RESEARCH PAPER

Role of endothelin-1 in the hyper-responsiveness to nitrovasodilators following acute NOS inhibition

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BACKGROUND AND PURPOSE

Acute NOS inhibition in humans and animals is associated with hypersensitivity to NO donors. The mechanisms underlying this phenomenon have not been fully elucidated. The purpose of the present study was to assess whether hypersensitivity to NOS-blockade is linked to endothelin-1 (ET-1) signalling.

EXPERIMENTAL APPROACH

Sprague Dawley rats were instrumented with indwelling arterial and venous catheters for continuous assessments of haemodynamic parameters and drug delivery, respectively. Mesenteric arteries were isolated and tested for reactivity by wire myography.

KEY RESULTS

NOS blockade with L-N^G-nitroarginine methyl ester (L-NAME) caused a pronounced increase in arterial blood pressure (BP) (~40 mmHg). In L-NAME-treated animals, the dose of sodium nitroprusside (SNP) required to cause a significant reduction in arterial BP was lower than in vehicle-treated rats ($P < 0.001$), and the magnitude of the reduction in BP was greater. Similar results were obtained with other NO mimetics, but not isoprenaline; moreover, decreasing the BP back to baseline levels with prazosin after L-NAME treatment did not attenuate the hyper-responsiveness to NO donors. The increased responsiveness to NO donors was abolished by pretreatment with the ET$_{A/B}$ receptor antagonist, PD145065, or the ET$_A$ receptor-specific antagonist ABT627. *Ex vivo*, L-NAME treatment potentiated the constriction induced by big endothelin-1 (bET-1), the precursor to active ET-1, but had no effect on the ET-1-mediated constriction.

CONCLUSIONS AND IMPLICATIONS

These data suggest that the increased sensitivity to NO donors is mediated, at least in part, by ET-1 *in vivo*, and the mechanism may involve the conversion of bET-1 to ET-1.

Abbreviations

ABT627, 2R-(4-methoxyphenyl)-4S-(1,3-benzodioxol-5-yl)-1-(N,N-di(n-butyl)aminocarbonyl-methyl)-pyrrolidine-3R-carboxylic acid; bET-1, big endothelin-1; ET-1, endothelin-1; GTN, glyceryl trinitrate; L-NAME, L-N^G-nitroarginine methyl ester; PD145065, N-acetyl-α-[10,11-dihydro-5h-dibenzo[a,d]cycloheptadien-5-yl]-d-gly-leu-asp-ile-ile-trp; SNP, sodium nitroprusside; Veh, vehicle

Introduction

NO is an endogenous free radical formed from the terminal guanidino nitrogen of the amino acid L-arginine by the enzyme NOS (Palmer *et al.*, 1988). Among its many physiological functions, NO plays an important role in regulating vascular resistance, and hence arterial BP (Rees *et al.*, 1989; Vallance *et al.*, 1989; Moncada and Higgs, 2006; Bian *et al.*,

2008). Indeed, *in vivo* inhibition of endogenous NO production with analogues of L-arginine [e.g. L-N^G-nitroarginine methyl ester (L-NAME)] results in a marked hypertensive response characterized by pronounced vascular constriction (Rees *et al.*, 1989).

Both humans and animals exhibit increased vascular responsiveness to NO donors following acute NOS inhibition (Alheid *et al.*, 1987; Busse *et al.*, 1989; Flavahan and Vanhoutte, 1989; Luscher *et al.*, 1989; Gardiner *et al.*, 1991; Moncada *et al.*, 1991). This phenomenon remains of interest to investigators because it provides important insights into the mechanisms of acute vascular control. Despite being first described over two decades ago, the mechanisms underlying the increased responsiveness to NO donors following NOS inhibition are still not completely understood. Studies have shown that the increased responsiveness to NO donors may stem, in part, from a vascular sensitization of second messenger cGMP following NOS inhibition (Moncada *et al.*, 1991), although whether this mechanism predominates *in vivo* is not known.

In addition to directly eliciting vasodilatation (Rees *et al.*, 1989; Vallance *et al.*, 1989), NO has been shown to regulate vascular tone via modulation of vasoconstrictor agents, including endothelin-1 (ET-1) (Banting *et al.*, 1996; Filep, 1997; Cardillo *et al.*, 2000). ET-1 is a potent vasoconstrictor peptide that is produced primarily within the endothelium. It elicits its vascular effects by binding to either ET$_A$ or ET$_B$ receptor subtypes. ET-1 binding to ET$_A$ and ET$_B$ receptors, located on smooth muscle cells, causes vasoconstriction, whereas binding to ET$_B$ receptors, located on endothelial cells, mediates NO release (Warner *et al.*, 1989). Previous studies in humans and animals have shown that antagonism of ET-1 receptors attenuates the hypertensive effect caused by NOS inhibition (Banting *et al.*, 1996; Filep, 1997; Cardillo *et al.*, 2000), suggesting that ET-1 signalling is an important mechanism by which NO maintains normal vascular tone. On the basis of this work, we hypothesized that the increased responsiveness to NO donors following NOS inhibition could involve ET-1 signalling. More specifically, we reasoned that NOS inhibition would unmask ET-1 actions, and subsequent addition of low doses of NO donors would oppose this effect, resulting in an exaggerated NO responsiveness. As such, the pharmacological antagonism of ET-1 receptors would attenuate this hyper-responsiveness to NO donors. The objectives of the present study were: (i) to assess and characterize the nature of BP responses to vasodilator agents in naïve and L-NAME-treated rats; (ii) to assess the involvement of the ET-1 system in these animals using endothelin receptor antagonists; (iii) to assess isolated vascular responses to vasoconstrictors in the presence of L-NAME treatment and ET-1 antagonists.

