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Endothelial nitric oxide modulates perivascular sensory neurotransmission in the rat isolated mesenteric arterial bed

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1 A possible role of nitric oxide (NO) as a modulator of capsaicin-sensitive sensory neurotransmission in blood vessels was investigated in the rat isolated mesenteric arterial bed.

2 Electrical field stimulation (EFS) of methoxamine-preconstricted mesenteric beds elicited frequency-dependent vasorelaxation mediated by capsaicin-sensitive sensory nerves. N^G-nitro-L-arginine methyl ester (L-NAME, 10 and 300 μM) and 7-nitroindazole (7-NI, 100 μM), inhibitors of nitric oxide synthase (NOS), augmented sensory neurogenic vasorelaxation. D-NAME (300 μM), 6-aminoindazole (100 μM) and N^{ω}-propyl-L-arginine (50 nM), a selective inhibitor of neuronal NOS, were without effect. The effect of 10 μM L-NAME was reversed by L-arginine (1 mM), the substrate for NOS.

3 L-NAME (300 μM) and 7-NI (100 μM) had no significant effect on vasorelaxations to calcitonin gene-related peptide (CGRP), the principal motor neurotransmitter of capsaicin-sensitive sensory nerves in rat mesenteric arteries, or to capsaicin, indicating a prejunctional action. The inhibitors of NOS had no effect on vasorelaxation to forskolin, but augmented vasorelaxation to sodium nitroprusside (SNP).

4 Removal of the endothelium augmented sensory neurogenic vasorelaxation, but did not affect vasorelaxation to CGRP, indicating a prejunctional action of endothelial NO.

5 In the absence of endothelium, L-NAME (300 μM) inhibited, and 7-NI (100 μM) caused no further augmentation of sensory neurotransmission.

6 SNP (100 nM), a nitric oxide donor, attenuated sensory neurogenic relaxations to EFS.

7 In rat isolated thoracic aortic rings, L-NAME (100 μM) and 7-NI (100 μM) attenuated concentration-dependent relaxations to acetylcholine.

8 These data show that NO modulates sensory neurotransmission evoked by EFS of the rat isolated mesenteric arterial bed, and that when NO synthesis is blocked sensory neurogenic relaxation is augmented. The source of NO is the vascular endothelium.

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Abbreviations: α,β-meATP, α,β-methylene ATP; ACh, acetylcholine; CGRP, calcitonin gene-related peptide; EFS, electrical field stimulation; L-NAME, N^G-nitro-L-arginine methyl ester; 7-NI, 7-nitroindazole; NO, nitric oxide; NOS, nitric oxide synthase; SNP, sodium nitroprusside

Introduction

Capsaicin-sensitive sensory nerves are widely distributed in the cardiovascular system (Barja *et al*., 1983; Wharton *et al*., 1986). They are found in the adventitia of blood vessels and are activated by a variety of chemical and mechanical stimuli that present challenges to homeostasis (Maggi & Meli, 1988; Holzer, 1992). Activation of the peripheral terminals of sensory nerves elicits an afferent function, whereby the perceived information is transmitted to the central nervous system to initiate voluntary and autonomic reflexes, and an efferent function, whereby neurotransmitters are released from the peripheral terminals of the nerves. The released sensory neurotransmitters evoke arteriolar vasorelaxation and an increase in venular permeability. Perivascular sensory nerves contain and release a variety of neurotransmitters, including the neuropeptides substance P and calcitonin gene-related peptide (CGRP). In addition to their involvement in 'defence' of the cardiovascular system, sensory nerves in the mesentery may have a physiological role in coordinating, by reflex activation, mesenteric blood flow with changes in gut motility (Meehan & Kreulen, 1992).

At the luminal surface of blood vessels the endothelium is also an important regulator of blood vessel calibre and permeability *via* release of a variety of substances including the potent vasorelaxant nitric oxide (NO). In many blood vessels there is a tonic release of NO from the endothelium that is important in regulating basal and evoked tone, such that removal of the endothelium, or inhibition of NO synthesis, causes an elevation of tone and potentiates responses to various vasoconstrictors (Martin *et al*., 1986; Li & Duckles, 1992; Reid & Rand, 1992; Vo *et al*., 1992; Cederqvist & Gustafsson, 1994). Despite being separated by up to several layers of smooth muscle cells, interactions can

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occur between the endothelium and perivascular nerves. For instance NO, which diffuses readily across cell membranes, can modulate the release of contractile neurotransmitter from sympathetic nerves in blood vessels (Greenberg *et al*., 1989; 1990; Yamamoto *et al*., 1993). Whether NO can modulate perivascular sensory neurotransmission is unclear (Amerini *et al*., 1993; Li *et al*., 1993).

The aim of the present study, therefore, was to investigate a possible role of NO as a modulator of sensory neurotransmission in the rat isolated mesenteric arterial bed. To this end 7-nitroindazole (7-NI), a non-selective NO synthase (NOS) inhibitor (Moore & Handy, 1997; Alderton *et al*., 2001), as well as the archetypal NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) were used. In rat mesenteric arteries CGRP is the principal sensory motor neurotransmitter, mediating pronounced vasorelaxation (Kawasaki *et al*., 1988) *via* $CGRP_1$ receptors on the smooth muscle (Lei *et al*., 1994). Thus, in order to determine whether the effects of NOS inhibition were mediated pre- or postjunctionally, responses to CGRP and other vasodilators were investigated. The endothelium is not the only source of NO in the vasculature, NO being utilized as a neurotransmitter in some perivascular nerves (Liu *et al*., 1991; Toda & Okamura, 1992; Brizzolara *et al*., 1993; Yoshida *et al*., 1993; Toda *et al*., 1993). Hence, the effects of N^{ω}-propyl-L-arginine, a selective neuronal NOS (nNOS) inhibitor (Zhang *et al*., 1997), and of endothelium removal on sensory neurotransmission, and on the actions of the NOS inhibitors, were additionally investigated.

Methods

Male Wistar rats (250–300 g) were killed by exposure to CO_2 and decapitation. Mesenteric beds and thoracic aortae were isolated for experimentation.

