMMA, consistent with the presence or absence of a nitro group in their structure and their nitrodilator-potentiating effects. Metabolic conversion of the nitro group to NO-related breakdown products was confirmed using isotopically-labeled L-NAME. Consistent with Fenton chemistry, transition metals and reactive oxygen species accelerated the release of NO from L-NAME. Both NO production from L-NAME and its nitrodilator-potentiating effects were augmented under inflammation. NO release by L-NAME can confound its intended NOS-inhibiting effects, possibly by contributing to a putative intracellular NO store in the vasculature.

1. Introduction

The identification of nitric oxide (NO) as endothelium-derived relaxing factor stimulated a surge of interest in studying this bioactive molecule produced endogenously from L-arginine by NO synthase (NOS). Due to their NOS inhibiting effects, several L-arginine analogues have been useful pharmacological tools in NOS-related research. Of all such NOS antagonists, L-N^G-nitro arginine methyl ester (L-NAME) has been the most widely used [1]. It has been used to create "NO-deficient hypertension" in many animal models, including rats. Several clinical trials (clinicaltrials.gov) have also been launched to test its therapeutic potential for diseases involving over production of NO (e.g. septic shock).

Despite its use as a NOS antagonist, there are numerous reports of L-NAME inducing responses that are inconsistent with NOS inhibition [2]. Several non-canonical actions of L-NAME have been proposed, including sympathetic activation [3], reactive oxygen species (ROS) generation [4], and paradoxically, increased NO production [2,5]. Supported by the up-regulation of NOS expression and activity measured in vitro, feedback NO production via NOS activation by L-NAME has been proposed, although it is unlikely that L-NAME increases the overall NO production from NOS in vivo [2]. On the other hand, the possibility of direct NO production from reduction of L-NAME has also been implied [6]. Some L-arginine analogues, including L-NAME, are notorious contaminants in the vanadium-based reductive assays of nitrite and nitrate [7], illustrating that this reduction possibility is at least chemically feasible. Indeed, production of NO from L-NAME was found in reactions with various agents, especially sodium ascorbate (NaAscH), a reducing agent that can also promote Fenton chemistry via production of H$_2$O$_2$ [8,9]. However, the chemical mechanism of L-NAME reduction, its biological relevance, and functional implications are unknown.

Nitroglycerin (NTG) [18] and NO congeners such as glutathione-liganded binuclear dinitrosyl iron complex (BDNIC; a candidate compound for treatment of hypertension [10]) and S-nitroso-glutathione (GSNO) all cause vasodilation via activation of soluble guanylate cyclase (sGC) [11,12]. These compounds are classified as nitrodilators and are often considered to be NO donors [13,14]. However, these nitrodilators represent a wide range of chemical classes, molecular sizes,

Abbreviations		L-NNA	L-N^G-nitro arginine
		MAP	mean arterial blood pressure
BDNIC	glutathione-liganded binuclear dinitrosyl iron complex	NaAscH	sodium ascorbate
D-NAME	D-N^G-Nitro Arginine methyl ester	NO	nitric oxide
EC$_{50}$	one-half maximal response concentration	NOS	NO synthase
E$_{max}$	maximal response	NOx	NO metabolites
EPR	Electron paramagnetic resonance	NTG	Nitroglycerin
GC-MS	Gas Chromatography-Mass Spectrometry	p.o.	oral
GSNO	S-nitroso-glutathione	ROS	reactive oxygen species
i.p.	intraperitoneal	sGC	soluble guanylate cyclase
L-NAME	L-N^G-nitro arginine methyl ester	SNO	S-nitrosothiols
L-NMMA	L-N^G-Monomethyl Arginine	SOD1	superoxide dismutase 1

and stoichiometries, and the mechanisms by which they lead to sGC activation remain unclear. Numerous attempts to ascribe their vasodilatory activity to the release of their NO moiety have failed [12,15,16], casting doubt on their role as NO donors or prodrugs. These discrepancies highlight a critical gap in our understanding of the mechanisms by which nitrodilators lead to sGC activation.

In this study, we tested the hypothesis that NO is released from the guanidino nitro group of L-NAME and participates in subsequent nitrodilator-mediated vasodilation. To test this hypothesis, we measured, in adult rats, whether four days of exposure to L-NAME would potentiate vasodilatory responses to NTG, BDNIC, and GSNO. By comparison with other L-arginine analogues, we tested whether the guanidino nitro group of L-NAME is required for the potentiation effects. Using isotopically-labeled L-NAME, we also examined the role of the nitro group in the production of NO via NaAscH-mediated reduction. In addition, based on evidence of a role for Fenton chemistry, we investigated whether ROS would facilitate both NO production from L-NAME and the nitrodilator-potentiating effects.

2. Material and methods

2.1. Chemicals

P-Rhod [20] was kindly provided by Dr. Nakagawa (Nagoya City University, Japan). CellROX™ was purchased from Thermo Fisher Scientific, Inc. (USA). L-N^G-Monomethyl Arginine (L-NMMA) was purchased from AG Scientific, Inc. (San Diego, CA). Two forms of ^{15}N-labeled L-NAME and L-N^G-nitro arginine (L-NNA) were synthesized in our laboratory: 1) an ^{15}N-nitro group on an ^{14}N-L-arginine backbone (^{15}N nitro + ^{14}N Arg), and 2) an ^{14}N-nitro group on an ^{15}N-L-arginine backbone (^{14}N nitro + ^{15}N Arg). Details of the synthesis are given in the **Supplementary Material**. Unlabeled L-NAME (with all nitrogens as ^{14}N) and other chemicals were obtained from Sigma Aldrich (St Louis, MO). HEPES buffer (pH = 7.40) contained 10 mmol/L HEPES, 10 mmol/L glucose, 137 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl$_2$, and 1 mmol/L MgCl$_2$. Phosphate-buffered solutions (PBS; 300 mOsM) at different pH were prepared with Na$_2$HPO$_4$ and NaH$_2$PO$_4$. All water solutions were prepared with ultrapure water from Millipore (Merck KGaA, Germany) at conductivity of ~0.050 μS/cm.

Contaminating amounts of NO metabolites (NOx), measured by triiodide based chemiluminescence, in 50 mmol/L of L-NAME, ^{15}N-nitro-L-NAME, and ^{15}N-Arg-L-NAME were 0.05 ± 0.01, 0.07 ± 0.01, and 0.62 ± 0.02 μmol/L, respectively (including a background of 0.02 ± 0.02 μmol/L in HEPES buffer). Using gas chromatography-mass spectrometry (GC-MS), contaminating amounts of ^{15}N-nitrite and ^{15}N-nitrate in 50 mmol/L ^{15}N-L-NAME with ^{15}N nitro + ^{14}N Arg (itself was not detectable in GC-MS) were measured to be below the limits of quantification at 5 and 500 nmol/L, respectively. The purity of synthesized ^{15}N-L-NAME with ^{15}N nitro + ^{14}N Arg was estimated to be ~99% by NMR. Further confirmation of successful ^{15}N-L-NAME

synthesis was achieved with LC-MS^2 (Supplementary Fig. 1; for ^{1}H, ^{15}N, and ^{13}C NMR results see **appendix 1** of **Supplementary Material**).