Methods

Animals and treatments

All animal care and experimental procedures were performed in accordance with standards established by the Canadian Council of Animal Care, and approved by the University of Alberta and Queen's University Animal Care Committees. The investigation complied with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996). Adult male Sprague-Dawley rats (approximately 12 weeks of age) were purchased from Charles River (St. Constant, QC for experiments done at Queen's University; Wilmingham, MA, USA for experiments done at the University of Alberta), and given at least 3 days to acclimatize to their surroundings before experimentation. Rats were housed in an animal care facility, which maintained a 12 h/12 h light/dark cycle and an ambient temperature of 23 ± 1°C. Rats had *ad libitum* access to food and water.

Conscious BP assessments

BP assessments were performed as described by Banting *et al.* (1996). Briefly, rats were anaesthetized with ketamine hydrochloride (70 mg·kg^{-1}, i.p.; Rogar/STB, London, Ontario, Canada) and xylazine (5 mg·kg^{-1}, i.p. Bayer, Etobicoke, Ontario, Canada), and a proper depth of anaesthesia was verified using a toe pinch. Rats were then implanted with four catheters (0.3 mm i.d., 0.76 mm O.D. Cole Parmer, Laval, Quebec, Canada); one in the aorta, distal to the kidneys, for BP assessments; two in the inferior vena cava distal to the kidneys and one in the intraperitoneal cavity, for drug administrations. All catheters were made of vinyl, with the exception of the venous catheters used for administration of glyceryl trinitrate (GTN), which were polyethylene (PE50: 0.58 mm i.d., 0.97 mm O.D., Becton Dickson, Sparks, MD, USA) to minimize drug absorption by the tubing. The catheters were filled with heparin-containing saline (10 IU·mL^{-1}), and held in place with a small amount of cyanoacrylate glue at the puncture site. All catheters were exteriorized and sutured into position after passing through the abdominal wall and subcutaneously tunneled towards the nape of the neck. Post-surgical pain was managed by s.c. administration of buprenorphine hydrochloride (3 mg·kg^{-1}, Reckitt and Colman Pharmaceuticals, Richmond, VA, USA). Rats were also administered trimethoprim and sulfadiazine (Tribrissen 24%, 0.3 mg·kg^{-1}, s.c.; Intervet/Schering-Plough, Kirkland, Quebec, Canada) for antibiotic prophylaxis. All BP data presented in this study were obtained from conscious animals. Rats were given at least 3 days to recover from the surgeries; this length of time was found to be sufficient for circulatory responses to recover from anaesthetic effects.

On the day of the experiment, BP was continuously recorded in conscious rats using a Powerlab® data acquisition system (ADInstruments, Colorado Springs, CO, USA), calibrated manually using a sphygmomanometer. Rats were given a washout period of at least 30 min before experimentation and between each series of experiments to establish a steady state baseline. Drugs were infused using a motorized syringe pump (Model 220, KD Scientific, Holliston, MA, USA). For experiments involving pretreatment with either L-NAME (100 mg·kg^{-1}, i.p., Sigma, St. Louis, MO, USA), PD145065 (10 mg·kg^{-1}·min^{-1} i.v., EMD Chemicals, Gibbstown, NJ, USA), or ABT627 (200 µg·kg^{-1}·min^{-1} i.v., for 10 min, followed by 100 µg·kg^{-1}·min^{-1} i.v. thereafter; Abott Laboratories, Abott Park, IL, USA), these maintenance drugs were administered after a steady haemodynamic [heart rate and mean arterial pressure (MAP)] baseline had been achieved, and infusions were continued until all concentration-response curves with vasodilators were

obtained. In the experiments where L-NAME and endothelin receptor antagonists were co-administered, endothelin receptor antagonists were administered first, followed by L-NAME. In the experiments where L-NAME and prazosin (2 mg·kg^{-1} i.p., Sigma, dissolved in 5% ethanol/95% saline) were co-administered, L-NAME was administered first followed by prazosin. Time-controlled rats received saline. Doses of sodium nitroprusside (SNP; 0.8–32·µg·kg^{-1}·min^{-1} i.v., Sigma), GTN (2–200 µg·kg^{-1}·min^{-1} i.v., Sabex, Boucherville, Quebec, Canada), methylamine hexamethylene methylamine NONOate (10–60 µg·kg^{-1}·min^{-1} i.v., Alexis Biochemicals, Plymouth Meeting, PA, USA), 8-bromo-cGMP (8Br-cGMP; 0.1–50 µg·kg^{-1}·min^{-1} i.v., Sigma), and isoprenaline (0.02–0.8 µg·kg^{-1}·min^{-1}, Sigma) were administered in a non-cumulative manner over a period of 30 s.

For assessments of circulating ET-1, rats were treated with L-NAME (100 mg·kg^{-1}, i.p.) or saline under isofluorance anaesthesia (dosed to effect by inhalation), and blood was collected after 15 min. Blood was kept on ice and allowed to clot, and was then centrifuged at 3650× g for 15 min. ET-1 from serum samples was purified, and quantified by enzyme immunoassay (Caymen Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's instructions. Sample yield was calculated based on recovery of [^{125}I]-ET-1 (>22 uCi·mmol^{-1}; Vitrax Radiochemicals, Placentia, CA, USA) from spiked samples.

Isolated vessel experiments

Rats were anaesthetized with isoflurane (dosed to effect by inhalation), and killed by exsanguination. Mesenteric vessels was rapidly excised and placed in iced HEPES-buffered physiological saline solution (in mmol·L^{-1}: NaCl 142, KCl 4.7, MgSO$_4$ 1.17, CaCl$_2$ 4.7, K$_2$PO$_4$ 1.18, HEPES 10 and glucose 5.5; pH 7.4). Second order mesenteric arteries were carefully isolated from the surrounding adipose tissue using a binocular microscope, and arteries with internal diameters ranging 150–250 µm were mounted in an isometric myograph system (DMT, Copenhagen, Denmark) using 40 µm tungsten wire. Vessels were normalized through a series of stepwise increases in diameter to determine their optimal resting tension, set to 0.8 × IC$_{100}$ (the internal circumference equivalent to a transmural pressure of 100 mmHg). Vessels were then given 30 min to equilibrate to their optimal resting tension.

