

# A Novel Role for HNO in Local and Spreading Vasodilatation in Rat Mesenteric Resistance Arteries

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## Abstract

Nitric oxide-mediated vasodilatation has previously been attributed to the uncharged form of the molecule ( $\text{NO}^\bullet$ ), but increasing evidence suggests that nitroxyl (HNO) may play a significant role in endothelium-dependent relaxation. The aim of this study was to investigate the mechanisms underlying HNO-mediated vasodilatation in phenylephrine pre-constricted pressurized (70 mmHg) mesenteric arteries, and on membrane currents in isolated smooth muscle cells using whole cell and perforated patch clamp recordings. Angeli's salt (AS: nitroxyl donor), evoked concentration-dependent vasodilatation that was insensitive to the  $\text{NO}^\bullet$  scavengers carboxy-PTIO and hydroxocobalamin (HXC), but sensitive to either the HNO scavenger L-cysteine, K-channel blockers (4-AP and iberiotoxin), raised  $[\text{K}^+]_o$ , or inhibition of soluble guanylyl cyclase (ODQ). AS-evoked smooth muscle hyperpolarization significantly augmented  $\text{K}_V$  current in an ODQ sensitive manner, and also increased the  $\text{BK}_{Ca}$  current. Importantly, 30  $\mu\text{M}$  AS initiated conducted or spreading vasodilatation, and following blockade of endothelial K-channels (TRAM-34 and apamin), ACh was able to evoke similar L-cysteine-sensitive conducted dilatation. These data show that vasodilatation induced by HNO is mediated by both  $\text{K}_V$  and  $\text{BK}_{Ca}$  channels, and suggest a physiological role in vasodilatation through the vasculature. *Antioxid. Redox Signal.* 14, 1625–1635.

## Introduction

THE ENDOGENOUS RELEASE OF NITRIC OXIDE (NO) by the endothelium modulates vascular tone and consequently regulates both blood pressure and flow. NO can exist in several different redox forms, including the uncharged free radical state ( $\text{NO}^\bullet$ ), in the oxidized state as nitrosonium cation ( $\text{NO}^+$ ), and in the one-electron reduced and protonated form as nitroxyl (HNO) (23).

The vasodilator action of NO has always been thought to be due to uncharged  $\text{NO}^\bullet$ , whether applied exogenously as an NO donor or after direct stimulation of the endothelium (7, 18). However, more recent evidence suggests that HNO may also be active endogenously (22, 33, 35, 38), and therefore likely to be of physiological significance. Indeed a number of potential biosynthetic pathways for HNO generation have been identified, including a) direct oxidation of the nitric oxide synthase (NOS) intermediates hydroxylamine (8) and N-hydroxy-L-arginine (19); b) nitrosothiol decomposition (44); c) direct reduction of  $\text{NO}^\bullet$  by either mitochondrial cytochrome *c*, ubiquinol, and/or xanthine oxidase (20, 36), and d) by NOS in the absence of the cofactor tetrahydrobiopterin ( $\text{BH}_4$ ) (1).

The potential importance of HNO in the vasculature was suggested by the work of Fukuto and colleagues (19),

who demonstrated that the HNO donor, Angeli's salt (AS; sodium trioxodinitrate), evoked a marked guanylyl cyclase-dependent vasodilatation in rabbit aorta and bovine intrapulmonary arteries. Further studies in the rat aorta (15, 25, 42), and the resistance vasculature (3, 16, 24), confirmed that like  $\text{NO}^\bullet$ , HNO induces vasodilation via stimulation of soluble guanylyl cyclase and an increase in cGMP. The possibility that HNO is an endothelium-derived relaxing factor (EDRF) then followed the demonstration that this reduced form of NO can be generated endogenously in sufficient amounts to affect vasodilatation, and that endothelium-dependent relaxation is sensitive to scavengers of both  $\text{NO}^\bullet$  and HNO (3, 15, 34, 42). At least in part, this effect of endogenous nitrogen oxides may be due to K channel activation, because in rat mesenteric arteries  $\text{NO}^\bullet$  can activate calcium-activated K channels ( $\text{K}_{Ca}$ ) and ATP-sensitive K channels ( $\text{K}_{ATP}$ ), causing hyperpolarization (29, 37). But in the same resistance arteries, Irvine and colleagues (24) have shown that HNO can mediate vasodilatation through the activation of  $\text{K}_V$  channels. Together with recent observations, these data suggest that HNO contributes to the endothelial hyperpolarizing effect of acetylcholine, and thus may be an endogenous endothelium-derived smooth muscle hyperpolarizing factor (EDHF) (3). However, it is not clear which K channels are key, and

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furthermore if HNO really does have a significant input to physiological responses in the vasculature.

The aim of the current study was therefore to address both of these points by investigating the role of HNO in the dilatation of rat resistance arteries under physiological pressure. Furthermore, as agents capable of evoking arterial hyperpolarization can also initiate spread of arterial dilatation over distance, we addressed the possibility that HNO may share this characteristic. Spreading or conducted vasodilatation is thought to be an important physiological mechanism to ensure local dilatation (e.g., of metabolic origin) is amplified sufficiently to ensure an adequate increase in blood flow. We have shown spreading vasodilatation occurs in pressurized mesenteric arteries (39, 43). Finally, we define directly the effect of HNO on specific ion channel currents recorded from individual mesenteric artery smooth muscle cells.

## Materials and Methods

### *Rat mesenteric artery isolation and cannulation*

Male Wistar rats (200–250 g) were killed by cervical dislocation in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986, UK. The entire mesenteric bed was removed and placed in cold MOPS buffer containing (mM): NaCl, 145.0; KCl, 4.7; CaCl<sub>2</sub> · 2H<sub>2</sub>O, 2.0; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 1.17; MOPS, 2.0; NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O, 1.20; glucose, 5.0; pyruvate, 2.0; EDTA, 0.02; NaOH, 2.75, and adjusted to pH 7.40 ± 0.02. Third-order branches of the superior mesenteric artery were dissected, isolated, and cannulated at each end with small glass pipettes (external diameter ~180 μm) and positioned near the base of a temperature-regulated chamber (RC-27 chamber, PH-6 platform, Warner Instruments, Hamden, CT) for observation on an IX71 (Olympus) inverted microscope, as described previously (28, 39, 43). The MOPS-buffered solution was heated to 36.5 ± 0.2 °C in all experiments. Vessels were pressurized to 70 mmHg by a gravity-fed inflow and outflow system, and the lack of small side-branches was ascertained by absence of diameter change following occlusion of perfusion inflow. Arteries were maintained at 70 mmHg for the duration of the experiment, and submaximally contracted with phenylephrine (0.5–2 μM) to generate a consistent, comparable level of tone in each experiment. Endothelial cell viability was assessed as > 95% relaxation to 1 μM ACh. Relaxation to AS was induced by bath application. Arterial outer diameter was measured online using motion analysis software (Vedi View v.1.2, DMT, Denmark). Dilatation was expressed as a percentage of the maximal diameter of the artery (~300–350 μm), and contraction (or tone) calculated as a percentage of the minimal artery diameter (near 100 μm). Data are summarized as means ± S.E.M. of *n* replicates, where *n* is quantity of vessels with each obtained from an individual animal. Statistical analyses were performed using Student's unpaired *t*-test or one-way ANOVA analysis followed by Bonferroni post test. A value of *p* < 0.05 was considered to be statistically significant.