2.2. Preparation of BDNIC and GSNO

BDNIC and GSNO were prepared as previously reported [10,21]. Details are given in the **Supplementary Material**.

2.3. Surgical procedures in rats

Rodent and sheep (below) protocols were pre-approved by the Institutional Animal Care and Use Committee of Loma Linda University, and were in accordance with guidelines of the American Physiological Society and the National Institutes of Health. Female Sprague-Dawley rats weighing 301 ± 5 g were surgically instrumented as previously reported [16], except that the flow probe was placed around the mesenteric instead of the femoral artery. After surgical instrumentation, isoflurane was discontinued and anesthesia was maintained with an intraperitoneal injection (i.p.) of urethane (800 mg/kg) that was supplemented thereafter as required. A bolus of hexamethonium (1 mg·kg^{-1}, iv) followed by a continuous infusion (2 mg·h^{-1}·kg^{-1}, iv) was given to limit neural influences on vascular tone. Details are given in the **Supplementary Material**.

2.4. Experimental protocols in rats

Rats were divided into one of several study groups. Each group received an i.p. injection daily for four days prior to and including the day of the experiment. The injectate was one of the following: L-NAME, D-N^G-Nitro Arginine methyl ester (D-NAME), L-NMMA, L-arginine, L-NAME + catalase, L-NMMA + catalase, and catalase or vehicle (saline; Control). An additional group received L-NAME by oral gavage (p.o.). L-arginine analogues and catalase were administered at 222 μmol·kg^{-1} and 16.5 mg·kg^{-1}, respectively.

After the surgery the rat was allowed to rest for 30 min to obtain a stable baseline. Then, BDNIC (25 μmol/L), NTG (50 μmol/L), or GSNO (50 μmol/L) was infused at rates of 0.05, 0.1, 0.2, and 0.4 ml/min, increasing every 3 min. An additional group of intact rats received a single i.p. injection of L-NMMA.

For wire myography and measurements of arterial tissue NOx concentrations, mesenteric arteries were isolated from rats that did not receive vasodilator infusion.

2.5. Wire myography

Both rat and sheep mesenteric arteries were dissected from a portion of the mesenteric artery supplying the duodenum. Rat mesenteric arteries (~150 μm diameter and 4 mm long) were used to measure the vasodilatory effects of BDNIC, NTG, GSNO, and NO, while those of sheep (2 mm diameter and 5 mm long) were used to test endothelium-

dependent relaxation induced by bradykinin. For both rat and sheep arteries the endothelium was kept intact. Arterial rings were mounted in organ bath chambers as described previously [16]. To inhibit endothelial NOS in rat mesenteric arteries, L-NAME (100 μmol/L) was added to the baths 15 min before contraction with 10 μmol/L serotonin. Sunlight and strong ambient light were avoided during the experiments. Further details are provided in **Supplementary Material**.

2.6. Cell experiments

Macrophage RAW 264.7 cells were cultured as reported [17]. Cells within 20 passages were incubated under normoxia (21% O_2/5% CO_2) for 10 h in the presence and absence of 1 mmol/L L-NAME, 1 mmol/L L-NMMA, 1 μmol/L antimycin A, 100 μmol/L deferoxamine, 10 μmol/L resveratrol, 800 U/ml PEG-catalase, or 800 U/ml catalase. A separate group (hypoxia group) of cells was incubated under two successive cycles of 2.5 h of hypoxic (1% O_2/5% CO_2) and 2.5 h normoxic conditions. Cells were harvested by scraping and lyzed in PBS for NOx measurement. To measure ROS levels, 2.5 μmol/L CellROX™ was added into the culture at the 6th hour, incubated for 0.5 h, and washed away with three PBS rinses before fluorescence measurement.

2.7. Analytical methodologies

NOx and nitrite concentrations were determined by triiodide-based chemiluminescence (280i, Sievers, Boulder, CO) as previously described [16]. Briefly, overall NOx concentrations were measured by direct injection of the sample into triiodide and nitrite concentrations were taken as the portion of the signal that was eliminated by prior reaction of the sample with acid sulfanilamide. In addition to measuring NO itself, the triiodide reagent facilitates detection of NO released from several types of NO adducts, including nitrite, S-nitrosothiols (SNO), and nitrosyl-iron complexes [17], but excluding nitrate.

Electron paramagnetic resonance (EPR) signals were recorded at room temperature using a Bruker X-Band EMX Plus EPR spectrometer with a cavity of high sensitivity as previously described [10]. The EPR was set to a microwave power of 20 mW, microwave frequency of 9.34 GHz, attenuator of 10 dB, modulation amplitude of 1 G, modulation frequency of 100 kHz, time constant of 20.48 msec, conversion

time of 81.92 msec, harmonic of 1, and number of scans of 2. $(MGD)_2Fe^{2+}$ (10 mmol/L MGD and 0.5 mmol/L Fe^{2+}) was prepared freshly in a glove box by dissolving $FeCl_2$ powder in Argon deoxygenated MDG solution.

^{15}N-labeled NOx were measured under negative-ion chemical ionization mode by GC-MS (Agilent; 6890-5973) as previously described [22]. Ferricyanide and $HgCl_2$ were added during sample preparation to facilitate conversion of NO, SNO, and nitrosyl-iron complexes into nitrite, which was derivatized as described [22]. Therefore, the term ^{15}N-NOx as used here represents any of several NO adducts that contribute to nitrite production by reacting with $HgCl_2$ and ferricyanide. Details of the method are provided in the **Supplementary Material**.

HNO and ROS were evaluated using fluorescence probes P-Rhod (10 μmol/L, Ex/Em = 491/526 nm) and CellROX™ (2.5 μmol/L, Ex/Em = 485/520 nm), respectively. Fluorescence intensity was measured using a Tecan Spark® platform.

2.8. Statistics

Average values are given as mean ± SEM. Two-way ANOVA was used to compare responses in the different infusion groups. One-way ANOVA with Tukey post hoc analysis was applied as noted in figure legends. Statistical analyses were carried out with Prism, v5.0c (Graphpad Software, La Jolla, CA) with significance accepted at $p < 0.05$.