Mesenteric arterial beds

Mesenteric beds were isolated and perfused, *via* the superior mesenteric artery, as described previously (Ralevic *et al*., 1996). In brief, the abdomen was opened and the superior mesenteric artery exposed and cannulated with a hypodermic needle. The superior mesenteric vein was cut, blood flushed from the preparation with 0.5 ml of Krebs' solution and the gut dissected carefully away from the mesenteric vasculature. The preparation was mounted on a stainless steel grid (7 × 5 cm) in a humid chamber and perfused at a constant flow rate of 5 ml min^{-1} using a peristaltic pump (model 7554-30, Cole-Parmer Instrument Co., Chicago, IL, U.S.A.). The perfusate was Krebs'–Bülbring solution of the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52 and glucose 7.8, gassed with 95% O_2–5% CO_2 and maintained at 37°C. The perfusate contained indomethacin (10 μM), to block cyclo-oxygenases that might have been generated during the experiment, and guanethidine (5 μM) to block sympathetic neurotransmission. Responses were measured as changes in perfusion pressure (mmHg) with a pressure transducer (model P23XL, Viggo-Spectramed, Oxnard, CA, U.S.A.) on a side arm of the perfusion cannula, and were recorded on a polygraph (model 7D, Grass Instrument Co., Quincy, MA, U.S.A.).

Experimental protocol–mesenteric beds

After 30 min equilibration, a submaximal concentration of methoxamine (10–50 μM) was added to the perfusate in order to increase the perfusion pressure of the preparations (by 40–80 mmHg) above baseline. In the presence of certain of the NOS inhibitors, a lower concentration of methoxamine had to be used in order to achieve this level of tone, and the possible effect of this was investigated in a latter part of the study. In the raised-tone preparations a frequency response curve was generated by electrical field stimulation (EFS; 1–12 Hz, 60 V, 0.1 ms, 30 s), and this was followed by generation of a dose–response curve to CGRP (0.05–50 pmol). The relaxant response to EFS can be abolished by *in vitro* and *in vivo* capsaicin pretreatment, indicating that it is mediated entirely by capsaicin-sensitive sensory nerves (Ralevic *et al*., 1995). The relaxant response to EFS in the presence of L-NAME was also shown to be abolished by capsaicin pretreatment in the present study ($n=2$), showing that the augmentation of responses in the presence of L-NAME is not due to the recruitment of a novel vasodilator mechanism. The protocol of EFS followed by CGRP was carried out under control conditions, or in the presence of NOS inhibitors, D-NAME, 6-aminoindazole or L-arginine, added during equilibration, at least 30 min prior to construction of the frequency response curves.

In separate experiments, the effect of SNP, a NO donor, on sensory neurotransmission was investigated. Preliminary experiments, in which concentration relaxation–response curves were constructed, had shown that SNP at 100 nM elicits approximately 75% relaxation of the methoxamine-constricted mesenteric arterial bed. In order to achieve raised tone in the presence of the vasodilator action of SNP, whilst avoiding addition of very high concentrations of methoxamine, α,β-methylene ATP (α,β-meATP, 0.1 μM) was added to the perfusate. Methoxamine was then added, as under control conditions, to raise tone to the working range. In separate experiments, also in methoxamine preconstricted preparations, 8 bromo cGMP and dibutyryl cAMP were added after generation of a first frequency response curve to EFS, 15 min before a second frequency response curve was carried out in the same preparation. The frequency–response curves are reproducible when carried out under control conditions. Endothelium removal was achieved by perfusion with distilled water (7–8 min) and was confirmed using acetylcholine (ACh, 5 nmol), which evoked a relaxation of $10.15 \pm 2.38\%$ ($n=17$). This dose normally elicits >80% relaxation in endothelially-intact preparations. In other methoxamine-preconstricted mesenteric beds, consecutive relaxation dose–response curves to bolus injections (50 μl) of doses of forskolin, SNP and capsaicin, in that order, were generated. In separate preparations the response curves were investigated in the presence of inhibitors of NOS.

Aortae

Thoracic aortae were cleaned of adherent tissue and cut into rings of about 4 mm in length. The rings were mounted in 10 ml organ baths containing Krebs'–Bülbring solution, saturated with 95% O_2/5% CO_2 and kept at 37°C. Two stainless steel hooks were inserted through the lumen; the lower hook was fixed and the upper one was attached to an isometric force transducer

(FT03, Grass, Quincy, MA, U.S.A.). The resting tension was set to 1 g and preparations allowed to equilibrate for 1 h, during which time the incubation medium was replaced twice, at 15 min intervals. After 1 h, 10 μM methoxamine was added to the medium to contract the tissues, and washed out with three changes of medium when the response had reached a maximum. After 30 min, L-NAME (10 and 100 μM), 7-NI (100 μM), ethanol (0.1% v v^{-1}) or no drug was added to the medium. After a further 25 min, methoxamine (0.2–10 μM) was added to the medium to precontract the preparations to about 0.5 g. When the contraction had plateaued ACh was added in a cumulative fashion to elicit a concentration–relaxation response curve. As there was no significant difference in relaxations elicited in the absence of agents ($n=3$) or in the presence of vehicle (ethanol; $n=3$), these control data were pooled.

Drugs

α,β-methylene ATP (lithium salt), acetylcholine (chloride), 8 bromo cGMP, calcitonin gene-related peptide, capsaicin (8-methyl-N-vanillyl-6-nonenamide), dibutyryl cAMP, forskolin, methoxamine (hydrochloride), L-NAME, 7-NI, L-arginine, D-NAME and SNP were from Sigma Chemical Co. N^{ω}-propyl-L-arginine was from Tocris (Avonmouth, U.K.). 6-Aminoindazole was from Aldrich (Gillingham, Dorset, U.K.), Guanethidine (Ismelin) was from Alliance Pharmaceuticals, Chippenham, Wiltshire, U.K. All drugs were made up in distilled water except for capsaicin and forskolin, which were made up as stock solutions of 10 mM in dimethyl suphoxide, and 7-NI and 6-aminoindazole, which were made up as stock solutions of 0.1 M in ethanol.

Data analysis

Vasorelaxant responses of the mesenteric arterial beds were measured as changes in perfusion pressure (mmHg) and expressed as percentage relaxation of the methoxamine-induced increase in tone above baseline. pD_2 values (negative logarithm of the dose or concentration of agonist required to elicit a half maximal response) were determined for each experiment where appropriate. ACh-induced relaxations of the aortic rings were measured at their maximum and expressed as a percentage of the methoxamine-induced contraction. Where the comparison was between more than two groups, data were compared by analysis of variance (ANOVA) with Tukey's *post hoc* test. A value of $P<0.05$ was taken to indicate a statistically significant difference. Data are expressed as mean $\pm$ s.e.mean and the number of preparations (n) in each group is given.