Following equilibration, mesenteric arteries were twice treated with phenylephrine (10 µM; Sigma) to assess viability. On the second phenylephrine dose, methacholine (3 µM, Sigma) was administered to all baths to assess integrity of the endothelium; vessels that did not achieve a minimum relaxation of 80% were excluded from all experiments. After several washes, vessels were treated for 30 min with antagonists: L-NAME (100 µM); PD145065 (5 µM). After incubation with antagonists, cumulative dose–response curves were generated for big endothelin-1 (bET-1; Anaspec, Fremont, CA, USA; 10 nM to 300 nM), ET-1 (1 nM to 300 nM), and SNP (0.01 nM to 1 µM). In the case of bET-1 and ET-1, vessels were allowed to incubate with each cumulative dose for a minimum of 5 min. For SNP dose–response curves, vessels were submaximally preconstricted (80% of maximum) using phenylephrine, and cumulative doses were added to the bath when vessels reached a plateau response (<2 min).

Statistical analyses

Data are presented as mean ± SEM. MAP values were calculated as peak responses, averaged over 5 s, within a dosing window. ΔMAP values were obtained by subtracting the MAP after each drug dose from the pretreatment baseline. Data from the dose–response curves were fitted to the Hill equation. The EC$_{50}$ was determined. For bET-1 dose–response curves, area under the curve was calculated, and compared between groups. MAP measurements were analysed by two-way ANOVA for effects of treatment and dose, followed by Bonferroni post-hoc test. Analyses with two comparisons were conducted using Student's t-test; where three or more comparisons were made, ANOVA was used, followed by Tukey's *post hoc* analysis. Data were analysed using Graph-Pad Prism 5 Software (La Jolla, CA, USA). P < 0.05 was considered significant.

Results

NOS inhibition causes enhanced responsiveness to NO donors

Concentration-response curves with the NO-donor SNP were performed in conscious rats in the absence and presence of the NOS inhibitor L-NAME (Figure 1). The doses of SNP were selected because they elicit minimal effects on BP under normal circumstances. As expected, treatment with L-NAME caused a hypertensive response (40 ± 4 mmHg) compared with vehicle (Veh) controls (P < 0.001), and this maximal effect was attained within 15 min of dosing. Subsequent administration of SNP to L-NAME-treated rats produced significant depressor effects at doses of 3.2 µg·kg^{-1}·min^{-1} (–17.8 ± 3.4 mmHg, P < 0.01; Figure 1) or greater. In contrast, in control rats, the dose of SNP required to produce a significant depressor effect was 8 µg·kg^{-1}·min^{-1} (–11.4 ± 2.8 mmHg,
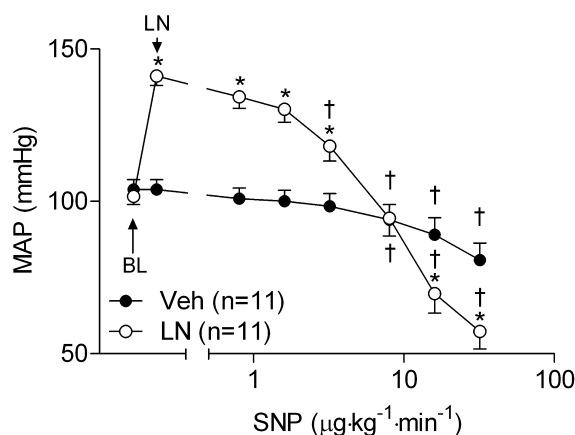
Figure 1

MAP depressor responses to SNP are exaggerated following NOS inhibition in conscious rats. Rats were treated with L-NAME (100 mg·kg^{-1}, i.p.) or saline, and subsequently administered the NO-donor SNP. *P < 0.05 compared with Veh-treated rats at the same dose of vasodilator. †P < 0.05 compared with MAP after administration of either L-NAME or saline in same group. LN, L-NAME.
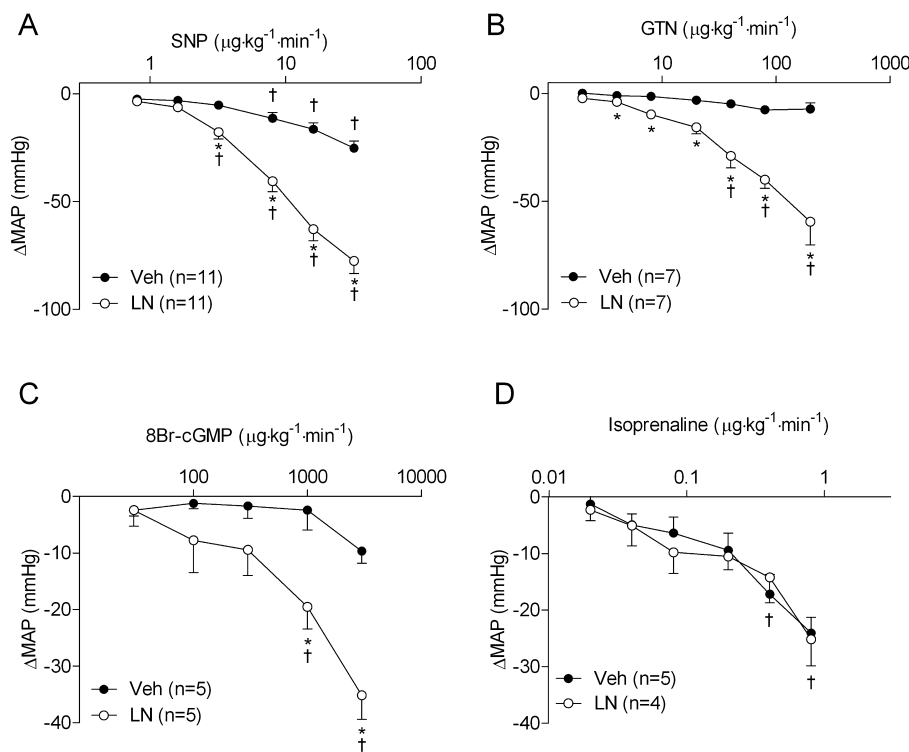
Figure 2