### *Rat mesenteric artery electrophysiology*

Third-order mesenteric arteries were mounted in a Mulvany-Halpern wire myograph (Model 400A, Danish MyoTech, Aarhus, Denmark) in either Krebs-buffered solution containing (mM): NaCl, 118.0; NaCO<sub>3</sub>, 25; KCl, 3.6;

MgSO<sub>4</sub> · 7H<sub>2</sub>O, 1.2; KH<sub>2</sub>O<sub>4</sub>, 1.2; glucose, 11.0; CaCl<sub>2</sub>, 2.5; and gassed with 95 % O<sub>2</sub> and 5 % CO<sub>2</sub> or MOPS buffered-solution as above, each at 36.5–36.8 °C. Following equilibration, arteries were set to a resting tension equivalent to that generated at 0.9 times the diameter of the vessel at 70 mmHg. Unless otherwise stated, only arteries with functional endothelium were used, assessed as over 95% relaxation to 1 μM ACh. Sharp glass microelectrodes were used to measure the membrane potential of individual smooth muscle cells or endothelial cells (filled with 2 M KCl, tip resistances 70–100 MΩ), as previously described (10). Rapid deflections in membrane potential to near –50 mV were observed upon cell impalement. The membrane potential was recorded via a pre-amplifier (Neurolog system, Digitimer Ltd., Welwyn, U.K.) linked to a MacLab data acquisition system (AD Instruments Model 4e) at 100 Hz. Drugs were added directly to the 10 ml bath, and mixed by gassing. There was no difference in resting membrane potential or in the response to agents with either buffer solution, so data were pooled.

### *Smooth muscle cell isolation from rat mesenteric artery*

Freshly dissected third-order mesenteric arteries were placed in ice-cold calcium-free isolation solution containing (mM): NaCl, 140; KCl, 4.7; MgCl<sub>2</sub>, 1.2; glucose, 10; HEPES, 10 (pH 7.4). After incubation on ice for 10 min, the arteries were transferred to the calcium-free isolation solution, containing 1 mg/ml papain (Sigma, St. Louis, MO), 1 mg/ml collagenase (Sigma), and 1 mM dithiothreitol, and allowed to digest for 20 min at 37 °C. Following digestion, the tissue was twice washed in isolation solution before gentle trituration to disperse cells. After centrifugation for 2 min at 1,000 rpm, the supernatant was removed, and the cells resuspended in fresh isolation solution. The concentration of extracellular calcium was increased over the next 30 min to 750 μM. Freshly isolated cells were maintained on ice for use on the same day.

### *Electrophysiological recordings*

Membrane potential and whole cell recordings were made mainly with voltage clamp electrodes, using the whole-cell and current-clamp modes and an Axopatch 200B amplifier and Digidata 1332A interface (Axon Instruments, Molecular Devices, Sunnyvale, CA), and piezoelectric (PCS-5000 series) precision micromanipulator (Burleigh, New York). Cells were placed in a recording chamber (RC-25F; Warner Instruments, Hamden, CT) and left to adhere to the cover glass for approximately 10 min. Cells were then continually superfused (~1 ml/min) with heated solution (SH-27B Inline Heater; Warner Instruments; 37 °C) via a multi-barrel gravity-fed perfusion system and observed using an inverted microscope (Olympus IX71). The physiological saline solution (PSS) used for superfusion contained (mM): NaCl, 140; KCl, 4; CaCl<sub>2</sub>, 1.5; MgCl<sub>2</sub>, 1.2; HEPES, 10; glucose, 5; pH 7.4. Membrane potential recordings were performed in current-clamp mode, using the perforated patch technique, and the pipette solution contained (mM): KCl, 130; NaCl, 10; HEPES, 10; MgCl<sub>2</sub>, 0.5; CaCl<sub>2</sub>, 0.5; amphotericin B (200 μg/ml), pH to 7.3.

Voltage-gated potassium channel current (I<sub>Kv</sub>) was recorded in whole cell patch clamp mode, and elicited by 10 mV step depolarization to more positive potentials from a holding potential of –80 mV, using the standard PSS described above. The pipette solution contained the following (mM) KCl, 140; MgCl<sub>2</sub>, 0.5; HEPES, 10; EGTA, 10; CaCl<sub>2</sub>, 0.5; Na<sub>2</sub>ATP, 1; GTP, 0.5; pH

7.3. These recordings were performed in the presence of 1  $\mu$ M paxilline and 10  $\mu$ M glibenclamide in the external PSS, in order to block calcium activated and ATP-sensitive K currents, respectively.  $I_{K_V}$  was inhibited by application of 1 mM 4-AP.

Experiments to record calcium-activated potassium channels utilized the standard PSS with the additional inclusion of 10  $\mu$ M glibenclamide to inhibit ATP sensitive potassium currents. The patch solution contained (in mM) KCl, 140;  $MgCl_2$ , 0.5; HEPES, 10;  $CaCl_2$ , 0.5;  $Na_2ATP$ , 1; GTP, 0.5; pH 7.3. Paxilline (1  $\mu$ M) was applied at the end of the protocol, to inhibit calcium activated potassium currents, and currents subtracted from the equivalent records in control and drug to give the paxilline-sensitive current.

The osmolarity of all solutions was measured and corrected to  $300 \pm 5$  mOsm using mannitol. All electrophysiological recordings were performed at 37°C, and using an agar bridge (2% agar filled with 3 M KCl). Cell membrane capacitance was measured using a 10 mV hyperpolarizing step pulse in order to normalize currents to cell size (pA/pF). Angeli's salt was infused with a BeeHive® syringe pump system (Bioanalytical Systems, Lafayette, IN) into an injection port in the superfusion line, directly upstream from the recording chamber.

Data were acquired using Clampex 8.0.2 and Axoclamp 9 (Axon Instruments) and analyzed offline using Clampfit (Axon Instruments). Values are expressed as means  $\pm$  SEM. Student's paired two-tailed *t* test was used to compare parameters obtained in control and test conditions in the same cell, followed by the Bonferroni post test. A nonpaired Student's *t*-test was used to compare the difference between groups of data. A value of  $p < 0.05$  was considered to be statistically significant.

#### Spreading dilatation to lumenally perfused agonists

In this series of experiments, three cannulation pipettes were used as previously described (43). The three ends of an isolated artery with a side branch were cannulated and mounted in a heated chamber and continuously superfused at 2 ml/min with heated MOPS solution. The upstream end of the artery (Feed artery) and one side of the bifurcation (Branch 2) were attached to the gravity-fed pressurizing syringe reservoirs. Phenylephrine was added to the superfusion solution, and each agonist used to study spreading dilatation was infused locally into Branch 1 for at least 2 min at 20  $\mu$ l/min using a BeeHive® syringe pump system. In all experiments, the movement of perfusion solution was monitored by including 0.1  $\mu$ M carboxyfluorescein in the agonist solution. Spread of dilatation upstream away from the area of local application could then be assessed throughout the feed artery. Arteries were visualized using a laser scanning confocal microscope (FV500-SU, Olympus, Japan, excitation 488 nm, emission 505 nm) to enable simultaneous fluorescence and brightfield imaging with a 4 $\times$ /0.13 NA objective (UplanFL, Olympus, Japan), and images were recorded at 1 Hz using Fluoview software (Olympus, USA). For all measurements of spreading dilatation, artery outer diameter was measured offline with motion analysis software (MetaMorph, Universal Imaging). This enabled simultaneous analysis of multiple, calibrated distances along the artery wall, and direct comparison of local dilatation (Branch 1) to spreading dilatation (0–2.0 mm upstream along the Feed artery) for a single application of agonist. Fluorescence intensity was also mea-

sured offline simultaneously at multiple positions in the lumen of arteries, which was temporally matched to diameter measurements. Feed artery diameter was only measured if no upstream flow of fluorescent indicator was detected, as previously shown (43). Values for summary data were taken at the time point where dilatation at the 0 mm position along the feed artery reached 80% of maximal diameter.