Results

Effects of L-NAME, D-NAME, 7-NI, 6-aminoindazole and N^{ω}-propyl-L-arginine on relaxation to EFS

EFS elicited frequency-dependent vasorelaxations of the mesenteric arterial beds that were augmented by L-NAME (300 μM) and 7-NI (100 μM) ($n=6-11$) (Figures 1 and 2). D-NAME (300 μM), the inactive enantiomer of L-NAME, and 6-aminoindazole (100 μM), an inactive analogue of 7-NI, had no significant effect on relaxations to EFS (Figure 3a,b). N^{ω}-propyl-L-arginine (50 nM) also had no significant effect on relaxations to EFS: responses at 4Hz were $48.8\pm8.8\%$ in controls ($n=6$) and $46.5\pm11.7\%$ ($n=6$) in the presence of N^{ω}-propyl-L-arginine; maximal relaxations (at 12 Hz) were $67.1\pm5.3\%$ ($n=6$) in controls and $66.2\pm6.6\%$ ($n=6$) in the presence of N^{ω}-propyl-L-arginine.

In the controls, the concentration of methoxamine used to raise the tone of the preparations was 37.82 ± 6.51 μM ($n=14$). This was significantly lower in the presence of L-NAME and 7-NI (3.44 ± 0.38 μM, $n=16$ and 14.31 ± 2.89 μM, $n=13$) ($P<0.001$). Compared to the controls, there was no significant difference in the concentration of methoxamine used to preconstrict the preparations in the presence of D-NAME (45 ± 4.08 μM, $n=6$), 6-aminoindazole (32.5 ± 4.23 μM, $n=6$) and N^{ω}-propyl-L-arginine (25.17 ± 6.47 μM, $n=6$).

Effects of L-NAME, D-NAME and 7-NI on relaxation to CGRP

There was no significant difference between dose-dependent relaxation response curves to exogenous CGRP under control conditions and in the presence of L-NAME (300 μM) and 7-NI (100 μM) (pD_2 values were 11.62 ± 0.1 ($n=6$), 11.48 ± 0.1 ($n=10$) and 11.81 ± 0.16 ($n=5$), respectively) (Figure 2b). D-NAME (300 μM) also had no significant effect on relaxations to CGRP (pD_2 value 11.11 ± 0.2, $n=6$) (Figure 3c).

Effect of L-arginine on modulation by L-NAME of relaxation to EFS

L-NAME (10 μM) augmented relaxations to EFS and this effect was reversed by L-arginine (1 mM) (Figure 3d). L-arginine alone had no significant effect on relaxation responses to EFS (Figure 3d) and CGRP (Figure 3c).

Effects of L-NAME and 7-NI on relaxation to capsaicin, forskolin and SNP

Dose-dependent relaxation to capsaicin was not significantly different in control conditions and in the presence of L-NAME (300 μM) and 7-NI (100 μM) (Figure 4a). The pD_2 values were 10.86 ± 0.05 ($n=8$), 10.95 ± 0.14 ($n=8$) and 10.97 ± 0.14 ($n=7$), respectively. The relaxation dose–response curve to SNP was leftward shifted in the presence of L-NAME. Although there was a trend for a shift in the response curve to SNP in the presence of 7-NI this did not reach statistical significance. pD_2 values in controls and in the presence of L-NAME and 7-NI were 9.98 ± 0.1 ($n=5$), 10.72 ± 0.13 ($n=7$) ($P<0.001$ *vs* control) and 10.17 ± 0.12 ($n=6$), respectively (Figure 4b). Dose-dependent relaxations to forskolin were not significantly different in control conditions and in the presence of L-NAME and 7-NI (Figure 4c). The pD_2 values were 9.21 ± 0.08 ($n=7$), 9.14 ± 0.11 ($n=7$) and 9.26 ± 0.13 ($n=6$), respectively.

Effects of L-NAME and 7-NI on relaxations to ACh in rat thoracic aortae

ACh elicited concentration-dependent relaxations of preconstricted thoracic aortae in control conditions (E_{max} $104.48\pm7.30\%$; pD_2 7.37 ± 0.07, $n=6$), in the presence of 10 μM L-NAME (E_{max} $87.19\pm7.14\%$; pD_2 7.26 ± 0.20, $n=7$),

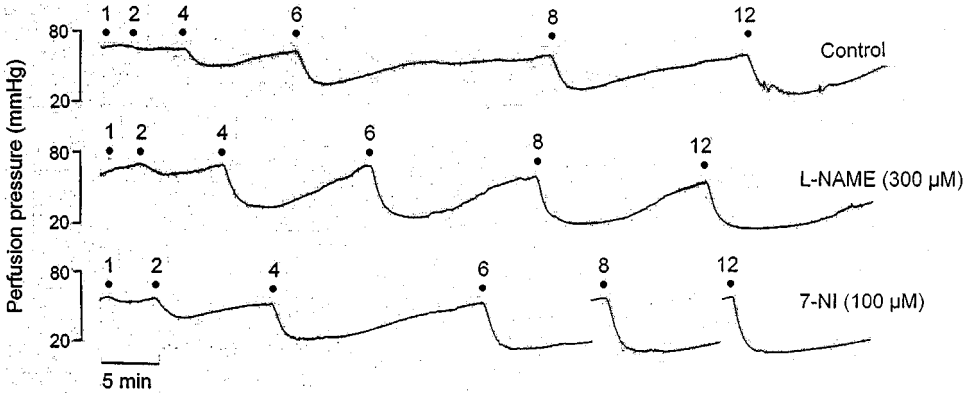

Figure 1 Representative traces showing effects of L-NAME and 7-NI on sensory neurogenic vasorelaxation to electrical field stimulation (EFS; 1–12 Hz) in methoxamine-preconstricted rat isolated mesenteric arterial beds. Frequency-dependent vasorelaxations to EFS are shown in preparations in control conditions (upper trace), in the presence of L-NAME (middle trace) and in the presence of 7-NI (lower trace). The NOS inhibitors augmented relaxations to EFS.