NOS inhibition causes increased BP responsiveness to the vasodilators (A) SNP, (B) GTN, (C) the cGMP analogue 8Br-cGMP, but not (D) isoprenaline. Rats were treated with L-NAME (100 mg·kg^{-1}, i.p.) or saline, and subsequently administered vasodilators. Data are shown as changes from steady state MAP levels after infusion of saline or L-NAME. *$P < 0.05$ compared with Veh-treated rats at the same dose of vasodilator. †$P < 0.05$ compared with baseline (BL) in same group. Note that in (D), treatment groups were combined since two-way ANOVA revealed no overall effect of L-NAME; therefore symbols reflect pooled data. GTN, glyceryl trinitrate; LN, L-NAME.

$P < 0.05$). The change in MAP from baseline levels is presented in Figure 2. At all doses tested, the magnitude of the decrease in BP induced by SNP was threefold greater in L-NAME-treated rats compared with controls (Figure 2A). Compared with controls, the BP in L-NAME-treated rats also exhibited increased responsiveness to the NO mimetics GTN (Figure 2B) and methylamine-hexamethylene-methylamine-NONOate (Hrabie *et al.*, 1993) (2.6- and 2.1-fold decrease in BP at doses of 40 and 50 μg kg^{-1}·min^{-1}, respectively; $P < 0.05$ for both parameters). Compared with Veh-treated rats, L-NAME-treated rats also exhibited increased responsiveness to the cGMP analogue 8Br-cGMP (Figure 2C). In contrast, NOS-inhibited rats showed no hyper-responsiveness to isoprenaline (Figure 2D) or PGE$_1$ (data not shown), which cause vasodilatation predominantly via cAMP-dependent signalling mechanisms.

As L-NAME caused a marked hypertensive response, we considered the possibility that the increased responsiveness to NO donors was related to mechanisms associated with this increase in BP (e.g. release of humoral factors, increased parasympathetic outflow, etc.), rather than an absence of NO *per se*. We therefore sought to determine whether the hyper-responsiveness to NO donors remained when BP was lowered to baseline levels (pre-L-NAME pressures). In a separate cohort, rats were treated with the α-adrenoceptor antagonist prazosin after L-NAME treatment, which lowered BP to a level

similar to naïve animals (Figure 3A). Despite the lack of BP difference compared with naïve animals, rats co-treated with L-NAME and prazosin exhibited a similar enhanced responsiveness to infused SNP (Figure 3B) as the rats treated with L-NAME alone (Figure 2A).

Increased responsiveness to NO donors following NOS inhibition is dependent on ET-1 signalling