#### Drugs

All drugs were obtained from Sigma (Poole, UK) with the exception of AS (sodium trioxodinitrate) from Axxora (Nottingham, UK) and iberiotoxin (Latoxan, Valence, France). AS was dissolved in 10 mM NaOH to prevent decomposition prior to use (17). Responses to AS and  $NO^*$  were recorded in the absence and presence of the HNO scavenger L-cysteine (3 mM, at least 5 min),  $NO^*$  scavengers hydroxocobalamin (HXC, 100  $\mu$ M, at least 15 min) or carboxy-PTIO (200  $\mu$ M). In experiments with the sGC inhibitor 1*H*-(1,2,4)oxadiazole(4,3-*a*)quinoxalin-1-one (ODQ), cells were incubated in 10  $\mu$ M ODQ for 15 min, and it was also included in the perfusion solutions. Responses to AS were also performed in the presence of the  $K_V$  channel inhibitor, 4-aminopyridine (4-AP, 1 mM). All other stock solutions were prepared using MQ water or DMSO.

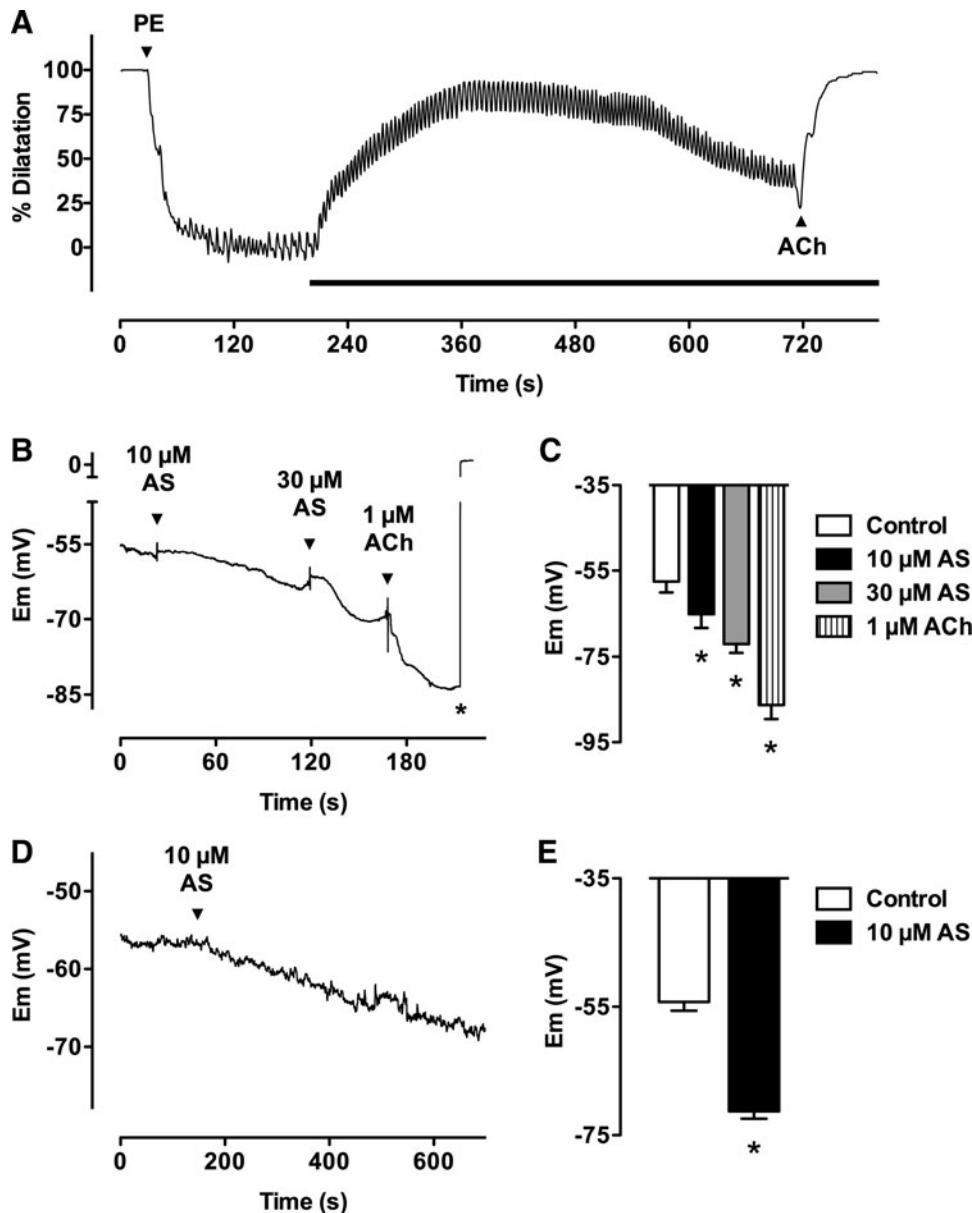
#### Results

##### *Effect of the HNO donor Angeli's salt in pressurized mesenteric arteries*

AS relaxes rat mesenteric arteries under isometric tension with a log  $EC_{50}$  of  $\sim -7.7$  (24). Herein, AS was administered to phenylephrine pre-constricted small mesenteric arteries under physiological pressure (70 mmHg), and a representative trace of vasodilatation to 30  $\mu$ M AS is shown in Figure 1A. 30  $\mu$ M AS evoked close to maximal dilatation within 5 min (relative to 1  $\mu$ M ACh). With longer periods of application, the magnitude of vasodilatation decreased. Mean data (not shown), demonstrated that the concentration-dependent vasodilatation to AS occurred over the concentration range of 100 nM–100  $\mu$ M and log  $EC_{50}$   $-4.7 \pm 0.03$  ( $n = 8$ ), a less potent effect than observed with wire myography. Furthermore, phenylephrine induced pre-constriction resulted in vasomotion that was consistently augmented upon application of AS.