3. Results

3.1. L-NAME potentiates BDNIC-, NTG-, and GSNO-mediated vasodilation of mesenteric arteries

Prior L-NAME treatment in rats potentiated the relaxation of mesenteric arteries by BDNIC, NTG, and GSNO both in vivo and in vitro (Fig. 1A-F), with unaltered one-half maximal response concentration (EC_{50}) but significantly larger maximal response (E_{max}) (Supplementary Table 1). However, the potentiation effects were not observed for NO itself (Fig. 1G), suggesting that the potentiation results from changes that lie upstream rather than downstream of sGC.
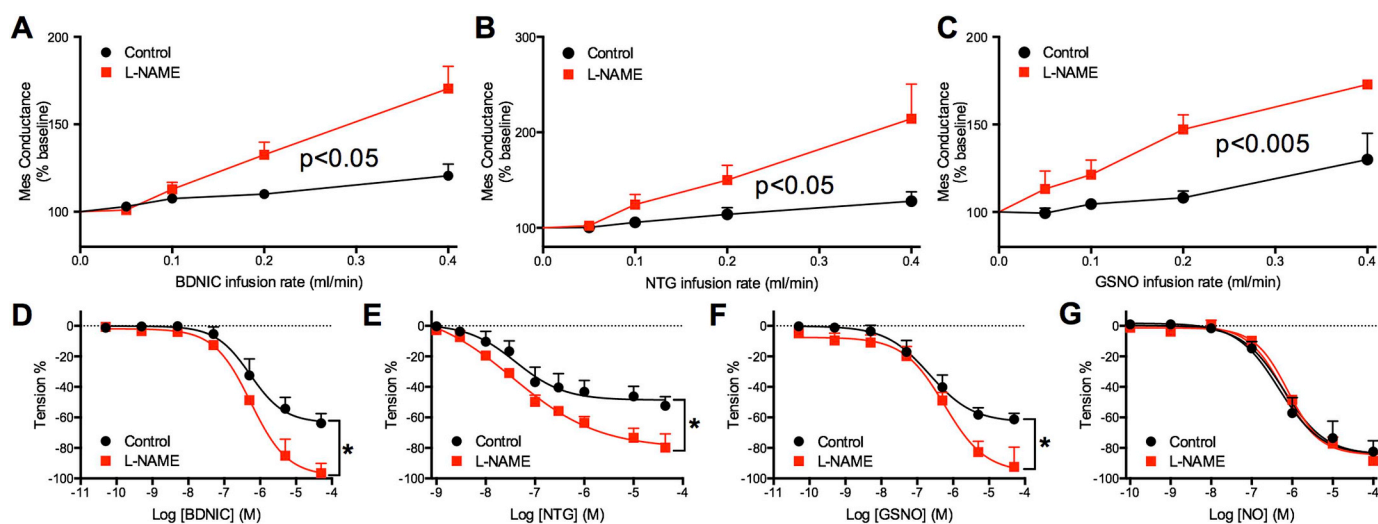

Fig. 1. L-NAME potentiates BDNIC-, NTG-, and GSNO-mediated vasodilation of rat mesenteric arteries both in vivo and in vitro. Rats were given L-NAME (222 μmol·kg^{-1}·day^{-1} for 4 days, i.p) and their results were compared to those given saline (control). **A-C)** L-NAME pretreatment increased mesenteric conductance (blood flow divided by pressure; an index of vasodilation) in response to stepwise-increased (3 min for each infusion rate) continuous infusion of **A)** BDNIC (25 μmol/L), **B)** NTG (50 μmol/L), and **C)** GSNO (50 μmol/L). Two-way ANOVA, $n \geq 5$. **D-G)** Prior L-NAME treatment in rats potentiates the relaxation of isolated mesenteric arteries induced by **D)** BDNIC, **E)** NTG, and **F)** GSNO, but not **G)** NO. *t* test, $n \geq 4$.

3.2. The nitro group of L-arginine analogues, rather than NOS-modulating activity, corresponds with the potentiation of BDNIC- and NTG-mediated vasodilation

To test for the mechanism by which L-NAME potentiates nitrodilator-mediated vasodilation, we examined the effects of pretreatment with other L-arginine analogues. These included the enantiomer D-NAME, generally considered as NOS-inactive and thus commonly used as a negative control of L-NAME, the NOS substrate L-arginine, and the NOS inhibitor L-NMMA [23–25]. L-NAME and D-NAME have a nitro group, L-arginine does not, and L-NMMA contains a guanidino methyl group instead of nitro group [23]. Similar to L-NAME, D-NAME pretreatment also potentiated subsequent BDNIC- and NTG-mediated vasodilation in rats, whereas L-arginine and L-NMMA did not (Fig. 2A-B). The nitrodilator-potentiating effects of L-NAME were also observed after oral, the most commonly used route of medicine administration in clinics, as well as after intraperitoneal administration (Fig. 2A-B).

3.3. Effects of L-arginine analogue pretreatment on baseline hemodynamics and nitrite levels

NOS inhibition has been shown to raise blood pressure, increase vascular tone, and reduce NO production [6,16,23,26]. We therefore measured the mean arterial blood pressure (MAP), mesenteric arterial conductance, and plasma nitrite concentrations in rats after four days of pretreatment with the L-arginine analogues listed above. After four days, MAP was found to be increased by i.p. L-NAME and D-NAME, but unaltered by L-NMMA or L-arginine (Fig. 2C). Mesenteric arterial conductance was decreased by L-NAME (both i.p. and p.o.) and D-NAME, but unaffected by L-NMMA or L-arginine (Fig. 2D). Plasma nitrite levels were decreased by L-NAME (both i.p. and p.o.), but not altered by other tested guanidines (Fig. 2E). In contrast to what would be expected based on the NOS-inhibiting activity of L-NAME, NOx levels in mesenteric arterial homogenates were increased by L-NAME (Fig. 2F). Likewise, the significant responses to D-NAME and the lack of responses to L-NMMA are in conflict with their purported NOS-inactive and NOS-inhibiting properties, respectively.

We next used wire myography to measure dose-responses to bradykinin in isolated endothelium-intact mesenteric arteries to verify the effects of these L-arginine analogues on NOS activity (Fig. 2G). We found that vasodilatory responses to bradykinin were similarly attenuated by L-NAME and by L-NMMA, unaltered by D-NAME, and potentiated by L-arginine. These results are all consistent with the general understanding of the NOS-modulating activity of these L-arginine analogues [23–25]. In addition, a single i.p. dose of L-NMMA increased the MAP within 1 h (Fig. 2H), consistent with L-NMMA acting as a NOS inhibitor in our rat model.