We subsequently investigated the involvement of ET-1 in the hyper-responsiveness to NO donors following NOS inhibition. In rats pretreated with the ET$_{A/B}$ receptor antagonist PD145065, L-NAME had only a modest hypertensive effect, corresponding to a blunting of 68% compared with L-NAME-treated rats (L-NAME: 41 ± 4 mmHg; L-NAME+PD145065: 13 ± 3 mmHg $P < 0.05$). Treatment with PD145065 also completely abolished the hypersensitivity to NO donors following NOS inhibition with L-NAME, and in fact resulted in diminished depressor responses even compared with controls (Figure 4A). To ascertain whether these effects were mediated by ET$_A$ receptors, we conducted similar experiments using the ET$_A$ receptor antagonist ABT627. Similar to PD145065, pretreatment with ABT627 attenuated the hypertensive response caused by L-NAME treatment by 53% (L-NAME: 40 ± 6 mmHg; L-NAME+ABT627: 19 ± 2 mmHg; $P < 0.05$), and abolished the hypersensitivity to NO donors (Figure 4B).
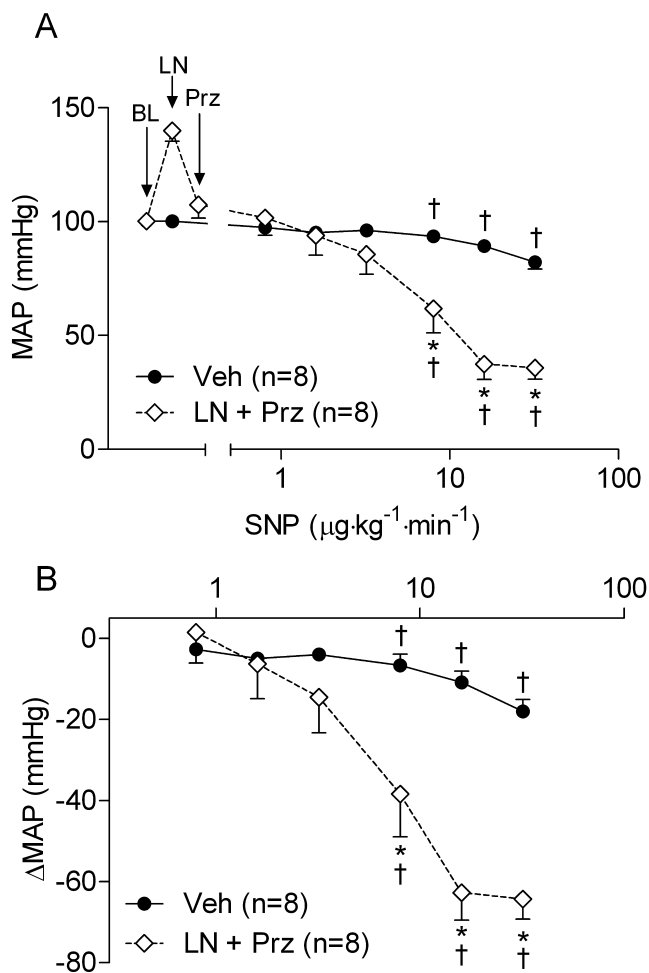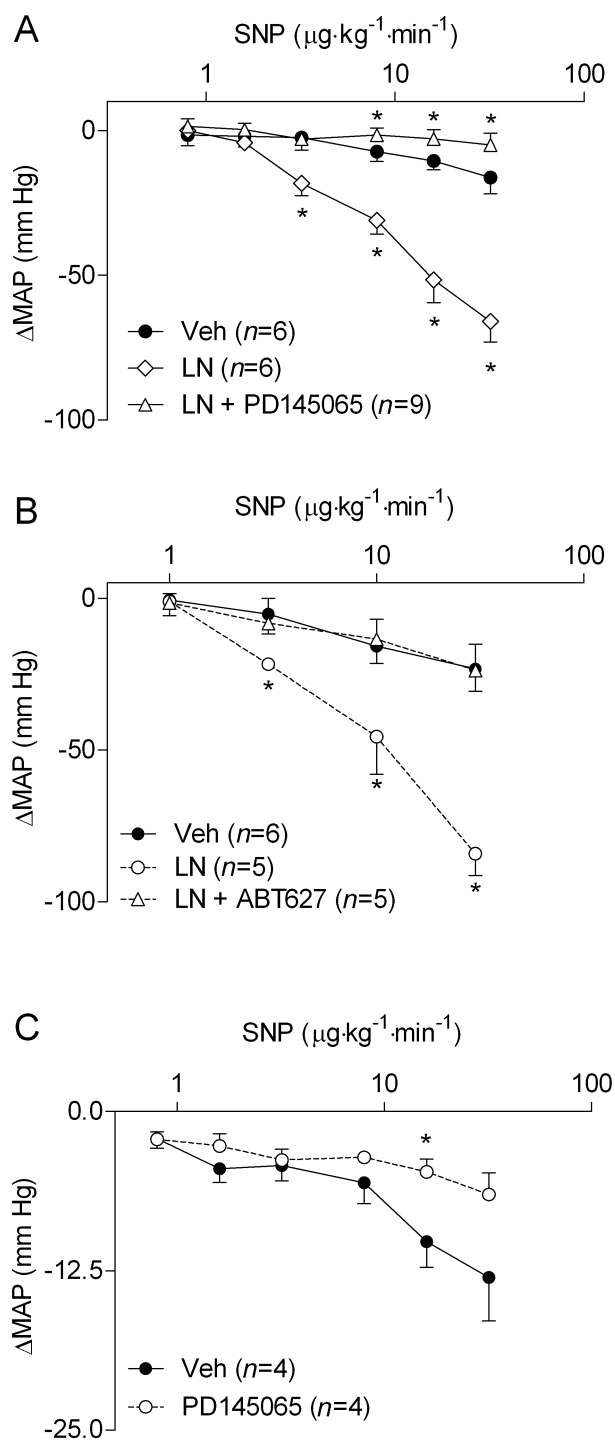
Figure 3
(A) MAP responsiveness to SNP following NOS inhibition is not dependent on initial MAP level. Rats co-treated with α-adrenoceptor antagonist prazosin (Prz; 2 mg·kg⁻¹, i.p.) and L-NAME (100 mg·kg⁻¹, i.p.) had similar MAP levels as Veh-treated rats, and had a similarly exaggerated responsiveness to SNP as L-NAME-treated rats. (B) Data from same experiments in (A) shown as changes from steady state MAP levels after infusion of saline, or combination of L-NAME and prazosin. *$P < 0.05$ compared with controls at the same dose of vasodilator. †$P < 0.05$ compared with baseline in same group. BL, baseline MAP; LN, L-NAME.

Interestingly, we also observed that PD145065 blunted the responsiveness to NO donors in the absence of L-NAME (Figures 4C, $P = 0.004$ for overall effect by two-way ANOVA). In a separate cohort of rats, we assessed circulating levels of immunoreactive ET-1 following L-NAME or saline treatment. Serum levels of ET-1 were not different between treatment groups at 15 min [saline: 375 ± 12 ng mL⁻¹ ($n = 4$); L-NAME: 364 ± 11 ng mL⁻¹ ($n = 5$); $P = 0.23$].

To gain insights into the mechanisms by which NO interacts with ET-1, we assessed vascular responses to vasoconstrictors in isolated vessels. Isolated mesenteric arteries were treated with bET-1, the inactive precursor to functional ET-1, in the presence or absence of L-NAME (Figure 5A). Treatment with L-NAME resulted in a potentiation of the bET-1-induced