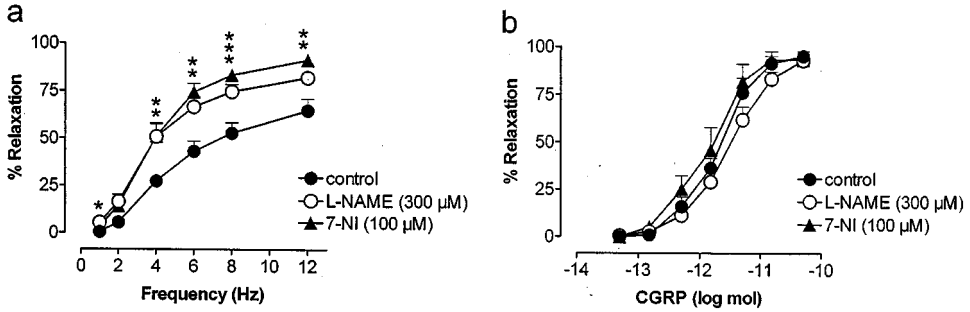

Figure 2 Effects of L-NAME and 7-NI on sensory neurogenic vasorelaxation to electrical field stimulation (EFS) and relaxations to calcitonin gene-related peptide (CGRP) in the rat isolated mesenteric arterial bed. (a) Relaxations to EFS in controls ($n=8$), and in the presence of the nitric oxide synthase inhibitors N^G-nitro-L-arginine methyl ester (L-NAME; $n=11$) and 7-nitroindazole (7-NI; $n=6$). (b) Relaxations to CGRP in controls ($n=6$) and in the presence of L-NAME ($n=10$) and 7-NI ($n=5$). Data are presented as means + s.e.mean. Significant differences between responses under control conditions compared to responses in the presence of both NOS inhibitors are denoted by $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

in the presence of 100 μM L-NAME (E_{max} 65.36 ± 12.29%; pD_2 6.91 ± 0.20, $n=8$) and in the presence of 100 μM 7-NI (E_{max} 95.24 ± 5.77%; pD_2 6.8 ± 0.08, $n=7$) (Figure 5). L-NAME at 100 μM attenuated the maximal relaxation to ACh compared to the control response ($P<0.01$). 7-NI alone inhibited the sensitivity of relaxation ($P<0.05$ *versus* control response).

There was no significant difference in the level of methoxamine-preconstricted tone that was achieved between the four groups of aortic rings: controls, 0.54 ± 0.03 g ($n=6$); 10 μM L-NAME, 0.60 ± 0.06 g ($n=7$); 100 μM L-NAME, 0.65 ± 0.04 g ($n=8$); 100 μM 7-NI, 0.50 ± 0.03 g ($n=7$). However, the concentration of methoxamine used to preconstrict the preparations was significantly greater in controls (10 ± 0 μM, $n=6$) and with 7-NI (9.17 ± 0.83 μM, $n=7$) than in the presence of 10 μM L-NAME (1.1 ± 0.20 μM, $n=7$) and 100 μM L-NAME (2.0 ± 1.16 μM, $n=8$) ($P<0.001$).

Effect of endothelium removal on responses to EFS and CGRP

Endothelium removal augmented sensory neurogenic relaxation to EFS, mimicking the effects of the NOS inhibitors (Figure 6 and 7a). Endothelium removal had no significant effect on dose-dependent relaxation to CGRP (Figure 7b). pD_2 values for responses to CGRP were 11.62 ± 0.1 ($n=6$) and 11.49 ± 0.09 ($n=7$) in the presence and absence of endothelium respectively.

In the absence of the endothelium, a lower concentration of methoxamine was needed to preconstrict the preparations (11.71 ± 1.78 μM, $n=7$) compared to the endothelium-intact controls. This concentration was not significantly different to the concentration of methoxamine used after addition of 7-NI in endothelially-denuded preparations (8.63 ± 0.8 μM, $n=4$). After endothelium removal, and in the presence of L-NAME, the concentration of methoxamine required to raise tone (4 ± 0.23 μM, $n=6$) was significantly lower than in the endothelially-denuded preparations alone, or in the presence of 7-NI ($P<0.05$).

Effect of endothelium removal on modulation by L-NAME and 7-NI of relaxation to EFS

In the absence of endothelium, there was no further augmentation by 7-NI of relaxation to EFS (Figure 7a). In contrast, L-NAME reversed the augmentation of relaxation to EFS caused by endothelium removal (Figure 7a). In the absence of endothelium and presence of L-NAME relaxation to CGRP was also slightly, but significantly, attenuated (pD_2 value 11.33 ± 0.07, $n=6$; $P<0.05$ *vs* control) (Figure 7b).