The combination of above results suggests that, albeit with confounding hemodynamic effects following four days of exposure, the nitrodilator-potentiating effects of L-arginine analogues seem to be independent of their respective NOS-modulating activities, instead corresponding to the presence or absence of a nitro group in their structure.
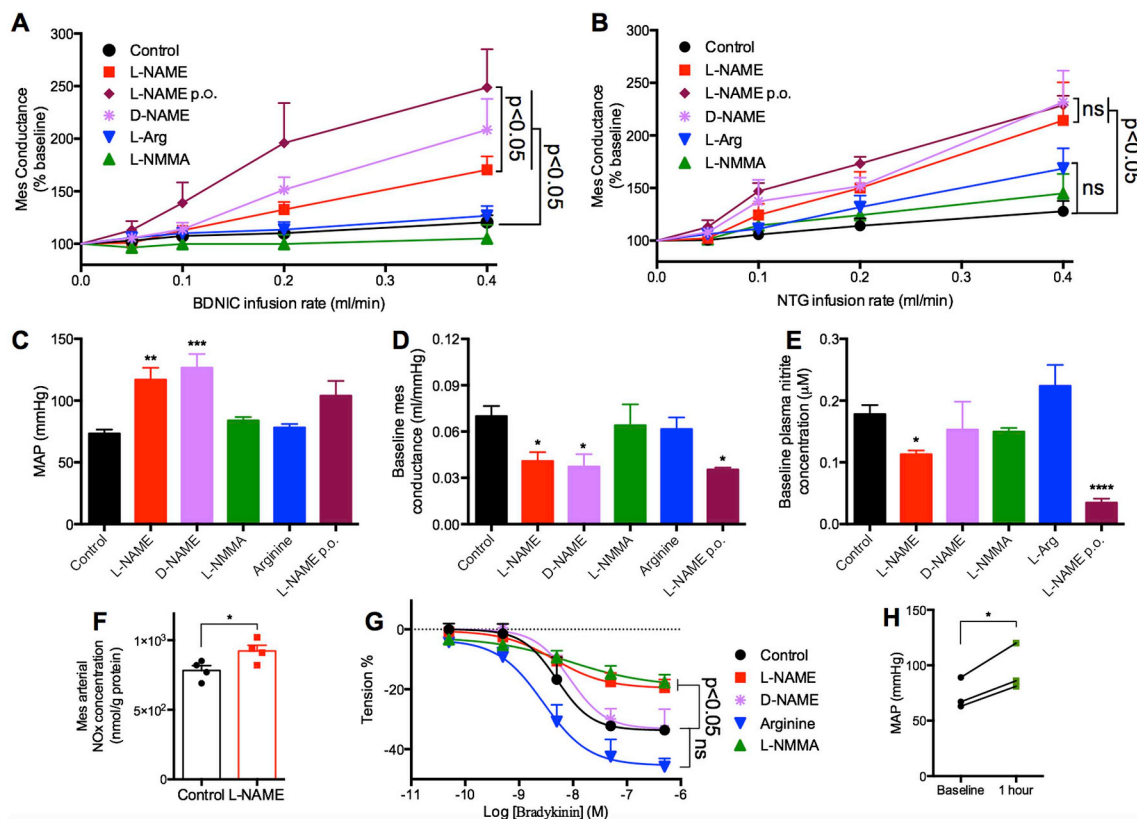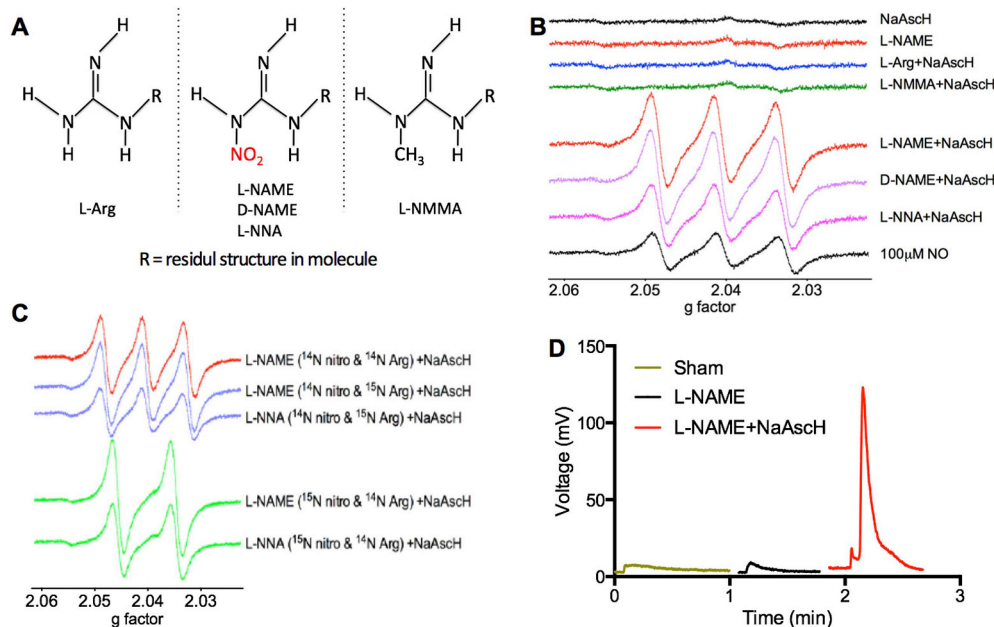

Fig. 2. L-arginine analogues containing a guanidino nitro group potentiate vasodilation by BDNIC and nitroglycerin (NTG). Rats were given L-NAME, D-NAME, L-Arg, or L-NMMA via i.p. injection for 4 days at 222 μmol·kg-1·day-1; another group was given L-NAME by oral gavage (p.o.) at the same dose. Blood pressure and flow responses in the mesenteric artery were then measured in response to nitrodilators. Vasodilation by **A)** BDNIC and **B)** NTG was potentiated by L-NAME and D-NAME, which contain a nitro group, but not by L-arginine and L-NMMA, which do not. A nitro group present in L-NAME and D-NAME but absent in L-arginine and L-NMMA potentiates dilation caused by **A)** BDNIC and **B)** NTG. Two-way ANOVA, $n \geq 5$. **C-E)** Effects of different L-arginine analogues on **C)** mean arterial blood pressure (MAP), **D)** mesenteric arterial conductance, and **E)** plasma nitrite concentration under baseline conditions. One-way ANOVA, $n \geq 5$. **F)** L-NAME pretreatment in rats increases baseline mesenteric arterial tissue NO metabolites (NOx) concentration. *t* test, $n = 4$. **G)** Effects of different L-arginine analogues on bradykinin (eNOS-dependent) mediated relaxation. One mM of each guanidine was applied 15 min before contraction. *t* test, $n \geq 4$. **H)** i.p. injection of a single dose of L-NMMA (222 μmol·kg^{-1}) increased MAP in intact rats. Paired *t* test, $n = 3$. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. Control.

3.4. Guanidino nitro group contributes to NO production

After finding L-NAME increased NOx in mesenteric arterial tissue, we next explored the possibility that the guanidino nitro group produces NO which participates in subsequent nitrodilator-mediated vasodilation. In agreement with a previous report [8], L-NAME, D-NAME, and L-NNA, which contain a guanidino nitro group, all had the potential to release NO (measured as $(MGD)_2FeNO$ by EPR) via reduction by NaAscH, while L-arginine and L-NMMA, which do not contain a nitro group, did not (Fig. 3A-B). To test whether it is the guanidino nitro group or nitrogen atoms in the L-arginine backbone that contribute to NO production, L-NAME and L-NNA were synthesized with two types of ^{15}N-labelling: 1) ^{15}N nitro + ^{14}N Arg; and, 2) ^{14}N nitro + ^{15}N Arg. Similar to our observations with unlabeled L-NAME, incubation of either type of synthesized ^{15}N-labeled L-NAME or L-NNA, in the absence of NaAscH, failed to produce a $(MGD)_2FeNO$ signal. After incubation with NaAscH, both L-NAME and L-NNA with an ^{15}N-nitro group contributed to a doublet EPR signal characteristic of ^{15}N-$(MGD)_2FeNO$ [27]. In contrast, L-NAME and L-NNA with ^{14}N-nitro group led to a triplet signal (of the same intensity as the ^{15}N doublet) that is characteristic of ^{14}N-$(MGD)_2FeNO$, regardless of whether the nitrogen atoms in the L-arginine backbone were ^{14}N or ^{15}N (Fig. 3C). These labeling experiments, in combination, demonstrate that the guanidino nitro group can be reduced in the presence of NaAscH to release free NO or HNO, either of which can react with $(MGD)_2Fe^{2+}$ to form $(MGD)_2FeNO$ [28]. To test whether the reaction of L-NAME and NaAscH produced HNO, we looked for the release of HNO with P-Rhod, an HNO-specific fluorescent probe [20]. No detectable HNO was found (Supplementary Fig. 2). In contrast, using direct measurement of NO with ozone-based chemiluminescence, which does not detect HNO [10], we confirmed that free NO was produced from reduction of the nitro group (Fig. 3D). Using ^{15}N-labeled L-NAME, the reaction between L-NAME and NaAscH was examined using LC-MS^2 (Supplementary Fig. 3). The proposed metabolism and fragmentation pathways of L-NAME are given in Supplementary Figs. 4 and 5.