Figure 4
Increased MAP responsiveness to SNP following NOS inhibition with L-NAME (100 mg·kg⁻¹, i.p.) is attenuated after pre-treatment with (A) the dual ET$_{A/B}$ receptor antagonist PD145065 (10 mg kg⁻¹ min⁻¹ i.v.) or (B) the ET$_A$ receptor antagonist ABT627 (200 µg·kg⁻¹·min⁻¹ i.v. for 10 min, followed by 100 µg·kg⁻¹·min⁻¹ i.v. thereafter). (C) BP responsiveness to SNP is also attenuated by PD145065 (10 mg·kg⁻¹·min⁻¹ i.v.) in NOS-intact rats. Data are shown as MAP changes from steady state baseline levels after infusion of saline, L-NAME, or combination of L-NAME and ET receptor antagonist. *$P < 0.05$ compared with controls at the same dose of vasodilator. LN, L-NAME.
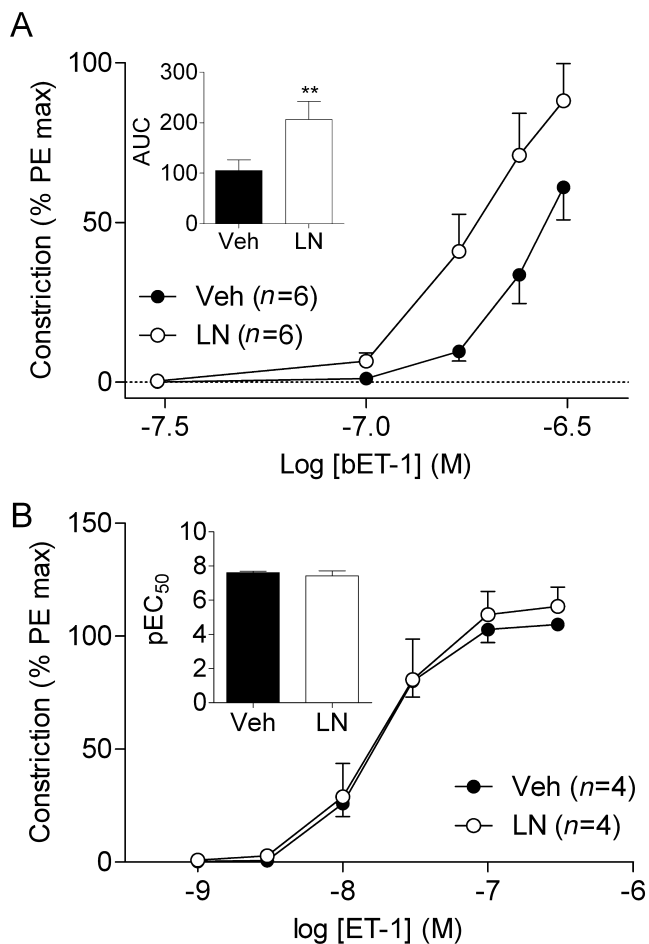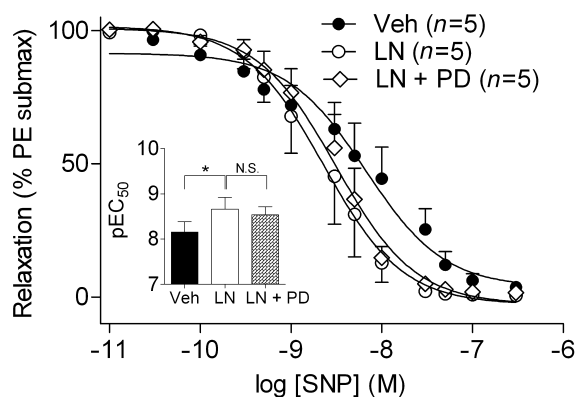
Figure 5

(A) bET-1 conversion to vasoactive ET-1 is increased after L-NAME treatment in isolated vessels. Inset: calculated AUC from bET-1 dose–response curves. *$P < 0.01$ compared with Veh AUC. (B) ET-1 mediated vasoconstriction is not affected by L-NAME (100 μM) treatment. Inset: pEC$_{50}$ values were derived from ET-1 dose–response curves. AUC, area under the curve; LN, L-NAME; PE, phenylephrine.

Figure 6

Increased vascular reactivity to SNP following NOS inhibition is not dependent on ET-1 in isolated mesenteric vessels. Isolated vessels were incubated with Veh, L-NAME (100 μM), or a combination of L-NAME (100 μM) and the ET$_{A/B}$ receptor antagonist PD145065 (5 μM). Inset: pEC$_{50}$ values were derived from dose–response curves. *$P < 0.05$ compared with Veh-treated vessels. LN, L-NAME, N.S., not significant, PD, PD145065.

the pEC$_{50}$ (Figure 6, inset). Responses to SNP in vessels treated with both L-NAME and the endothelin receptor antagonist PD145065 were superimposed on those induced in L-NAME-treated vessels, corresponding to an unaltered pEC$_{50}$.

Discussion and conclusions

The role of NO as a critical mediator of vascular tone is well established. The importance of NO in the acute regulation of BP is underscored by the fact that NO-mimetics, such as GTN and SNP, represent first-line treatments in a host of circulatory pathologies, including ischaemic heart disease and acute hypertensive crises. As such, hyper-responsiveness to NO-mimetics resulting from diminished NO synthesis could be an important clinical issue. In the present study, we investigated the contribution of ET-1 signalling in this phenomenon, and sought to gain additional insights into the interaction between NO and ET-1 in the regulation of vascular tone, and hence, BP. To summarize, we demonstrated, using a conscious rat model that: (i) acute treatment with L-NAME causes increased sensitivity to NO-mimetics and the cGMP analog 8Br-cGMP, but not to the other vasodilators tested; (ii) hypersensitivity to NO-mimetics is abolished after dual antagonism of ET$_{A/B}$ receptors, or selective antagonism of ET$_A$ receptors; furthermore, in isolated vessels, (iii) NOS inhibition potentiated the conversion of bET-1 to active ET-1, but had no effect on ET-1-mediated vasoconstriction; and finally, (iv) whereas treatment of isolated mesenteric resistance arteries vessels results in enhanced responsiveness to NO donors following NOS inhibition, this effect is not dependent on endothelin signalling.

The involvement of ET-1 in the hypertensive response, observed after acute NOS inhibition, has been demonstrated by several groups (Banting *et al.*, 1997; Filep, 1997; Cardillo *et al.*, 2000). The present study extends these findings by

constriction, resulting in a 96% increase in the area under the curve compared with Veh-treated vessels (Figure 5A inset). Treatment with the endothelin converting enzyme phosphoramidon significantly inhibited the constriction induced by bET-1, corresponding to an 80% reduction in the area under the curve ($P < 0.001$; data not shown), confirming that bET-1 must be converted to active ET-1 to elicit vasoconstriction. These data demonstrate that NO modulates conversion of bET-1 to active ET-1. In contrast, treatment with L-NAME had no effect on the dose–response curve to ET-1 (Figure 5B).