##### *Hyperpolarization of the resting membrane potential to HNO*

Measurement of change in smooth muscle membrane potential, recorded either with sharp microelectrodes (Figs. 1B and 1C) or patch electrodes in current-clamp mode (Fig. 1D and 1E) in the presence of the  $NO^*$  scavengers hydroxocobalamin (HXC) or carboxy-PTIO, revealed hyperpolarization of *circa* 15–20 mV. The patch clamp recordings were made from cells perforated with amphotericin B, to minimize changes to the intracellular milieu. In the sharp electrode studies, the mean resting membrane potential was  $-57.6 \pm 2.5$  mV ( $n = 7$ ), increasing by  $7.3 \pm 2.0$  mV and  $14.5 \pm 1.6$  mV to 10 and 30  $\mu$ M AS, respectively ( $n = 6, 7$ ). In the perforated patch experiments, the mean resting membrane potential was  $-54.2 \pm 1.2$  mV ( $n = 5$ ). Rapid bath infusion of 10  $\mu$ M AS induced marked hyperpolarization by  $17.0 \pm 1.0$  mV ( $n = 5$ ), measured after 5 minutes application ( $p < 0.05$ ) (Fig. 1C).



**FIG. 1. Angeli's salt induces vasodilation and smooth muscle cell hyperpolarization.** (A) Typical trace illustrating the dilatation in response to Angeli's salt. A mesenteric artery mounted in a pressure myograph was precontracted with phenylephrine (PE,  $0.8 \mu\text{M}$ , 73% tone), and Angeli's salt (AS,  $30 \mu\text{M}$ ) added to the bath during the period indicated by the bar. ACh ( $1 \mu\text{M}$ ) was added at the end. Following 5 min exposure to AS, almost complete vasodilatation was achieved, and over a longer period of time the degree of vasodilatation was reduced, perhaps resulting from decomposition of AS. Note that vasomotion was observed in these experiments, and was consistently augmented by Angeli's salt. The  $\text{NO}^*$  scavenger carboxy-PTIO ( $200 \mu\text{M}$ ) was present throughout. Maximum and minimum outer diameters were  $320$  and  $110 \mu\text{m}$ . (B) Typical trace showing hyperpolarization to AS in an artery. Smooth muscle cell membrane potential ( $E_m$ ) was recorded using sharp microelectrodes in a mesenteric artery mounted in a wire myograph. Both AS ( $10$  and  $30 \mu\text{M}$ ) and ACh ( $1 \mu\text{M}$ ) evoked hyperpolarization from resting membrane potential. The microelectrode was manually pulled out of the cell at the asterisk. Data are summarized in (C),  $n=6-7$  from three arteries;  $*p < 0.05$  vs control. The  $\text{NO}^*$

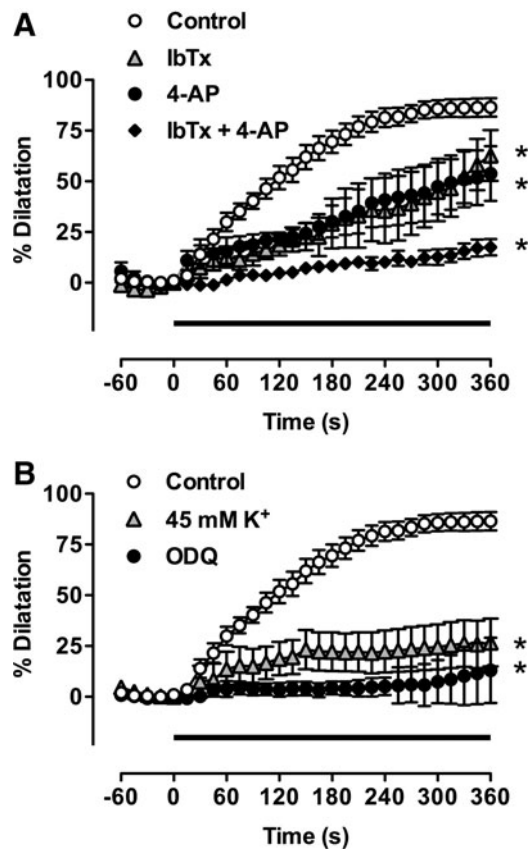
scavenger carboxy-PTIO ( $200 \mu\text{M}$ ) was present throughout all experiments. (D) Typical trace showing hyperpolarization to AS in a single smooth muscle cell. Perforated patch-clamp recording in current-clamp mode of membrane potential in a mesenteric artery smooth muscle cell in the presence of the  $\text{NO}^*$  scavenger HXC ( $100 \mu\text{M}$ ). The arrow indicates where  $10 \mu\text{M}$  AS's was applied, resulting in a significant hyperpolarization. Data are summarized in (E),  $n=5$  cells from four animals;  $*p < 0.05$  vs control.

AS was diluted in  $10 \text{ mM}$  NaOH to prevent decomposition (17), and  $10 \text{ mM}$  NaOH vehicle had no significant effect on vascular tone in phenylephrine pre-constricted vessels ( $p > 0.05$ ,  $n=2$ ).

#### Augmented K channel activity with Angeli's salt

4-AP potently inhibits  $\text{K}_v1$  and  $\text{K}_v3$  type voltage-gated potassium channels (GRAC) (2). When applied in low concentrations ( $150 \mu\text{M}$ ), 4-AP inhibited relaxation to the submaximal concentration of AS ( $30 \mu\text{M}$ ), and slowed the time-course of the response (Fig. 2A,  $n=4$ ). Previous work in mesenteric artery by Irvine *et al.* (24) indicated AS-induced

vasodilatation was insensitive to iberitoxin (IbTx), suggesting that  $\text{BK}_{Ca}$  channels may not provide a significant contribution to HNO-evoked hyperpolarization/dilatation. However, in pressurized mesenteric arteries these channels seemed clearly to contribute to AS vasodilatation. Pre-incubation with the specific  $\text{BK}_{Ca}$  inhibitor IbTx ( $100 \text{ nM}$ ) not only caused further constriction of the vessel (to  $35.3 \pm 8.4\%$  of the maximal response), but also induced vasomotion in four of seven experiments ( $n=7$ ). After 5 min application of AS, in the presence of HXC ( $100 \mu\text{M}$ ) and IbTx, vasodilatation was significantly attenuated (Fig. 2A,  $n=4$ ;  $p < 0.05$ ), and the time-course of the persistent vasodilatation considerably slower than with AS alone. In combination, 4-AP and IbTx almost completely



**FIG. 2.** The vasodilatation to Angeli's salt is mediated by  $BK_{Ca}$  and  $K_V$  channels and soluble guanylyl cyclase. Summary data showing the effect of K-channel blockers (A) and high  $K^+$  and ODQ (B) on dilatation to Angeli's salt (AS). AS ( $30 \mu M$ ) was added to the bath during the period indicated by the bar (0–360 s). The dilatation under control conditions ( $n = 13$ ) was significantly reduced in the presence of either iberiotoxin (IbTx,  $100 nM$ ,  $n = 5$ ) or 4-AP ( $150 \mu M$ ,  $n = 5$ ) at the 5 min point. The combination of IbTx and 4-AP almost fully inhibited dilatation to AS ( $n = 4$ ). This block was matched in the presence of either  $45 mM K^+$  ( $n = 5$ ) or the sGC inhibitor ODQ ( $10 \mu M$ ,  $n = 5$ ). \* $p < 0.05$  vs control, 5 min.

inhibited the vasodilatation to AS, demonstrating significant input through both  $BK_{Ca}$  and  $K_V$  channels (Fig. 2A). Increasing external  $K^+$  ( $45 mM$ ), which causes vasoconstriction following smooth muscle membrane depolarization and abolishes ACh-evoked hyperpolarization (40), almost totally abolished vasodilatation to AS in pressurized arteries (Fig. 2B,  $n = 3$ ).