3.5. Possible role for Fenton chemistry in the production of NOx from reduction of L-NAME

Further experiments were performed to investigate the redox reaction by which the guanidino nitro group in L-NAME is reduced in the presence of NaAscH. L-NAME was incubated with various redox-active reaction constituents followed by assay of the products with triiodide-based chemiluminescence. This assay detects NO and its primary products (NOx) which include nitrite, SNO, and nitrosyl-iron complexes, but not nitrate or L-NAME [17]. Consistent with our EPR data (Fig. 3), we observed NOx production when L-NAME and NaAscH were incubated together (Fig. 4A peak #3). Intriguingly, the NOx production was increased by the addition of metal ions (Fe^{2+}, Fig. 4A peak #4; Cu$^{1+/2+}$ and the copper-containing protein ceruloplasmin, Supplementary Fig. 6A). NOx production was also increased by the addition of superoxide dismutase 1 (SOD1), which converts superoxide to H_2O_2, whereas it was diminished by catalase, which converts H_2O_2 to H_2O (Fig. 4A, peaks #5-7). Addition of either xanthine and xanthine oxidase as sources of superoxide and H_2O_2 (Supplementary Fig. 7), or H_2O_2 itself, respectively (Fig. 4A peaks, 8-9), also dose-dependently increased the NOx production from L-NAME. These results strongly suggest that NO production from the reaction between L-NAME and NaAscH involves Fenton chemistry (Fig. 4A inset) [9], which relies on the presence of H_2O_2 and trace amount of transition metals.

To test whether the release of NO can occur in biological tissues, NOx production was measured following incubation of L-NAME in various biomatrices (Fig. 4B-D). NOx production was most pronounced in peritoneal fluid and plasma, and least in femoral muscle homogenates and blood (Fig. 4C). The NOx production in different biomatrices was largely ablated by addition of catalase (Fig. 4C-D), consistent with a role for H_2O_2 and Fenton chemistry. Notably, although chemiluminescence signals measured in most biomatrices provided sharp peaks (Fig. 4B), the signal from liver homogenates presented as a plateau (Supplementary Fig. 6B), possibly due to formation of different NO metabolites that have a slower rate of NO release in triiodide. The

Fig. 3. Guanidino nitro groups are essential to the production of NO from reduction by sodium L-ascorbate (NaAscH). $n = 3$. **A)** Structures of the guanidino compounds tested with the nitro group highlighted in *red*. **B)** Representative EPR spectra showing that only guanidines with nitro groups in their structure contribute to the production of NO from reduction by NaAscH as captured by $(MGD)_2Fe^{2+}$. Twenty-five mmol/L guanidine was reacted with 25 mmol/L NaAscH in 10 mmol/L HEPES for 15 min, and then mixed at 1:1 ratio with 10:0.5 mmol/L $(MGD)_2Fe^{2+}$. Spectra were obtained after standing for 1.5 h at room temperature. **C)** L-NAME or L-NNA with ^{15}N nitro group produced a doublet EPR signal, characteristic of ^{15}N $(MGD)_2FeNO$, whereas that with ^{14}N nitro group produced a triplet signal, characteristic of ^{14}N $(MGD)_2FeNO$, regardless of the isotopic type of L-arginine backbone nitrogen atoms. Experiments were performed in the same manner as **(B)**. **D)** Representative direct measurement of authentic NO from reaction of L-NAME 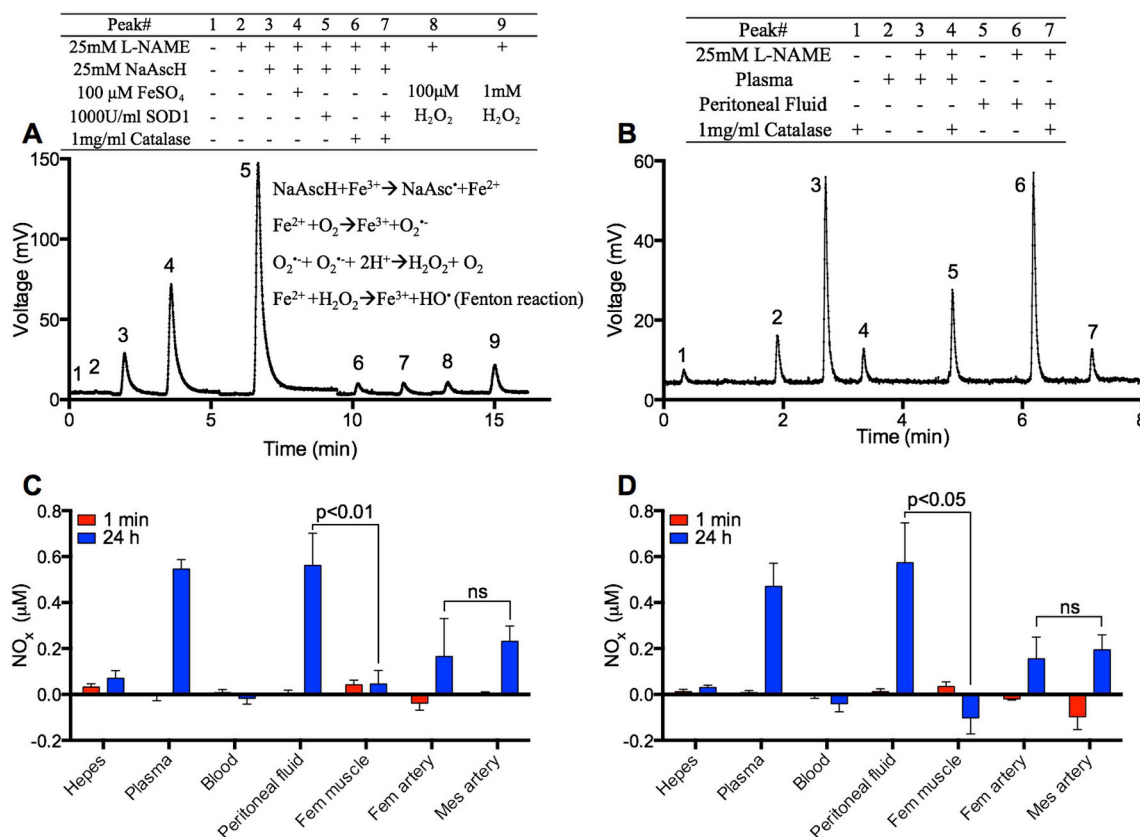and NaAscH. HEPES buffer containing 25 mmol/L L-NAME and 25 mmol/L NaAscH (pH = 7.42 ± 0.03) was deoxygenated by argon in a purge vessel, never previously used for triiodide reagent, for 0.5 h, and then allowed to react for 12 h at room temperature, in the dark, and while anaerobic (sealed; to avoid reaction of NO with atmosphere O_2). To measure NO the headspace gas was carried by a stream of argon to the chemiluminescence NO analyzer. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 4. Role of Fenton chemistry in the production of NOx from L-NAME. **A-B)** NOx production from incubation of L-NAME in **A)** NaAscH or H_2O_2 in the presence and absence of Fe^{2+}, SOD1, and catalase, in HEPES buffer for 3 h or in **B)** plasma or peritoneal fluid (centrifuged at 3000 rpm for 10 min to remove cells) in the presence and absence of catalase for 24 h. Chemicals, as given in each column of the table, were mixed and incubated. Proposed Fenton-related reaction equations are shown as inserts in **(A)**. **C)** NOx production from incubation of L-NAME in different biomatrices. Fresh tissues from adult sheep were homogenized in HEPES buffer (1 g tissue in 8 ml). The supernatant obtained after centrifugation at 3000 rpm for 10 min was incubated with 25 mmol/L L-NAME for 24 h. For each biomatrix, a time-control sample without L-NAME was subtracted to calculate NOx production from L-NAME. **D)** NOx production from incubation of L-NAME in different biomatrices that could be eliminated by catalase. **(D)** is the difference between **(C)** and a parallel experiment that was performed in the same manner as **(C)** except for the addition of 1 mg/mL catalase before incubation. One-way ANOVA for **(C-D)**. All incubations were in HEPES at 37 °C in the dark while exposed to room air, and measured by triiodide-based chemiluminescence. $n \geq 3$.