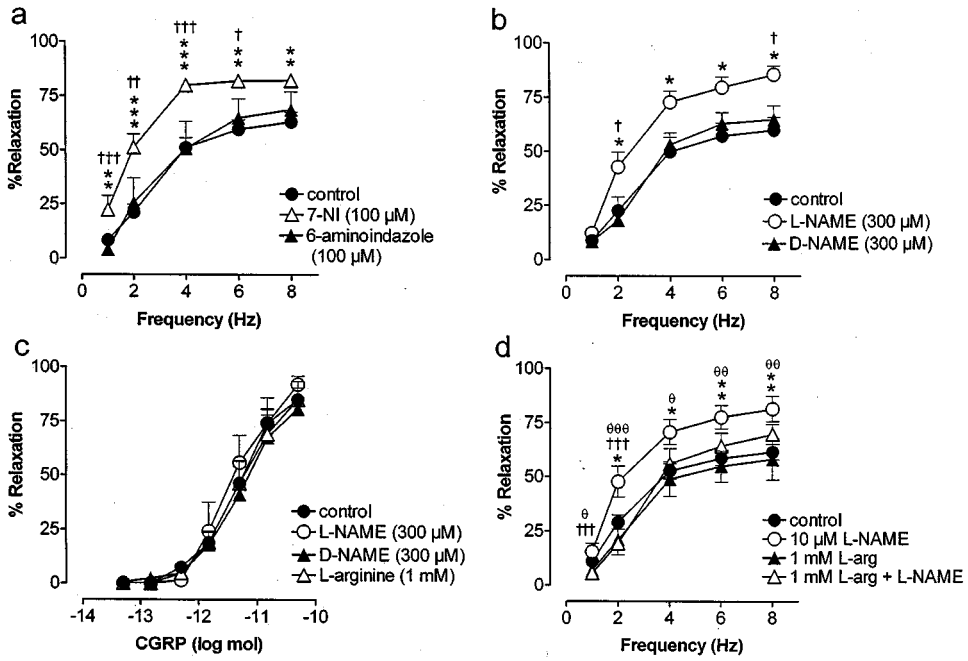

Figure 3 Effects of 6-aminoindazole, D-NAME and L-arginine on sensory neurogenic relaxation in the rat isolated mesenteric arterial bed. (a) Relaxation responses to electrical field stimulation (EFS; 1–8 Hz) in the presence of 6-aminoindazole ($n=5$), 7-nitroindazole (7-NI; $n=7$) and in control condition ($n=14$). Significant difference of 7-NI compared to the control group is denoted by $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. Significant difference of 6-aminoindazole compared to the control group is denoted by † $P<0.05$, †† $P<0.01$ and ††† $P<0.001$. (b) Effect of N^G-nitro-L-arginine methyl ester (L-NAME) and D-NAME on relaxations to EFS ($n=5-7$). Significant difference between L-NAME and control group is denoted by $^*P<0.05$, and between L-NAME and D-NAME is denoted by †$P<0.05$. (c) Effect of L-NAME, D-NAME and L-arginine on relaxation responses to calcitonin gene-related peptide (CGRP) ($n=5-7$). (d) Effect of 10 μM L-NAME, in the absence and presence of L-arginine (L-arg), on sensory neurogenic relaxations to EFS. There was no significant effect of L-arginine alone ($n=6$). Significant difference between responses in the presence of L-NAME ($n=9$) and control group ($n=15$) are denoted by $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. Significant difference between responses in the presence of L-NAME and in the presence of L-arg + L-NAME ($n=7$) are denoted by †$P<0.05$, ††$P<0.01$ and †††$P<0.001$. Significant difference between responses in the presence of L-NAME and L-arg are denoted by $\theta P<0.05$, $\theta\theta P<0.01$ and $\theta\theta\theta P<0.001$. Data are presented as means + s.e.mean.

Effect of SNP and methoxamine on responses to EFS

SNP (100 nM) attenuated relaxation to EFS (Figure 8). In the presence of the NOS inhibitors lower concentrations of methoxamine were required to preconstrict the preparations. In order to investigate whether augmentation of relaxation to EFS was due to the lower concentration of methoxamine, α,β-meATP (0.1 μM) was added to reduce the concentration of methoxamine required to preconstrict the preparations. α,β-meATP at up to 10 μM has been shown not to affect sensory neurogenic relaxation in the rat isolated mesenteric arterial bed (Ralevic, 2001). The concentration of methoxamine in control preparations was 51.67 ± 10.77 μM ($n=6$). In the presence of α,β-meATP the concentration of methoxamine was 11.67 ± 0.7 μM ($n=6$), which was not significantly different from the methoxamine concentration used in the presence of 7-NI (12.29 ± 2.28 μM; $n=7$). In the low methoxamine control preparations, relaxations to EFS were not significantly different from those in control preparations (Figure 8).

Effect of 8-bromo-cGMP and dibutyryl cAMP on responses to EFS

There was no significant difference between frequency response curves carried out in the absence and presence of 8-bromo-cGMP (10 μM) ($n=5$). At 8 Hz vasorelaxations were $62.70\pm8.71\%$ ($n=5$) and $58.30\pm4.80\%$ ($n=5$). Relaxation-response curves in the presence of dibutyryl cAMP (10 μM) were slightly, but significantly ($P=0.038$, $n=7$) attenuated, but further analysis did not show a significant difference at any individual frequency. At 8 Hz vasorelaxations were $52.14\pm6.48\%$ ($n=7$) and 42.27 ± 7.0 ($n=7$) in the absence and presence of dibutyryl cAMP, respectively.

Discussion

The present study has shown that 7-NI and L-NAME, inhibitors of NOS, augment sensory neurogenic relaxation to EFS, but not relaxation to exogenous CGRP, in the rat isolated mesenteric arterial bed. The inhibitory effect of L-NAME was reversed by excess L-arginine, the substrate for NOS, and D-NAME, the enantiomer of L-NAME, and 6-aminoindazole, an inactive analogue of 7-NI, were inactive. Endothelium removal also augmented relaxation to EFS, and in the additional presence of 7-NI or L-NAME there was no further enhancement of relaxation to EFS. Furthermore, N^{ω}-propyl-L-arginine, a selective inhibitor of neuronal NOS, had no effect on sensory neurogenic relaxations. These data indicate that NO is an inhibitory modulator of sensory neurotransmission in the rat mesenteric arterial bed, and that the source of NO is the vascular endothelium.

Inhibition of NOS with 7-NI and L-NAME augmented vasorelaxant responses to EFS of the mesenteric arterial beds, indicating that a tonic release of endogenous NO inhibits the neurogenic response under control conditions. The NOS inhibitors had no significant effect on relaxation to CGRP, indicating that NO is likely to be acting prejunctionally to inhibit neurotransmitter release. These data are in agreement with the findings of Li *et al.* (1993) who also showed that L-NAME prejunctionally augments sensory neurotransmission in the rat isolated mesenteric arterial bed. In contrast, Amerini *et al.* (1993) and Moll-Kaufmann *et al.* (1998) concluded that NO does not modulate sensory neurotransmission in this vascular preparation. The reason for the difference between these studies is not entirely clear. However, neither Amerini *et al.* (1993) or Moll-Kaufmann *et al.* (1998) controlled for the augmenting effect of inhibitors of NOS on responses to vasoconstrictors, raising the possibility that functional antagonism (the vascular smooth muscle is more contracted) might have opposed relaxation. Amerini *et al.* (1993) used a fixed and maximal concentration of methoxamine (100 μM) to preconstrict mesenteric preparations, and there was a further increase in tone of approximately one hundred per cent when NOS inhibitors were added. Moll-Kaufmann *et al.* (1998) also used a fixed concentration of methoxamine to generate tone, and tone was similarly greater in the presence of a NOS inhibitor than in the controls. In the present study tone was kept constant by using a lower concentration of