#### HNO and whole cell potassium channel activity

To probe further the ion channels involved in vasodilatation to HNO, experiments were performed in the whole-cell mode of the patch clamp technique using intracellular and extracellular solutions designed to minimise contamination from interfering conductances (see Materials and Methods). The  $NO^*$  scavenger HXC ( $100 \mu M$ ) was also included in all externally applied solutions. Families of  $BK_{Ca}$  channel currents were elicited by sequential step depolarization to positive test potentials, from a holding potential of  $-80 mV$ . Currents under control conditions are shown in Figure 3A (i). Application of  $10 \mu M$  AS significantly enhanced these

currents, as shown in the typical traces in Figure 3A (ii). Mean data in Figure 3C show enhanced  $BK_{Ca}$  channel activity at positive test potentials in 6 cells ( $p < 0.05$ ), confirming directly that HNO enhances  $BK_{Ca}$  activity in small mesenteric artery smooth muscle cells.

Figure 3B (i) shows a family of  $K_V$  currents elicited by step depolarization from a holding potential of  $-80 mV$  to more positive potentials, under control conditions. Rapid bath infusion of  $10 \mu M$  AS significantly augmented current amplitude (Fig. 3B (ii)). The mean current voltage relationship for 15 cells is shown in Figure 3D. Potentials marked by an asterisk in the presence of  $10 \mu M$  AS are significantly greater than control ( $p < 0.05$ ,  $n = 15$ ). These data support the pressurized artery studies, indicating that HNO induced vasodilatation is mediated by enhancement of both  $K_V$  and  $BK_{Ca}$  channel activity.

#### HNO augments $K_V$ activity via soluble guanylyl cyclase

Pre-incubation of pressurized mesenteric vessels with ODQ ( $10 \mu M$ ,  $n = 3$ ), completely abolished vasodilatation. This indicated a central role for soluble guanylyl cyclase (Fig. 2B). Experiments with isolated smooth muscle cells confirmed these observations. The presence of ODQ ( $10 \mu M$ ) completely inhibited the augmentation of  $K_V$  current normally induced by AS in mesenteric artery smooth muscle cells (Fig. 4,  $n = 9$ ).

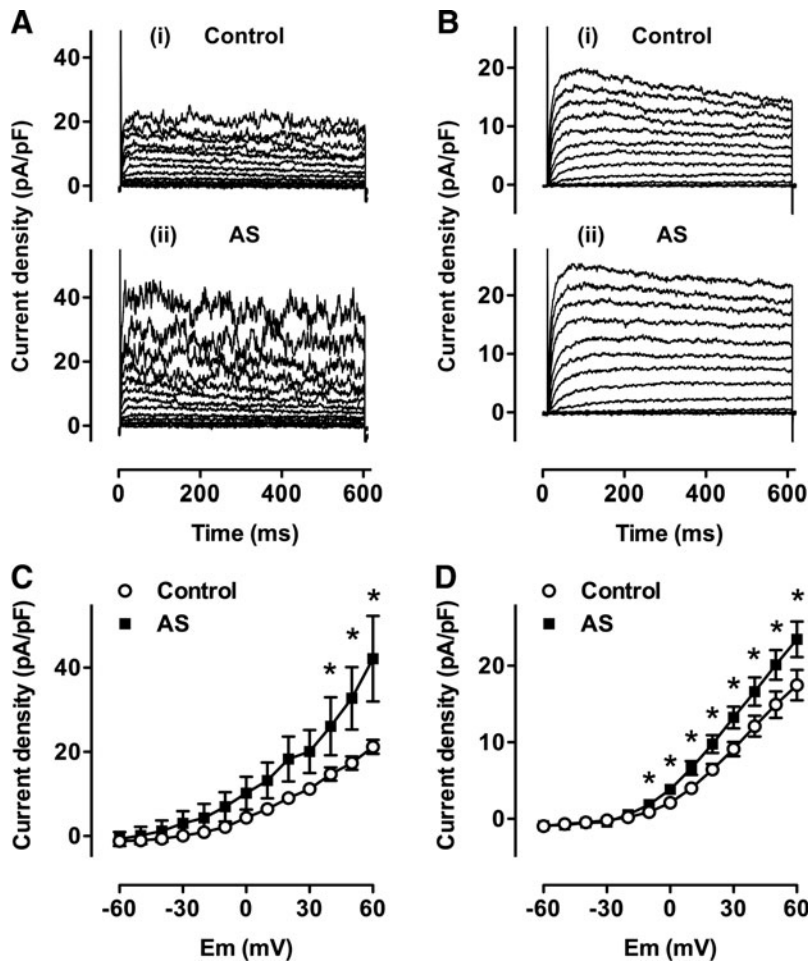
#### L-Cysteine ameliorates the response to Angeli's salt

To demonstrate directly if the current augmentation observed in the presence of AS was due to HNO, and not partially due to  $NO^*$ , experiments were performed in the presence of the nitroxyl scavenger L-cysteine. Figure 5A shows a cumulative concentration-response curve to AS in pressurized mesenteric arteries. Whilst the  $NO^*$  scavenger carboxy-PTIO did not alter vasodilatation to AS,  $3 mM$  L-cysteine, applied together with carboxy-PTIO (24), markedly inhibited this response ( $n = 6$ ). Similar responses were also observed using an alternate  $NO^*$  scavenger, hydroxycobalamin (HXC) (4, 34), data not shown.

In single smooth muscle cells, AS significantly augmented  $K_V$  currents as described previously (Fig. 5B upper panel). In the presence of L-cysteine and HXC, the same concentration of AS now failed to augment current amplitude (Fig. 5B lower panel). There was no significant difference in current amplitude in the presence or absence of AS in five cells incubated with L-cysteine and HXC, indicating that current augmentation is explained solely by the action of HNO.

#### Angeli's salt evokes spreading vasodilatation in pressurized mesenteric arteries

As previous studies have demonstrated that conducted vasodilatation is usually associated with agonists that either directly hyperpolarize the smooth muscle layer (39), or act indirectly by stimulating endothelium-dependent hyperpolarization (12, 39, 43), we investigated whether AS could evoke spreading responses in this preparation. Figure 6 shows that AS ( $30 \mu M$ ), infused locally into branch 1 of a triple-cannulated artery, evoked local dilatation (of phenylephrine-induced precontraction) that then spread upstream in the feed artery. Each individual trace shows the dilatation developed during 6 min luminal application of AS and



**FIG. 3.** Angeli's salt activates both  $BK_{Ca}$  and  $K_V$  channels in single smooth muscle cells. **(A-i)** Family of  $BK_{Ca}$  currents in an isolated mesenteric artery smooth muscle cell. Control currents shown are those sensitive to  $1 \mu M$  paxilline, and elicited upon  $10 mV$  step depolarizations to more positive potentials from holding potential of  $-80 mV$  in the presence of  $10 \mu M$  glibenclamide and  $100 \mu M$  HXC. **(A-ii)** Currents elicited under the same conditions as above, but in the presence of  $10 \mu M$  AS. **(B-i)** A family of  $K_V$  currents recorded in an isolated mesenteric artery smooth muscle cell. Control currents shown are those sensitive to  $1 mM$  4-AP, and elicited upon  $10 mV$  step depolarizations to more positive potentials from holding potential of  $-80 mV$  in the presence of  $1 \mu M$  paxilline,  $10 \mu M$  glibenclamide, and  $100 \mu M$  HXC. **(B-ii)** Currents elicited under the same conditions as above, but in the presence of  $10 \mu M$  AS. **(C, D)** Mean current-voltage relationship showing an augmentation in the  $BK_{Ca}$  current **(C,  $n = 7$ )** and  $K_V$  current **(D,  $n = 15$ )** to  $10 \mu M$  AS at test potentials indicated by an asterisk ( $p < 0.05$  vs control).