kinetics of degradation of endogenous NOx and that of consumption of exogenous NOx in different biomatrices are shown in Supplementary Fig. 8.

A cursory assessment of the specificity, dose-dependence, pH-dependence, and kinetics of the NOx-producing reactions of L-NAME and NaAscH or H_2O_2 was also performed (Supplementary Fig. 9). Of note, NOx production from L-NAME in PBS was 5 to 10 fold greater than in HEPES buffer (Supplementary Fig. 9D), possibly because of the catalysis of iron autoxidation by PBS during incubation [29].

3.6. Production of NOx from L-NAME in vivo and in cells, and its correlation with subsequent nitrodilator-mediated vasodilation

The production of NO from L-NAME was also investigated using isotopically labeled L-NAME in rats. ^{15}N-NOx was measured by GC-MS in samples from animals that had been given L-NAME with ^{15}N nitro $+ ^{14}N$ Arg (Fig. 5A-D). Plasma concentrations of ^{15}N-NOx were approximately one third of the total plasma NOx measured by triiodide-based chemiluminescence. Thus, although L-NAME pretreatment results in a significant decrease in plasma NOx levels (Fig. 2E), a significant portion of the remaining plasma NOx is derived from the nitro group of L-NAME. In addition, ^{15}N-NOx was also detected in homogenates of various organs. Possibly due to some limitations of GC-MS methodology (Supplementary Fig. 10), the measured ^{15}N-NOx only accounted for ~2% of total NOx in mesenteric arteries, much less than would be expected based on the significant increase of total NOx in

these tissues following L-NAME treatment (Figs. 5D and 2F). In contrast to our findings in mesenteric arteries, no ^{15}N-NOx was detected in femoral artery homogenates. The reason for this difference is unclear but might be attributable to the higher yield of NOx from L-NAME in the peritoneal fluid surrounding mesenteric vessels than in muscles surrounding femoral vessels (Fig. 4C). Another possibility is a role for macrophages within the abdomen (Fig. 5E-F) as shown below.

Given the evidence that the nitro group of L-NAME can be reduced into NO via Fenton chemistry in biological matrices, we determined the possibility for this reaction in macrophages, a cell type that is prone to generate ROS. Exposure of cultured RAW264.7 macrophages to L-NAME led to a significant increase of NOx in cell lysates (Fig. 5F), but not in media (Supplementary Fig. 11A). NOx production was absent if L-NAME alone, in the absence of cells, was added to the culture media (0.30 ± 0.01 vs. 0.30 ± 0.01 μmol/L in media), suggesting that the cells were required for NO production from L-NAME. Substitution of L-NMMA for L-NAME resulted in no increase in NOx in either cell lysates or media. Stimulation of macrophages by hypoxia or antimycin A, which increases intracellular ROS levels, resulted in increased NOx production in the presence of L-NAME. In contrast, the addition of deferoxamine or resveratrol, which decreases intracellular ROS, decreased NOx production (Fig. 5E-F). These combined results are consistent with our earlier findings (Fig. 4) and demonstrate ROS-dependent production of intracellular NOx from L-NAME.

To further characterize the role of H_2O_2 in intracellular NOx production in macrophages, PEG-catalase, a membrane-permeable form of
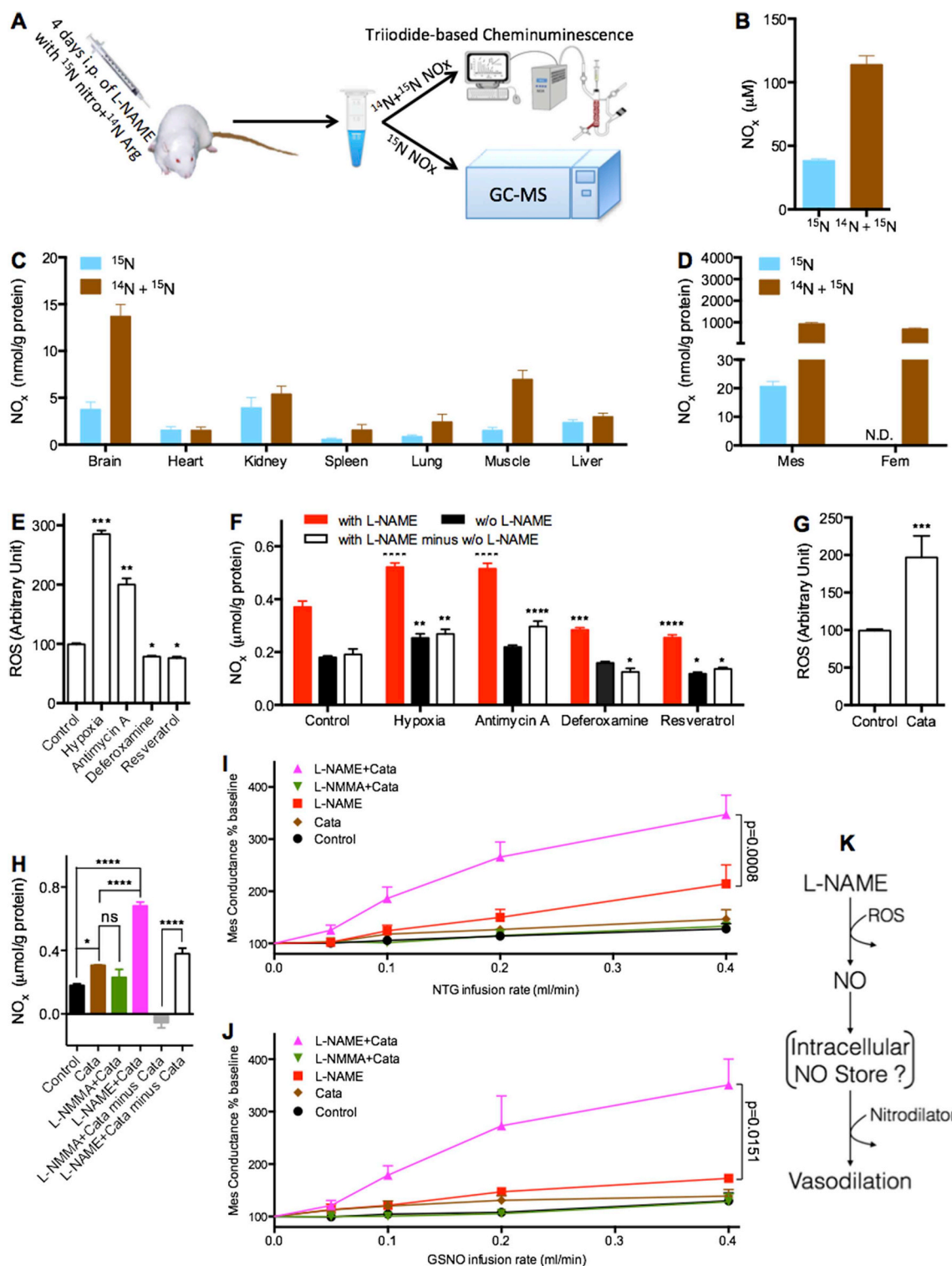
Fig. 5. Production of NOx from L-NAME in vivo and in cells, and its relation to nitrodilator-mediated vasodilation. **A)** Experimental protocol for measurements of ^{15}N and ^{14}N + ^{15}N NOx levels in rats. **B-D)** ^{15}N and ^{14}N + ^{15}N NOx levels in **B)** plasma, and homogenates of **C)** various tissues and **D)** mesenteric and femoral arteries ($n = 4$). **E)** ROS levels in RAW264.7 macrophages that had been incubated in the presence and absence of hypoxia, Antimycin A, deferoxamine, or resveratrol. One-way ANOVA, $n = 3$. **F)** NOx levels in lysates of cells that had been incubated with or without L-NAME (1 mmol/L) under conditions of (**E**). NOx levels in cell lysates were measured by triiodide-based chemiluminescence. White columns in (**F**) show values for NOx production from L-NAME. One-way ANOVA, $n = 3$. **G)** ROS levels in macrophages incubated in the presence and absence of catalase ($n = 3$). **H)** NOx levels in cell lysates and changes with incubation. One-way ANOVA with Tukey's test for left four columns; t test for right two columns. **I-J)** Co-administration of catalase (16.5 mg·kg^{-1}·day^{-1}; i.p.; 4 days) augments the potentiation effects of prior L-NAME treatment on **I)** NTG ($n = 5$-6) and **J)** GSNO ($n = 3$) mediated vasodilation. Note the null effects of L-NMMA + catalase. Two-way ANOVA. **K)** Proposed schema for the relation between L-NAME's NO contributing property and subsequent nitrodilator-mediated vasodilation. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. Control.