Finally, because the increased responsiveness to NO donors following acute L-NAME treatment was observed in isolated vessels, we tested whether this phenomenon was also dependent on ET-1 signalling. We further assessed the responsiveness of isolated vessels to SNP in the presence and absence of L-NAME (Figure 6). Pretreatment of vessels with L-NAME resulted in an increased responsiveness to SNP, corresponding to a leftward shift in the dose–response curve, and increase in

demonstrating that low doses of NO, which have little effect on BP in naïve animals, have marked effects on BP in NOS-inhibited animals. These observations may indicate that concentrations of NO below those that directly elicit vasodilatation effectively inhibit ET-1-mediated vasoconstriction. It is possible, therefore, that under normal circumstances, the predominant role for NO is to suppress the actions of vasoconstrictors such as ET-1. This is consistent with the observation that PD145065 inhibited the response to SNP even in the absence of L-NAME (Figure 4C). The observation that circulating ET-1 levels were not altered following L-NAME treatment may reflect the fact that ET-1 release is predominantly abluminal, and therefore changes in circulating levels do not necessarily indicate ET-1 signalling at the level of vascular receptors. It is clear, however, that more extensive studies are needed to fully test this hypothesis. Nevertheless, it is clear that ET-1 is involved in the hyper-responsiveness to NO donors following NOS inhibition. The finding that ABT627 could also attenuate this effect is consistent with the results of Richard et al. (1995) who demonstrated that L-NAME, used at a similar dose as in the present study, induced a hypertensive response that could be attenuated by ET_A receptor antagonism.

To gain additional insights into the mechanisms by which NO may modulate the ET-1 system in the vasculature, we performed similar experiments in isolated resistance arteries. We demonstrated that NO and ET-1 directly interact at the level of the vasculature. Specifically, the absence of endogenous NO potentiated the conversion of bET-1 to active ET-1, confirming that NO mediates an inhibitory effect on ET-1 signalling. We considered the possibility that blockade of NOS could simply be potentiating the vasoconstrictor effects of newly formed ET-1 by inhibiting the NO produced following autocrine activation of ET_B receptors (Warner et al., 1989). However, NOS blockade failed to potentiate the vasoconstrictor effects of ET-1, which is consistent with other studies (Fernandez et al., 1995; Ferrero et al., 2008), suggesting that NO derived from ET_B activation does not appreciably oppose vasoconstriction. In this regard, it is tempting to further speculate that autocrine activation of ET_B receptors in vivo produces low quantities of NO that are sufficient to inhibit further ET-1 production, but do not contribute directly to modulation of vascular tone.

The increased responsiveness to NO donors in isolated tissues is consistent with previous reports (Alheid et al., 1987; Busse et al., 1989; Flavahan and Vanhoutte, 1989; Luscher et al., 1989; Moncada et al., 1991). However, the inability of PD145065 to reverse the L-NAME-mediated hyper-responsiveness to SNP in isolated tissues suggests that this mechanism is different when studied in vivo and ex vivo. Whereas the hyper-responsiveness in vivo implicates ET-1, the increased responsiveness to NO donors in isolated tissues does not, and is more likely attributed to enhanced sGC activity and sensitization of downstream pathways following NOS inhibition, which has been reported previously (Moncada et al., 1991; Gupta et al., 2008). The apparent disparity between in vivo and ex vivo involvement of ET-1 in the hyper-responsiveness to NO donors is easily reconciled. As removal of resistance arteries from the rat results in loss of vascular modulatory systems – including sympathetic, parasympathetic and nitrergic innervations as well as

actions by local and humoral factors – vascular tone is generated ex vivo 'artificially' by the addition of PE (i.e. pre-constriction). It is therefore perhaps not surprising that hyper-responsiveness observed in the wake of NOS inhibition is not dependent on ET-1 signalling. In contrast, in a fully integrated system (i.e. conscious rat) vascular tone is established by a host of signalling systems acting in concert, resulting in the perpetual release of ET-1 and NO (among several other vasoactive systems). However, we must also acknowledge the possibility that the interaction between NO and ET-1 observed in the present experiments may be occurring in other tissues, or could be due to regional vascular differences in responsiveness to NO donors and NOS inhibition, which has been described previously (Gardiner et al., 1991).

A point that is particularly noteworthy in the context of the present study is the time course in which NOS is inhibited. Although increased responsiveness to NO donors has been observed in both acute and chronic NOS inhibition models (Alheid et al., 1987; Busse et al., 1989; Flavahan and Vanhoutte, 1989; Luscher et al., 1989; Gardiner et al., 1991; 1993; Moncada et al., 1991), the mechanisms underlying these effects may be different (Bourque et al., 2011). The present experiments, which provide evidence for a complex interaction between NO and ET-1, were performed on a relatively short time scale (acute NOS inhibition), and may not be applicable in models of chronic NOS inhibition. Indeed, in studies in which ET-1 antagonists are administered before or immediately after NOS blockade, the hypertension is preventable or reversible (Richard et al., 1995; Banting et al., 1997; Filep, 1997; Cardillo et al., 2000; present study). However, after several weeks of NOS blockade, the hypertension is not reversible by ET-1 antagonism (Bank et al., 1994; Fujita et al., 1995). Similarly, whereas L-arginine, the precursor to NO, readily reverses the hypertension associated with L-NAME when administered simultaneously with the NOS inhibitor (Salazar et al., 1992), it was shown to have progressively less effect when administered several days (Ribeiro et al., 1992) or several weeks after L-NAME treatment began (Erley et al., 1995; Qiu et al., 1995).

In summary, the present study offers insights into the mechanisms by which NOS inhibition causes enhanced sensitivity to NO. Perhaps more importantly, it also offers insights into the general mechanisms of vascular control. That is, the present results, together with other studies, suggest that NO maintains vascular tone largely through an ET-1-dependent signalling mechanism (Bourque et al., 2011). The paradigm we see emerging is one in which vascular control by NO occurs by several mechanisms, including opposing the actions of vasoconstrictor agents, as well as direct vasodilatation, among others.

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Conflict of interest

None.

References

Alheid U, Dudel C, Forstermann U (1987). Selective inhibition by gossypol of endothelium-dependent relaxations augments relaxations to glyceryl trinitrate in rabbit coeliac artery. Br J Pharmacol 92: 237–240.