measured at  $500 \mu m$  intervals along the feed artery away from the point of infusion. Spreading dilatation was decremental, but was still significant beyond  $1.5 mm$  (Fig. 6C). Note also that the presence of AS in Branch 1 was associated with synchronized vasomotion observed along the length of the artery. In the presence of elevated external  $K^+$  ( $45 mM$ ) or the K-channel blockers TEA ( $1 mM$ ) and 4-AP ( $150 \mu M$ ), the spreading response to AS was completely abolished (Fig. 6C).

#### Spreading dilatation with HNO is sensitive to L-cysteine

Previous work has shown that ACh, by increasing endothelial cell  $Ca^{2+}$ , stimulates small conductance ( $SK_{Ca}$ , apamin sensitive) and intermediate conductance ( $IK_{Ca}$ , TRAM-34 sensitive) calcium-activated potassium channels (9, 10, 13, 14, 41), leading to smooth muscle cell hyperpolarization and consequent dilatation/relaxation. This hyperpolarization is observed in the presence of NOS inhibitors, which are routinely utilized in studies investigating EDHF responses, and would therefore preclude effects mediated by NO. As a recent study (3) has demonstrated that ACh, following blockade of  $SK_{Ca}$  and  $IK_{Ca}$  channels, but in the absence of NOS inhibitors, can evoke hyperpolarization and relaxation of rat mesenteric arteries via the release of HNO, we sought to establish whether this response was associated conducted vasodilatation. Figure 6D shows summary data for dilatation at  $500 \mu m$

distances along the feed artery, following luminal perfusion of ACh ( $10 \mu M$ ) and in the presence of apamin ( $50 nM$ ) and TRAM-34 ( $1 \mu M$ ) ( $n = 4$ ). As with responses to AS, robust spreading vasodilatation was stimulated, and decreased with distance along the feed artery. It too was accompanied by significant vasomotion. The local dilatation in Branch 1 was reduced by  $3 mM$  L-cysteine, and spreading dilatation was not evoked. The residual dilatation to ACh was further reduced by the NOS inhibitor L-NAME ( $n = 4$ ). These data indicate that the spreading responses induced by both Angeli's salt and ACh are likely to be initiated by HNO-dependent hyperpolarization of the smooth muscle.

#### Discussion

This study provides direct functional evidence from mesenteric resistance arteries, under physiological pressure, that HNO can evoke significant vasodilatation. Electrophysiological data from both whole tissue and single smooth muscle cells support the suggestion that HNO affects dilatation by augmenting current flow through both voltage gated and large conductance calcium-activated potassium channels. Finally, we provide the first direct observations to suggest a physiological role for endogenous HNO, in initiating spreading vasodilatation.

A key finding in the present study is that AS can induce vasodilatation in resistance arteries under physiological

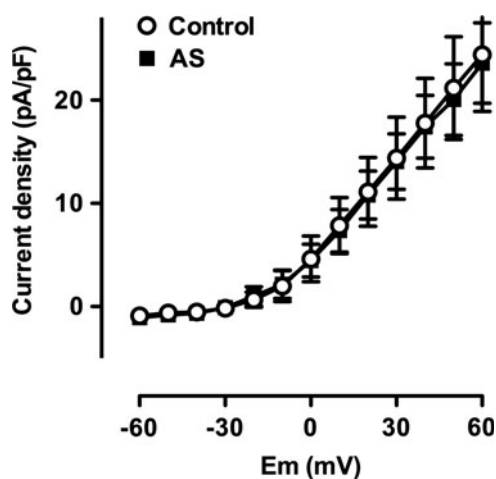


FIG. 4. Activation of  $I_{K_V}$  depends on soluble guanylyl cyclase. Mean current-voltage relationship showing full block of the augmentation in the  $K_V$  current (same conditions as in Figs. 3B and 3D) to  $10 \mu M$  AS in the presence of ODQ ( $10 \mu M$ , paired data,  $n = 9$ ).

pressure, and that this effect correlates with smooth muscle hyperpolarization. The fact that both hyperpolarization and dilatation were abolished in the presence of raised extracellular  $K^+$  suggests a cause and effect relationship. These data are consistent with previous observations in a variety of arterial preparations assessed mainly by wire myography (15, 16, 19, 24), demonstrating that nitroxyl, derived from AS, mediates vasodilatation in both the intact circulation and isolated arteries.

The hyperpolarization evoked by HNO appeared to be explained by the activity of both  $K_V$  and  $BK_{CA}$  channels in the smooth muscle layers. A central role for  $K_V$  channels was suggested originally from sharp electrode and isometric tension measurements in the mesenteric artery (17, 24).  $K_{V1.2}$ ,  $K_{V1.3}$ , and  $K_{V1.5}$  isoforms are expressed in these arteries and may therefore underlie both the resting membrane potential and the hyperpolarization (45). As 4-AP significantly attenuated responses to AS, our data confirm an action through  $K_V$  channels. Furthermore, selective recording of whole cell  $K_V$  currents showed AS induced a significant increase in whole current density, without altering the voltage dependent activation. Taken together, these observations demonstrate that  $K_V$  channels mediate a significant part of the hyperpolarization and dilatation to AS and thus HNO.

In the coronary vasculature,  $K_{ATP}$  channels contribute to AS-mediated vasodilatation (16), and in the mesenteric artery they underlie hyperpolarization from resting potential to NO gas or acidified sodium nitrite (21). However, we did not observe any significant effect of AS on direct recordings of  $K_{ATP}$  currents in isolated smooth muscle cells (data not shown), indicating that neither vasodilatation nor the underlying hyperpolarisation receive input through  $K_{ATP}$  activation. The ability of NO to activate  $K_{ATP}$  is lost once the muscle cells are depolarized, so this characteristic may explain our observations in the pressurized arteries (21).