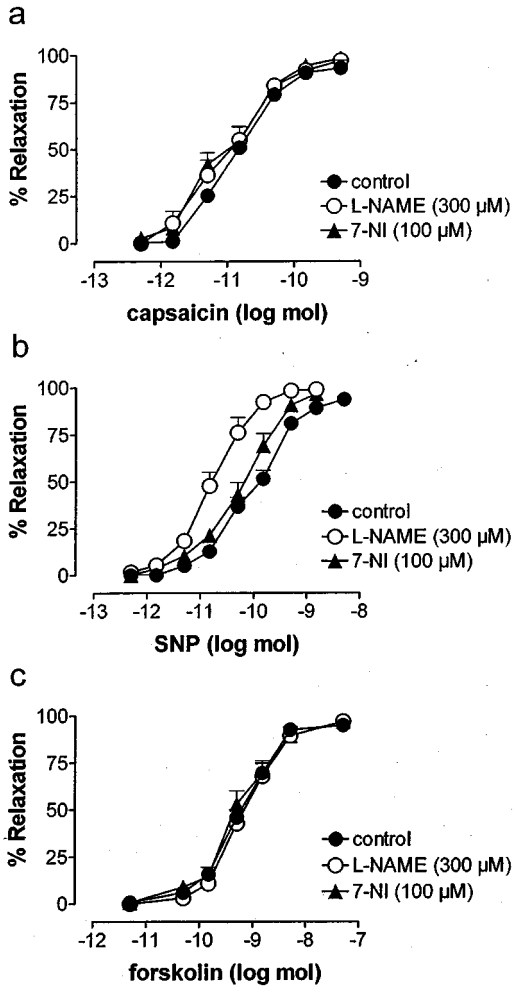

Figure 4 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole (7-NI) on dose-dependent relaxations to: (a) capsaicin ($n=7-8$), (b) sodium nitroprusside (SNP; $n=5-7$) and (c) forskolin ($n=6-7$) in the rat isolated mesenteric arterial bed. Data are presented as means + s.e.mean. Only the dose–response curve to SNP in the presence of L-NAME was significantly different compared to the control ($P<0.001$).

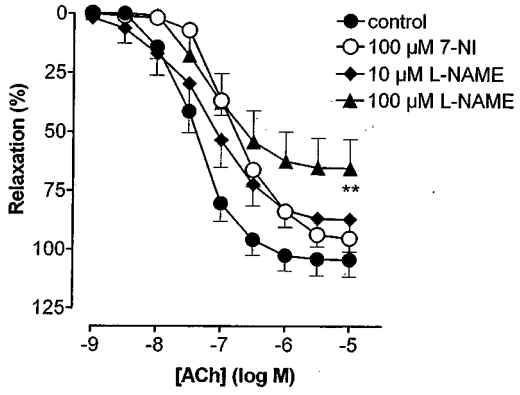

Figure 5 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole (7-NI) on concentration-dependent relaxations to acetylcholine (ACh) in rat isolated aortae. Relaxations were elicited under control conditions ($n=6$), in the presence of 10 μM L-NAME ($n=7$), 100 μM L-NAME ($n=8$) and 100 μM 7-NI ($n=7$). Data are presented as means + s.e.mean. Significant difference between maximal relaxations in the presence of 100 μM L-NAME and in controls is denoted by $^{**}P<0.01$. There was also a significant difference in pD_2 values between relaxations in controls and in the presence of 7-NI ($P<0.05$).

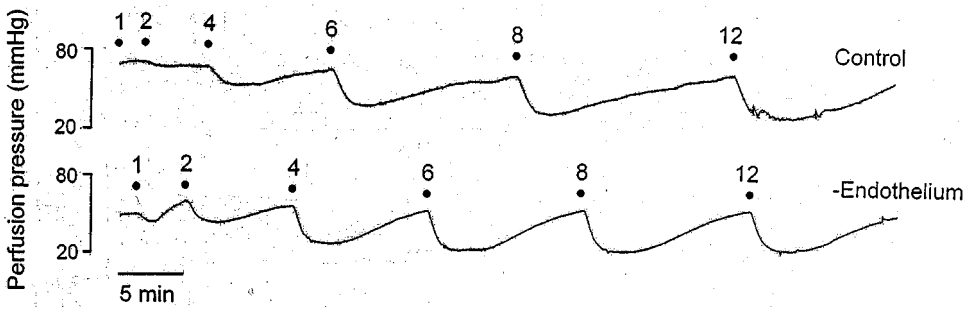

Figure 6 Representative traces showing effect of endothelium removal on sensory neurogenic relaxations to electrical field stimulation (EFS; 1–12 Hz) of methoxamine preconstricted mesenteric arterial preparations in control conditions (upper trace), and after endothelium removal with distilled water (lower trace). Endothelium removal augmented relaxations to EFS.

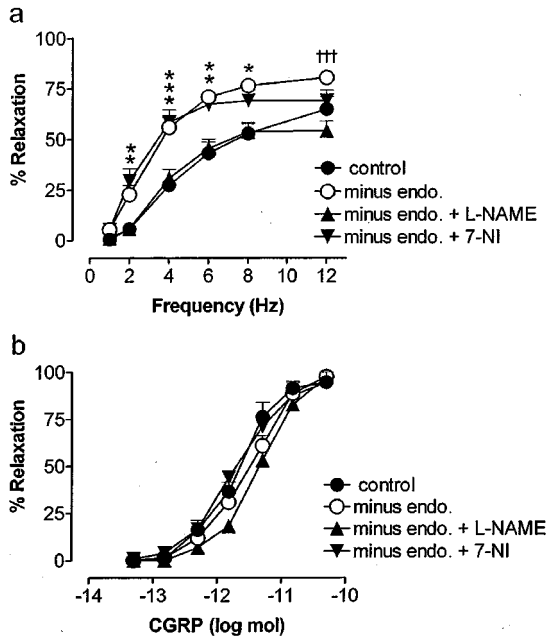

Figure 7 Effect of endothelium removal (minus endo.), N^G-nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole (7-NI) on: (a) responses to electrical field stimulation ($n=4-8$) and (b) relaxations to calcitonin gene-related peptide (CGRP) ($n=4-7$) in the rat isolated mesenteric arterial bed. Data are presented as means+s.e.mean. A significant difference between endothelially-denuded preparations and endothelially-denuded preparations plus 7-NI compared to the other two groups at 2–8 Hz is denoted by $*P<0.05$, $**P<0.01$ and $***P<0.001$. At 12 Hz a significant difference between endothelially-denuded preparations with and without L-NAME is denoted by $\dagger\dagger\dagger P<0.001$.