Bank N, Aynedjian HS, Khan GA (1994). Mechanism of vasoconstriction induced by chronic inhibition of nitric oxide in rats. Hypertension 24: 322–328.

Banting JD, Friberg P, Adams MA (1996). Acute hypertension after nitric oxide synthase inhibition is mediated primarily by increased endothelin vasoconstriction. J Hypertens 14: 975–981.

Banting JD, Thompson KE, Friberg P, Adams MA (1997). Blunted cardiovascular growth induction during prolonged nitric oxide synthase blockade. Hypertension 30: 416–421.

Bian K, Doursout MF, Murad F (2008). Vascular system: role of nitric oxide in cardiovascular diseases. J Clin Hypertens (Greenwich) 10: 304–310.

Bourque SL, Davidge ST, Adams MA (2011). The interaction between endothelin-1 and nitric oxide in the vasculature: new perspectives. Am J Physiol Regul Integr Comp Physiol 300: R1288–R1295.

Busse R, Pohl U, Mulsch A, Bassenge E (1989). Modulation of the vasodilator action of SIN-1 by the endothelium. J Cardiovasc Pharmacol 14 (Suppl 11): S81–S85.

Cardillo C, Kilcoyne CM, Cannon RO,3rd, Panza JA (2000). Interactions between nitric oxide and endothelin in the regulation of vascular tone of human resistance vessels in vivo. Hypertension 35: 1237–1241.

Erley CM, Rebmann S, Strobel U, Schmidt T, Wehrmann M, Osswald H et al. (1995). Effects of antihypertensive therapy on blood pressure and renal function in rats with hypertension due to chronic blockade of nitric oxide synthesis. Exp Nephrol 3: 293–299.

Fernandez N, Monge L, Garcia-Villalon AL, Garcia JL, Gomez B, Dieguez G (1995). Endothelin-1-induced in vitro cerebral venoconstriction is mediated by endothelin ETA receptors. Eur J Pharmacol 294: 483–490.

Ferrero E, Labalde M, Fernandez N, Monge L, Salcedo A, Narvaez-Sanchez R et al. (2008). Response to endothelin-1 in arteries from human colorectal tumours: role of endothelin receptors. Exp Biol Med (Maywood) 233: 1602–1607, 10.3181/0802-RM-69.

Filep JG (1997). Endogenous endothelin modulates blood pressure, plasma volume, and albumin escape after systemic nitric oxide blockade. Hypertension 30: 22–28.

Flavahan NA, Vanhoutte PM (1989). Mechanism underlying the inhibitory interaction between the nitrovasodilator SIN-1 and the endothelium. J Cardiovasc Pharmacol 14 (Suppl. 11): S86–S90.

Fujita K, Matsumura Y, Miyazaki Y, Takaoka M, Morimoto S (1995). Role of endothelin-1 in hypertension induced by long-term inhibition of nitric oxide synthase. Eur J Pharmacol 280: 311–316.

Gardiner SM, Kemp PA, Bennett T (1991). Effects of NG-nitro-L-arginine methyl ester on vasodilator responses to acetylcholine, 5′-N-ethylcarboxamidoadenosine or salbutamol in conscious rats. Br J Pharmacol 103: 1725–1732.

Gardiner SM, Kemp PA, Bennett T (1993). Effects of chronic treatment with nitric oxide synthase inhibitors on regional haemodynamic responses to vasodilators in conscious Brattleboro rats. Br J Pharmacol 109: 222–228.

Gupta PK, Subramani J, Singh TU, Leo MD, Sikarwar AS, Prakash VR et al. (2008). Role of protein kinase G in nitric oxide deficiency-induced supersensitivity to nitrovasodilator in rat pulmonary artery. J Cardiovasc Pharmacol 51: 450–456, 10.1097/FJC.0b013e31816949ca.

Hrabie JA, Klose JR, Wink DA, Keefer LK (1993). New nitric oxide-releasing zwitterions derived from polyamines. J Org Chem 58: 1472–1476.

Luscher TF, Richard V, Yang ZH (1989). Interaction between endothelium-derived nitric oxide and SIN-1 in human and porcine blood vessels. J Cardiovasc Pharmacol 14 (Suppl 11): S76–S80.

Moncada S, Higgs EA (2006). Nitric oxide and the vascular endothelium. Handb Exp Pharmacol 176: 213–254.

Moncada S, Rees DD, Schulz R, Palmer RM (1991). Development and mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis in vivo. Proc Natl Acad Sci U S A 88: 2166–2170.

Palmer RM, Ashton DS, Moncada S (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 333: 664–666, 10.1038/333664a0.

Qiu C, Engels K, Baylis C (1995). Endothelin modulates the pressor actions of acute systemic nitric oxide blockade. J Am Soc Nephrol 6: 1476–1481.

Rees DD, Palmer RM, Moncada S (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. Proc Natl Acad Sci U S A 86: 3375–3378.

Ribeiro MO, Antunes E, de Nucci G, Lovisolo SM, Zatz R (1992). Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. Hypertension 20: 298–303.

Richard V, Hogie M, Clozel M, Loffler BM, Thuillez C (1995). In vivo evidence of an endothelin-induced vasopressor tone after inhibition of nitric oxide synthesis in rats. Circulation 91: 771–775.

Salazar FJ, Pinilla JM, Lopez F, Romero JC, Quesada T (1992). Renal effects of prolonged synthesis inhibition of endothelium-derived nitric oxide. Hypertension 20: 113–117.

Vallance P, Collier J, Moncada S (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. Lancet 2: 997–1000.

Warner TD, Mitchell JA, de Nucci G, Vane JR (1989). Endothelin-1 and endothelin-3 release EDRF from isolated perfused arterial vessels of the rat and rabbit. J Cardiovasc Pharmacol 13 (Suppl. 5): S85–S88; discussion S102.