$BK_{CA}$  channels in vascular smooth muscle contribute to both the repolarization and relaxation to NO gas, and the NO donors 3-morpholino-sydnominimide (SIN-1), sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) (5, 31,

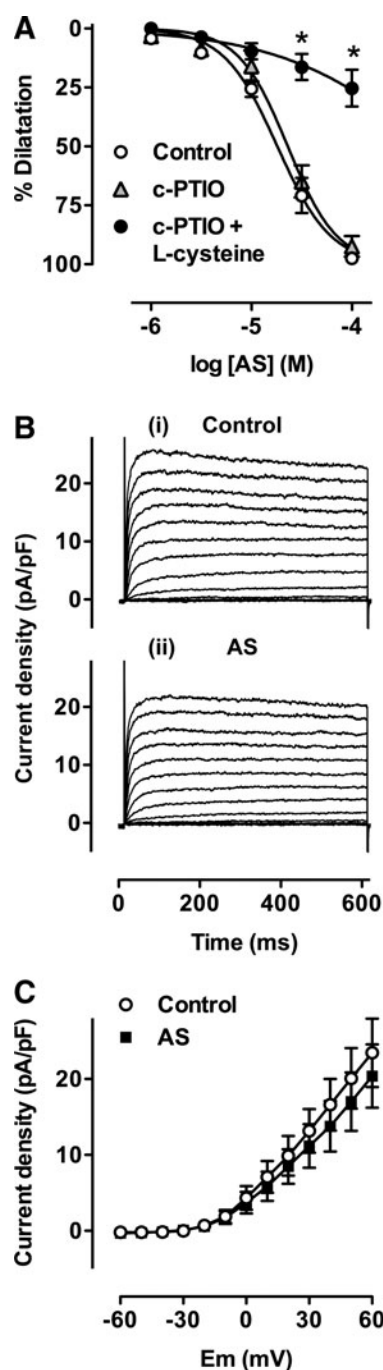
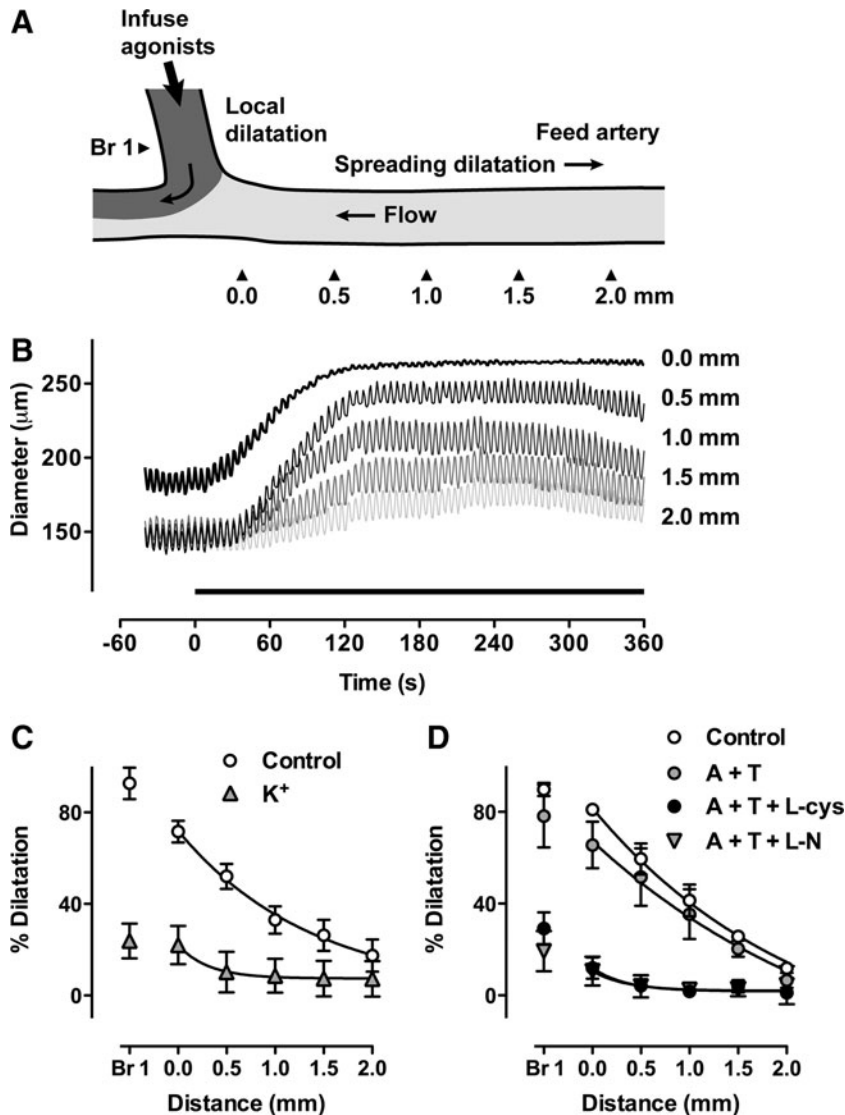


FIG. 5. Angeli's salt induced  $K_V$  augmentation is ameliorated by L-cysteine. (A) The effects of the NO $^*$  scavenger carboxy-PTIO (c-PTIO,  $200 \mu M$ ) alone ( $n = 2$ ) or together with the HNO scavenger L-cysteine ( $3 mM$ ,  $n = 6$ ), on the cumulative concentration response curves to Angeli's salt (AS).  $*p < 0.05$  vs control. (B-i) A family of  $K_V$  currents recorded in an isolated mesenteric artery smooth muscle cell. Control currents shown are those sensitive to  $1 mM$  4-AP, and elicited upon  $10 mV$  step depolarizations to more positive potentials from holding potential of  $-80 mV$  in the presence of  $1 \mu M$  paxilline,  $10 \mu M$  glibenclamide, and  $100 \mu M$  HXC. (B-ii) Currents elicited under the same conditions as above, but in the presence of both L-cysteine ( $3 mM$ ) and  $10 \mu M$  AS. (C) Mean current-voltage relationship showing full block of the augmentation in the  $K_V$  current to  $10 \mu M$  AS in the presence of L-cysteine ( $3 mM$ , paired data,  $n = 5$ ).



**FIG. 6.** HNO induces spreading dilatation responses in pressurized rat mesenteric arteries. (A) Using a third pipette, one branch at an arterial bifurcation (Br 1) was cannulated, through which perfusate containing an agonist and fluorescent marker was infused. (B) Simultaneous traces of outer diameter in response to luminal infusion of  $30\ \mu\text{M}$  Angeli's salt (AS) and  $0.1\ \mu\text{M}$  carboxyfluorescein into Branch 1 (Br 1). The arrowheads in (A) indicate the positions of diameter and fluorescence measurement in Br 1 and upstream from the bifurcation into the Feed artery (0–2.0 mm). Phenylephrine ( $0.5\ \mu\text{M}$ ) was present in the superfusion solution, resulting in 79% of maximum tone and synchronized vasomotion along the entire arterial segment. AS was infused during the period indicated by the bar (0–360 s) and clearly evoked spreading dilatation that decayed with distance from the bifurcation, but was associated with continued synchronized vasomotion (compare to Fig. 1A). (C) Summary data showing local (Br 1) and spreading dilatation (0–2.0 mm) to luminal perfusion of  $30\ \mu\text{M}$  AS into Br 1 ( $n=3$ ). The data are normalized to  $\sim 80\%$  dilatation at 0 mm. The local and spreading dilatation were markedly reduced in the presence of either  $45\ \text{mM}$   $\text{K}^+$  ( $n=1$ ) or TEA ( $1\ \text{mM}$ ) and 4-AP ( $150\ \mu\text{M}$ ) ( $n=2$ ), and these data were combined ( $\text{K}^+$ ,  $n=3$ ). (D) Summary data showing local (Br 1) and spreading dilatation (0–2.0 mm) to luminal perfusion of ACh ( $10\ \mu\text{M}$ ) into Br 1. Responses were obtained in the absence (Control) and presence of apamin ( $50\ \text{nM}$ ) and TRAM-34 ( $1\ \mu\text{M}$ , A + T) ( $n=4$ ), and were normalized to  $\sim 80\%$  dilatation at 0 mm. The local and spreading dilatation were markedly reduced by the additional presence of either L-cysteine ( $3\ \text{mM}$ , A + T + L-cys,  $n=4$ ), or the NOS inhibitor L-NAME ( $100\ \mu\text{M}$ , A + T + L-N,  $n=4$ ).