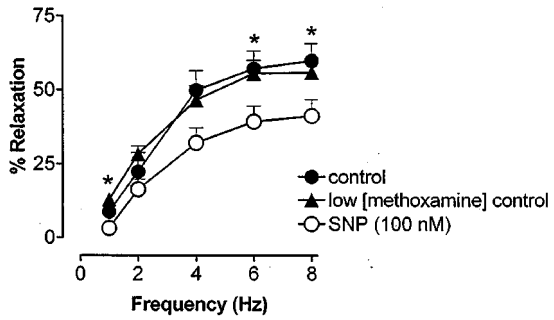

Figure 8 Effect of reducing the methoxamine concentration (low (methoxamine) control, $n=6$) and sodium nitroprusside (SNP, $n=8$) on relaxation under control conditions ($n=6$) of electrical field stimulation of the rat isolated mesenteric arterial bed. In both cases 0.1 μM α,β-methylene ATP was added to reduce (low (methoxamine) control) or maintain (in the presence of the vasodilator SNP) the concentration of methoxamine used to preconstrict the preparations. Data are presented as means+s.e.mean. A significant difference between control preparations and those with SNP at 1, 6 and 8 Hz, and low (methoxamine) control (at 1 Hz) is denoted by $*P<0.05$.

methoxamine to preconstrict preparations in the presence of the NOS inhibitors.

The actions of the NOS inhibitors were further investigated using forskolin and SNP, activators of adenylyl cyclase and guanylyl cyclase respectively. CGRP mediates relaxation of rat mesenteric arteries primarily *via* smooth muscle $CGRP_1$ receptors (Lei *et al*., 1994). This does not involve potassium channel opening, but likely involves generation of cAMP (Lei *et al*., 1994). 7-NI and L-NAME had no effect on relaxation to forskolin, consistent with an involvement of cAMP in CGRP-mediated vasorelaxation. This is in line with the suggestion that the NOS inhibitors modulate prejunctionally sensory neurotransmission. However, the NOS inhibitors did have postjunctional actions, as relaxation to SNP was augmented by L-NAME and there was a trend for augmentation of relaxation by 7-NI. This is believed to be due to an increase in the available pool of guanylyl cyclase/cGMP for relaxation following inhibition of endogenous NO formation (Busse *et al*., 1989; Ralevic *et al*., 1991).

As endothelium removal mimicked the effect of the NOS inhibitors, but had no effect on responses to CGRP, the source of NO modulating sensory neurotransmission is likely to be the vascular endothelium. Moreover, following endothelium removal there was no further augmentation of sensory neurotransmission when the NOS inhibitors were added, indicating that there is likely no additional source of NO, consistent with the endothelium as the principal source of NO in rat mesenteric arteries. NO is released tonically from endothelial cells in the mesenteric arterial bed, as evidenced by augmentation of constriction to methoxamine by NOS inhibitors and endothelium removal. This, to our knowledge, is the first indication that endothelial NO, which readily crosses cell membranes, may diffuse to the adventitial layer to modulate sensory neurotransmission. In contrast, Li *et al*. (1993) concluded that the source of NO modulating sensory neurotransmission was not endothelial cells, as in that study endothelial denudation did not potentiate relaxations to EFS. Different methods, however, were used by the two studies to remove the endothelium. Li *et al*. (1993) used saponin, which might have caused damage to sensory nerves (indeed, in their study there was a trend for neurogenic relaxations to be smaller after this treatment), whilst the present study used distilled water as a more gentle procedure for endothelial denudation.

The lack of effect of N^{ω}-propyl-L-arginine on relaxations to EFS is consistent with the suggestion that the endothelium is the source of NO modulating sensory neurotransmission in rat mesenteric arteries. This compound is 149-fold selective as an inhibitor of nNOS versus eNOS at its K_i value of 57 nM (Zhang *et al*., 1997). Furthermore, an involvement of nNOS seems unlikely, because of a lack of evidence for NOS-containing nerves in rat mesenteric arteries. Indeed, in the rat mesenteric arterial bed, the CGRP receptor antagonist CGRP(8-37) abolishes neurogenic vasorelaxation induced by periarterial nerve stimulation (Han *et al*., 1990). In contrast, nNOS-immunoreactivity has been localized in mesenteric arteries of the guinea-pig and monkey, and in both of these vasculatures NOS inhibitors block a non-adrenergic non-cholinergic nerve-evoked vasodilatation (Yoshida *et al*., 1994; Zheng *et al*., 1997).

The main aim of the present study was to investigate the effect of endogenous NO on sensory neurotransmission, and to this end two different inhibitors of NOS were used. Augmentation by 7-NI of contractions to methoxamine indicated that it was acting to block endothelial NO production in the mesenteric arterial bed. Although 7-NI is not selective for isoforms of NOS (Alderton *et al*., 2001), it has widely been used as a selective inhibitor of neuronal NOS (see Moore & Handy, 1997), so an evaluation of its ability to

functionally antagonise endothelial NOS (eNOS) was warranted. As there is a large NO-independent component of endothelially-mediated relaxation in the rat mesenteric arterial bed (Adeagbo & Triggle, 1993), the assay was carried out in rat aortic rings, although the results showed that a significant component of the ACh-mediated relaxation is also resistant to the effects of NOS inhibitors in this preparation. 7-NI blocked relaxations to ACh, as did L-NAME, indicating an action at eNOS. 7-NI has also been shown to inhibit, with micromolar activity, the activity of eNOS in homogenates of endothelial cells (Bland-Ward & Moore, 1995) and endothelium-dependent relaxations in rat aorta (Guilmard *et al*., 1998), rabbit aorta (Chinellato *et al*., 1998) and monkey cerebral arteries (Ayajiki *et al*., 2001). It is unclear why, in the present study, 7-NI produced a rightward shift of the concentration–response curve, whilst L-NAME reduced the maximal response.

Interestingly, in the absence of endothelium, L-NAME, but not 7-NI, attenuated sensory neurogenic relaxation. This may involve a postjunctional effect of L-NAME, as relaxations to CGRP were also slightly attenuated. NOS inhibitors are generally very specific in their actions. However, it has been shown that a NOS inhibitor that is structurally-related to L-NAME, N^G-nitro-L-arginine, contracts endothelially-denuded rat aortic vascular smooth muscle (Wang & Pang, 1994), raising the possibility that functional antagonism due to smooth muscle constriction was involved in this effect. There is also evidence that 7-NI causes an endothelium- and NO-independent relaxation of smooth muscle (Medhurst *et al*., 1994), which may have been involved in differences observed between the effects of 7-NI and L-NAME. It is interesting that in the aorta a higher concentration of methoxamine was required to raise the tone in the presence of 7-NI than in the presence of L-NAME, which is consistent with a possible smooth muscle vasorelaxant action of 7-NI.