catalase, was used with the intention of reducing intracellular H_2O_2 and thus decreasing the NOx production from L-NAME. Unexpectedly, addition of PEG-catalase to the cultured cells resulted in significantly increased NOx levels in both cells and media, even in the absence of L-NAME (Supplementary Fig. 12). In addition, production of NOx from L-NAME in macrophages was also increased by co-incubation with PEG-catalase. Although unexpected at first, these findings are in agreement with previous reports that, similar to lipopolysaccharide, catalase acts to stimulate iNOS in macrophages resulting in NO production [30,31]. Catalase also activates NADPH oxidases resulting in enhanced ROS production [32]. Notably, NOx produced from PEG-catalase stimulation appeared in both cells and media, whereas NOx from L-NAME was only found in the cells. These results suggest that the production of NO from PEG-catalase stimulation resulted in different NOx products than the NO produced from L-NAME (Supplementary Figs. 11–12). Like PEG-catalase, catalase also increased cellular ROS and NOx (Fig. 5G-H), with the latter slightly decreased by L-NMMA, possibly through inhibition of the activated iNOS [1]. In contrast, despite its NOS inhibiting function, L-NAME did not decrease cellular NOx levels in the presence of catalase but rather increased it to an extent greater than that of L-NAME alone (Fig. 5H and Supplementary Fig. 11B). These results are consistent with the idea that NO production from L-NAME is favored in the presence of inflammation.

We further tested the effects of intraperitoneal co-administration of catalase and L-NAME on nitrodilator-mediated vasodilation in rats. Our data demonstrated that catalase augmented the potentiation effects of L-NAME on NTG- and GSNO-mediated vasodilation. In contrast, co-administration of catalase with L-NMMA had null effects (Fig. 5 I-J; baseline hemodynamic information given in Supplementary Fig. 13). These results suggest that inflammation, which promotes NO production from L-NAME, augments the nitrodilator-potentiating effects of L-NAME.

4. Discussion

NOS-inhibiting L-arginine analogues such as L-NAME and L-NMMA have been a mainstay tool in characterizing the role of NOS in the regulation of vascular tone [1]. The effects of L-arginine analogues on NOS activity are enantiomer-specific, and thus D-NAME is often used as a negative control for L-NAME [23–25]. However, consistent with previous works [33,34], we observed that D-NAME administration to rats for four days resulted in increased MAP and mesenteric vasculature vasoconstriction that was comparable to that observed with L-NAME (Fig. 2C-D). In addition, we found differential responses to four days of treatment with L-NAME and L-NMMA, as L-NMMA had no observable effect on MAP or mesenteric vascular tone. Likewise, plasma nitrite concentrations, an index of endogenous eNOS activity [26], were significantly lowered by orally and intraperitoneally administered L-NAME, but were not altered by four days of L-NMMA injections. These findings are in contrast to our observation that a single i.p. dose of L-NMMA significantly increased MAP, and differ from our wire myography data demonstrating that the eNOS-dependent vasodilatory responses to bradykinin were attenuated to a similar extent by L-NAME and L-NMMA, but not D-NAME. Notably, the significant shorter half-life of L-NMMA than L-NAME (1 h vs 23 h in humans) [35,36] may not fully explain the lack of effect of prolonged L-NMMA treatment, because the measurements were made within the effective time of the last dose of L-NMMA. Discrepancies such as these are not uncommon in protocols that utilize prolonged application of L-arginine analogues, and highlight the likelihood that the canonical understanding of their function as NOS-modulating agents may be incomplete. We herein propose a novel non-canonical action of L-arginine analogues.

While the metabolism of L-NAME has been investigated by multiple groups, attention has primarily focused on the cleavage of the amino acid skeleton while the nitro group has been considered stable both chemically and biologically [35]. Our current data generated using ^{15}N-labeled L-NAME definitively demonstrate that NO can be released from the nitro group of L-NAME both in vitro and in vivo. This release of NO from L-NAME is surprising, as it would seem to directly counteract its widely intended use as a NOS-inhibitor. Our results indicate that while the effects of NOS inhibition can be observed as an increase in blood pressure within minutes, NO release proceeds slowly requiring hours or days to have physiological effects. Understanding of the rate and mechanisms of the underlying reactions is of importance in determining the biological significance of our findings.