32). However, vasorelaxation induced by AS appears insensitive to the  $\text{BK}_{\text{Ca}}$  inhibitor IbTx (24). However, in this previous study IbTx was tested alone, so AS-evoked vasorelaxation might still be mediated through  $\text{K}_{\text{V}}$  activation. We demonstrated a partial attenuation of the AS-evoked vasodilatation with IbTx. This observation was consistent with our single cell experiments, where AS clearly activated  $\text{BK}_{\text{Ca}}$  channels at positive test potentials. Furthermore, in combination 4-AP and IbTx abolished the effect of AS. So it seems reasonable to conclude that both channel types contribute to vasodilatation and the underlying smooth muscle hyperpolarization to AS.

Previous studies have suggested that NO in the form of  $\text{NO}^*$  is responsible for stimulating soluble guanylyl cyclase (7). However, as discussed in a recent review by Irvine *et al.* (26), these studies were carried out under high-thiol-containing conditions that would be predicted to scavenge HNO. Consistent with this suggestion, is the fact that vasodilatation to AS in aorta (15, 42), anococcygeus (27), urethral (6), and mesenteric artery smooth muscle (24), among others, is sen-

sitive to the soluble guanylyl cyclase inhibitor ODQ, indicating that HNO can affect vasodilatation by increasing intracellular cGMP. Recently, ODQ has also been shown by one of us to block both the AS-evoked repolarization and relaxation of mesenteric arteries, and to have far less effect against NO gas. These observations are therefore consistent with previous data and strongly indicate that soluble guanylyl cyclase is the signaling mechanism linking HNO to activation of  $\text{K}_{\text{V}}$  (17). Albeit that the possibility of significant intracellular oxidation of HNO to  $\text{NO}^*$  still remains to be discounted.

Our data appear to be quite clear. Vasodilatation to AS was effectively blocked with ODQ, suggesting that soluble guanylyl cyclase is the central signaling transducer in the activation of both  $\text{K}_{\text{V}}$  and  $\text{BK}_{\text{Ca}}$ . In patch-clamp experiments, ODQ blocked the augmented  $\text{K}_{\text{V}}$  current with AS, consistent with our data in Favalaro and Kemp-Harper (17). We are currently investigating whether  $\text{BK}_{\text{Ca}}$  channels are subject to a similar influence. However, the activation of  $\text{BK}_{\text{Ca}}$  with NO, presumably as  $\text{NO}^*$ , appeared not to be sensitive to ODQ (29).



To ensure, as far as reasonably possible, that we were recording only the effect of HNO on pressurized arteries and isolated smooth muscle cells, we used pharmacological agents to distinguish between the different redox states of NO. The free radical scavengers carboxy-PTIO and HXC, both inactivate  $NO^*$ , with no effect against HNO (15, 27, 42), whereas mM concentrations of L-cysteine scavenge only HNO (15, 30, 42). We found that carboxy-PTIO did not modify vasodilatation to AS, consistent with this agent acting purely via HNO, not  $NO^*$ . This conclusion is supported by the fact that L-cysteine (4) applied together with carboxy-PTIO did eliminate vasodilatation. Further support comes from the single cell electrophysiological experiments, where the direct effect of AS on  $K_V$  current was markedly reduced in the presence of L-cysteine.

Probably the most important observation in the present study is the demonstration that AS stimulates spreading or conducted vasodilatation. To achieve the increases in tissue blood flow necessary to meet changing metabolic demand, the restricted local action of vasodilator agents must be conducted upstream in order to provide a significant drop in vascular resistance. Whether initiated in the endothelium or the smooth muscle, hyperpolarization can spread axially through the walls of small arteries and arterioles to affect this response (see Dora, (11) for a recent review). In small mesenteric arteries, increasing endothelial cell calcium with agonists such as ACh as well as generating NO also activates the  $SK_{Ca}$  and  $IK_{Ca}$  channels within these cells causing hyperpolarization. The hyperpolarization spreads radially into the smooth muscle to affect 'EDHF'-mediated dilatation, but in addition somehow initiates a calcium-independent hyperpolarization at upstream sites (39). This axial hyperpolarization forms the basis of spreading dilatation. A similar response occurs when hyperpolarization is initiated in the smooth muscle by directly activating  $K_{ATP}$  channels with levcromakalim (39). Interestingly, a fundamental feature of spreading dilation is that it has not been observed following local application of NO (likely  $NO^*$  with NONOate) (43), presumably because hyperpolarization was not induced. By blocking the 'EDHF' response to ACh specifically with blockers with apamin and TRAM-34 together, we now show that an *endogenous* nitrogen oxide can actually cause spreading dilatation, and that this ability appears to be explained by the generation and action of HNO. Also of note is the fact that the dilatation to HNO was superimposed with vasomotion. The possibility that HNO has an important role in vasomotion, a physiological response that remains to be fully defined, is an exciting development that merits further investigation.

In conclusion, we show that AS can induce robust and sustained vasodilatation in mesenteric resistance-size arteries under physiological pressure. This response is caused by muscle hyperpolarization, and appears to reflect the activation of both  $K_V$  and  $BK_{Ca}$  channels possibly via soluble guanylyl cyclase. Based on the sensitivity of hyperpolarization and dilatation to the thiol L-cysteine, and resistance to block with carboxy-PTIO and HXC, the effects appear to be due solely to HNO. Finally, it appears that HNO can be generated endogenously by the endothelium in quantities sufficient to initiate spreading vasodilatation, suggesting an important physiological role for this reduced form of NO.

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## Author Disclosure Statement

No competing financial interests exist.

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**Abbreviations Used**

4-AP = 4-aminopyridine  
Ach = acetylcholine  
AS = Angeli's salt  
 $BK_{Ca}$  = big conductance calcium activated potassium channel  
cGMP = cyclic-guanosine-monophosphate  
EDHF = endothelium derived hyperpolarizing factor  
EDRF = endothelium derived relaxation factor  
HNO = nitroxyl  
HXC = hydroxocobalamin,  $NO^\bullet$  scavenger  
IbTx = Iberiotoxin,  $BK_{Ca}$  inhibitor.

$I_{Kv}$  = voltage-gated potassium channel current  
 $K_V$  = voltage-gated potassium channel  
NO = nitric oxide  
 $NO^\bullet$  = uncharged free radical state of NO molecule  
 $NO^+$  = the nitrosonium cation in oxidized state  
 $NO^-$  = the nitroxyl anion in reduced state  
NOS = nitric oxide synthase  
ODQ = soluble guanylyl cyclase inhibitor (1H-(1,2,4)oxadiazole(4,3-a)quinoxalin-1-one)  
sGC = soluble guanylyl cyclase  
 $SK_{Ca}$  = small conductance calcium-activated potassium channel