In the presence of NOS inhibitors, and after endothelium removal, responses to vasoconstrictors are augmented, as observed in the present study and by others (Martin *et al*., 1986; Li & Duckles, 1992; Reid & Rand, 1992; Vo *et al*., 1992; Cederqvist & Gustafsson, 1994). Hence, a lower concentration of methoxamine was required to preconstrict the preparations to match preconstricted tone under control conditions. A lack of effect of NOS inhibitors on relaxations to exogenous CGRP indicates that the lower concentration of methoxamine did not affect postjunctionally sensory neurogenic relaxation. However, a prejunctional action was still possible (relaxations might be smaller with a higher concentration of methoxamine, for instance because of possible non-selective actions of methoxamine at inhibitory prejunctional α_2-adrenoceptors). In order to investigate this, α,β-meATP was added to the perfusate at a concentration which does not itself affect sensory neurotransmission in the rat mesenteric arterial bed (Ralevic, 2001), but which augmented contractions to methoxamine, allowing a lower concentration to be used to preconstrict control preparations. In these experiments, the 'low' methoxamine concentration was not significantly different from that used to raise tone in the presence of 7-NI. Importantly, there was no significant difference in sensory relaxations to EFS in the presence of the different concentrations of methoxamine. Hence, augmented sensory neurogenic vasorelaxation by NOS inhibitors is not due to the lower concentration of methoxamine required to preconstrict the preparations.

Inhibition of sensory neurotransmission by SNP, a NO donor, is consistent with an inhibitory action of NO on sensory neurotransmission in rat mesenteric arteries. A possible involvement of cGMP was investigated using a cell permeable analogue of cGMP, 8-bromo-cGMP. NO is a powerful stimulator of soluble guanylyl cyclase causing an increase in cGMP levels in cells, and there is evidence for an involvement of cGMP in NO-mediated inhibition of responses of primary afferent fibres in the rat spinal cord (Kurihara & Yoshioka, 1996). Moreover, Li *et al*. (1993) showed that methylene blue, an inhibitor of guanylyl cyclase, augmented sensory neurogenic relaxation to EFS in the rat mesenteric arterial bed. In the present study, however, there was no effect of 8-bromo-cGMP on sensory neurogenic relaxation. Cross-talk between cyclic nucleotides can occur, and cGMP has been shown to down-regulate cAMP synthesis (Kim *et al*., 2001). Hence, a decrease in basal levels of cGMP might elevate levels of cAMP, and activation of the cAMP transduction pathway has been shown to sensitize afferents (Kress *et al*., 1996; Brunsden & Grundy, 1999; Bolyard *et al*., 2000). However, dibutyryl cAMP inhibited sensory neurotransmission in the present study, as described for neurogenic plasma extravasation in rat airways (Morikawa *et al*., 1993). Hence, the present study does not support an involvement of cyclic nucleotides in NO inhibition of sensory neurotransmission in rat mesenteric arteries. NO-mediated inhibition of evoked neurotransmission was similarly not mimicked by 8-bromo-cGMP in central neuronal cultures (Pan *et al*., 1996).

One possible mechanism by which NO inhibits sensory neurotransmission is that NO (or NO^+) interacts with critical regulatory protein thiols on cysteine residues (*S*-nitrosylation) leading to modulation of protein structure, and hence gating of various ion channels, as seems to be involved in NO-mediated inhibition of neurotransmission in central neuronal cultures (Pan *et al*., 1996; Lipton *et al*., 1998). This action of NO is akin to that of an oxidising agent by allowing disulphide bond formation between two closely located *S*-nitrosylated thiols in the channel protein. Along this line, NO donors have been shown to reduce Ca^{2+} and Zn^{2+} entry through voltage-gated Ca^{2+} channels in mouse neocortical cultures, which may involve oxidization of key thiol groups (Snider *et al*., 2000). In the present study, the NOS inhibitors augmented specifically electrically-evoked neurotransmitter release, relaxation to capsaicin being unaffected. This is consistent with a lack of effect of L-NAME on capsaicin responses reported previously in the rat mesenteric arterial bed (Potenza *et al*., 1994) and in rat dorsal root ganglion cells (Lopshire & Nicol, 1997). This indicates that the neuromodulatory mechanism of NO does not involve transmitter release activated by the vanilloid VR1 receptor and related mechanisms. A clue to the mechanism may come from the fact that ω-conotoxin, a selective blocker of N-type voltage-sensitive Ca^{2+} channels, can block the electrical activation of sensory nerves mediated by propagated action potentials, but not the excitatory effect of capsaicin on the same nerves (Maggi *et al*., 1988a, b; Lou *et al*., 1992; Evans *et al*., 1996). Hence, NO inhibition of neurotransmitter release from sensory nerves may involve inhibition of voltage-sensitive Ca^{2+} currents *via* N-type channels.

Regional differences in NO modulation of sensory neurotransmission in peripheral tissues have been described, and non-endothelial sources of NO described. For example, in rat paw skin NOS inhibitors attenuate sensory neurotransmitter release and neurogenic oedema formation (Kajekar *et al*., 1995), whereas L-NAME increases neural discharge and enhances responsiveness to bradykinin in articular sensory nerves (Kelly *et al*., 2001). In some blood vessels NO is released as a neurotransmitter from non-adrenergic non-cholinergic nerves, and attenuation of neurogenic vasodilatation by NOS inhibitors can be explained by inhibition of the postjunctional actions of NO (Liu *et al*., 1991; Toda & Okamura, 1992; Brizzolara *et al*., 1993).

In conclusion, these data have shown that inhibition of NOS augments sensory neurotransmission in the rat isolated mesenteric arterial bed, and this appears to be mediated via a prejunctional action. Endothelium removal mimics the effect of the NOS inhibitors, and there is no further augmentation of sensory neurotransmission with addition of NOS inhibitors, indicating that the likely source of the NO is the endothelium. One implication of these findings is that when there is damage to the endothelium, with loss of associated vasorelaxant mechanisms and potentiation of contractile responses, sensory nerves assume a greater importance in the vasodilator regulation of blood vessel tone.

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