We herein provide evidence that reduction of the guanidino nitro group can proceed via Fenton chemistry, which is promoted by transition metals and H_2O_2 (Fig. 3, Supplementary Figs. 6, 7, and 9). In vitro, with only trace contaminating transition metals and H_2O_2, this reaction proceeds slowly enough that it would not be expected to significantly confound the NOS-inhibiting effects of L-NAME (Fig. 4). However, the potential physiological and pathological significance of the NO production from L-NAME should not be disregarded, especially given the long half-life of L-NAME [35]. First, metal and metalloproteins, which can catalyze the Fenton reaction, are abundant in biological environments. Indeed, considerable NO production from L-NAME was measured in vivo. Four days of ^{15}N-nitro-L-NAME administration to rats at a commonly used experimental dose resulted in plasma ^{15}N-NOx levels that were approximately one third of endogenous levels, as well as significant amounts of NOx in the liver (Fig. 5B-C), which was barely detectable in intact rats [17]. Second, the involvement of Fenton chemistry in NO production from L-NAME leads to the expectation of augmented NO release under conditions of heightened oxidative stress, such as inflammation (Fig. 5E-H). Therefore, it is reasonable to speculate that NO release from L-NAME may play a more significant and possibly confounding role in settings of increased oxidative stress.

It is important to note that the detailed mechanisms underlying the NO releasing reaction of L-NAME and H_2O_2 are still not clear, due to the incomplete knowledge of Fenton chemistry [37]. H_2O_2 is highly oxidative, and thus is not a reasonable reductant [38]. It is possible that the reduction was mediated via unidentified secondary intermediates that decrease the thermodynamic gradient. One possible explanation is given in Supplementary Fig. 14. Alternatively, it is possible that the nitro group was oxidized into nitrogen oxide species of higher states such as N_2O_3 and N_2O_4 (ON-NO_3), which then decomposed into NO [39]. Regardless of the chemical mechanism, the apparent production of NO from the nitro group by H_2O_2 via Fenton chemistry adds a new convergence between reactive nitrogen species and ROS to the current paradigm, which holds that ROS scavenges NO.

The potentiation effects of L-NAME on nitrodilator-mediated vasodilation were observed both in vitro and in vivo (Fig. 1). In the dose response curves of nitrodilators, L-NAME did not alter the EC_{50}, an index of relaxation sensitivity, but increased the E_{max}, an index of relaxation potency. These results suggest that the L-NAME did not affect the sensitivity but rather the bioavailability of some effectors in the vasodilatory signaling cascade. In contrast to the augmented vasodilatory responses of isolated arteries to NTG, BDNIC, and GSNO, the vasodilatory effects of NO *per se* were unaffected (Fig. 1G), suggesting that the potentiating effect of prolonged treatment of L-NAME lies upstream rather than downstream of sGC activation. Notably, acute exposure of L-NAME also enhanced responsiveness to nitrodilators [40]. However, different from the current study, this enhanced vasodilation had altered EC_{50} and unchanged E_{max}, was accompanied with enhanced constriction, and could also be induced by L-NMMA. Therefore, the enhancements were alternatively attributed to the mechanisms secondary to the NOS-inhibition, including sensitization of sGC [40–43].

Possible mechanisms by which L-NAME can potentiate nitrodilator-mediated vasodilation by acting upstream from sGC are not readily discernable, partly due to a lack of general understanding of the mechanisms by which these nitrodilators activate sGC. While these nitrodilators are often assumed to cause NO-mediated vasodilation by simply acting as an NO precursor or donor, much evidence argues

against their NO-donating properties [12,15,16]. For example, GSNO and BDNIC do not readily release free NO or cross the plasma membrane [10,16,17]. Similarly, the bioactivation of NTG by enzymes such as mitochondrial aldehyde dehydrogenase produces nitrite rather than NO [15,19]. The possibility that the NO moiety of these nitrodilators is the sole source of NO equivalent involved in activation of sGC is difficult to reconcile with the fact that they all cause vasodilation with efficacy comparable to free NO itself (Supplementary Table 1 and [16]).

Notably, the potentiation of nitrodilator-mediated vasodilation following pretreatment with L-NAME or D-NAME is not likely to have been explained by a decrease in baseline arterial conductance (Fig. 2D) because similar potentiation effects were observed in myography experiments in which isolated arteries were pre-contracted to the same baseline tensions (Fig. 1 and Supplementary Table 1). In addition, L-NAME and catalase co-administration resulted in similar baseline conductances but augmented the potentiation effects more than L-NAME alone (Supplementary Fig. 12B and Fig. 5I-H), also suggesting that the potentiation is independent of baseline conductance in our experiments. Besides, the nitrodilator-potentiating effect of L-NAME is also unlikely associated with alterations in redox signaling. Prolonged treatment of L-NAME may result in increase of ROS [4]. However, oxidative stress generally hampers NO-cGMP dependent vasodilation. Therefore, this potential alteration in redox signaling seems counter to the nitrodilator-potentiating effects of L-NAME observed in the current study. In addition, the dose response curve of NO-mediated vasodilation was not changed with L-NAME pretreatment in our experiments with isolated arteries (Fig. 1G), indicating an intact NO-cGMP pathway in these animals.

We have previously noted that the vasodilatory effects of GSNO are potentiated by pre-exposure of the vessels or animals to nitrite, and proposed the possibility that GSNO-mediated vasodilation involves the mobilization of an intracellular vascular smooth muscle reservoir of NO moieties to which nitrite contributes [16]. In the context of this paradigm, it is possible that the NO released from the nitro group of L-NAME is incorporated into this "NO reservoir", resulting in a potentiated subsequent response to nitrodilators (Fig. 5K). The results of the current experiments are consistent with this idea in several ways. First, as mentioned above, all nitrodilators caused vasodilation with efficacy comparable to free NO itself. This suggests that NO equivalents from sources other than the nitrodilators themselves are necessary in order to stimulate the equivalent sGC activation. Second, L-NAME increased the E_{max} of the nitrodilators but did not alter the EC_{50}, suggesting that the bioavailability of NO equivalent for sGC activation was increased by L-NAME while the sensitivity of sGC activation was unchanged. Third, the potentiated vasodilation of mesenteric arteries in response to L-NAME pretreatment was associated with an increase in the NOx content of the vessels. Fourth, only L-arginine analogues containing a nitro group that can contribute to NO production potentiated nitrodilator-mediated vasodilation. Finally, inflammation that promoted NO production from L-NAME augmented the nitrodilator-potentiating effects of L-NAME, whereas inflammation together with L-NMMA had null effects.

5. Conclusions

In summary, this study highlights caution in prolonged application of L-arginine analogues as NOS-modulating agents. Our experiments demonstrate that nitro-group-containing L-arginine analogues such as L-NAME potentiate nitrodilator-mediated vasodilation, and that NO is released from the nitro group of L-arginine analogues via Fenton reactions. In addition, the current study raises the possibility that the NO released from a guanidino nitro group may participate in subsequent nitrodilator-mediated vasodilation by contributing to an intracellular store of NO within the vasculature.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101238.

Author contributions

TM.L. contributed to the overall concept, designed, performed and analyzed experiments, and wrote the manuscript. MJ.Z. designed, performed, and analyzed chemiluminescence, wire myography, and rats experiments. G.T.M. synthesized isotopic L-arginine analogues. D.B. performed and analyzed NMR experiments. Q.L. and T.E.T contributed to data interpretation and manuscript preparation. A.S.I.A. performed cell experiments. G.G.P. and A.B.B. contributed to the overall concept, experimental design, and manuscript preparation.

Conflicts of interest

None.

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