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Vascular natriuretic peptide receptor-linked particulate guanylate cyclases are modulated by nitric oxide–cyclic GMP signalling

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1 The sensitivity of the particulate guanylate cyclase–cyclic guanosine-3′,5′-monophosphate (cGMP) system to atrial (ANP) and C-type (CNP) natriuretic peptides was investigated in aortae and mesenteric small arteries from wild-type (WT) and endothelial nitric oxide synthase (eNOS) knockout (KO) mice.

2 ANP and CNP produced concentration-dependent relaxations of mouse aorta that were significantly attenuated by the natriuretic peptide receptor (NPR)-A/B antagonist HS-142-1 (10^{-5} M). Both ANP and CNP were more potent in aortae from eNOS KO mice compared to WT.

3 The potency of ANP and CNP in aortae from WT animals was increased in the presence of the NOS inhibitor, N^G-nitro-L-arginine (3×10^{-4} M) and the soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolol[4,3,a]quinoxalin-1-one (5×10^{-6} M).

4 In contrast, the potency of ANP and CNP in aortae from eNOS KO animals was reduced following pretreatment of tissues with supramaximal concentrations of the NO-donor, glyceryl trinitrate (3×10^{-5} M, 30 min) or ANP (10^{-7} M, 30 min).

5 Responses to acetylcholine in aortae from WT mice (dependent on the release of endothelium-derived NO) were significantly reduced following pretreatment of tissues with GTN (3×10^{-5} M, 30 min) and ANP (10^{-7} M, 30 min).

6 CNP and the NO-donor, spermine-NONOate caused concentration-dependent relaxations of mesenteric small arteries from WT animals that were significantly increased in eNOS KO mice compared to WT. ANP was unable to significantly relax mesenteric arteries from WT or eNOS KO animals.

7 In conclusion, both NPR-A- and NPR-B-linked pGC pathways are modulated by NO–cGMP in murine aorta and mesenteric small arteries and crossdesensitisation occurs between NPR subtypes. The biological activity of endothelium-derived NO is also influenced by the ambient concentration of NO and natriuretic peptides. Such an autoregulatory pathway may represent an important physiological homeostatic mechanism and link the paracrine activity of NO and CNP with the endocrine functions of ANP and BNP in the regulation of vascular tone and blood pressure.
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Keywords: Natriuretic peptides; nitric oxide; cyclic GMP; vasodilatation; soluble guanylate cyclase; particulate guanylate cyclase

Abbreviations: ACh, acetylcholine; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; cGMP, cyclic guanosine-3′,5′-monophosphate; GTN, glyceryl trinitrate; KO, knockout; NPR, natriuretic peptide receptor; NO, nitric oxide; NOS, nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; ODQ, 1H-[1,2,4]-oxadiazolol-[4,3-a]quinoxalin-1-one; pGC, particulate guanylate cyclase; PE, phenylephrine; sGC, soluble guanylate cyclase; SPER-NO, spermine-NONOate; pEC_{50}, $-\log [EC_{50}\,(M)]$; U46619, 9,11-dideoxy-11$^{\alpha}$,9$^{\alpha}$-epoxymethano-prostaglandin $F_{2\alpha}$; WT, wild-type

Introduction

Nitric oxide (NO) and natriuretic peptides play important roles in cardiovascular homeostasis and disease (Moncada *et al*., 1991; Maack, 1996; Chen & Burnett, 1998; Melo *et al*., 1998; Vallance, 1998). The mammalian natriuretic peptide family comprises atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and urodilatin (Levin *et al*., 1998; Kone, 2001). ANP and BNP are produced predominantly in the cardiac atria and ventricles, respectively. Both peptides are released into the circulation in response to hypervolaemia and act in an endocrine manner to regulate blood pressure and body fluid homeostasis (Maack, 1996). In contrast, CNP is found in several peripheral tissues, including endothelial cells, where it appears to be regulated by local factors (Stingo *et al*., 1992; Suga *et al*., 1992). As a consequence, CNP has been suggested to regulate vascular tone in a paracrine manner (Chinkers *et al*., 1989; Chen & Burnett, 1998). In a similar fashion, urodilatin is thought to represent a paracrine intrarenal regulator of sodium and water homeostasis (Forssmann *et al*., 2001).

To date, three natriuretic peptide receptor (NPR) subtypes have been cloned and characterised and designated NPR-A,

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NPR-B and NPR-C (Maack, 1996; Chen & Burnett, 1998; Levin *et al*., 1998; Kone, 2001). NPR-A binds ANP and BNP with equal affinity, whereas CNP appears to be the endogenous ligand for NPR-B. Both NPR-A and NPR-B are coupled to (particulate) guanylate cyclase which generates the intracellular second messenger cyclic guanosine-3′,5′-monophosphate (cGMP) upon natriuretic peptide binding/activation. NPR-C has a similar affinity for all three peptides, but is not linked to guanylate cyclase. This protein is thought to act as a clearance receptor and remove natriuretic peptides from the circulation (Maack *et al*., 1987). Recently, however, it has been demonstrated that NPR-C activates GTP-binding proteins that regulate adenylate cyclase and phosphoinositide turnover, intimating a signal-transduction role for this receptor (Murthy & Makhlouf, 1999; Murthy *et al*., 2000).

Both NO and natriuretic peptide share significant commonality in that they function as vasodilators, *via* activation of the guanylate cyclase/cGMP pathway. NO activates the cytoplasmic heterodimeric haemoprotein, soluble guanylate cyclase (sGC) (Hobbs, 1997), while natriuretic peptides activate the membrane-bound protein, particulate guanylate cyclase (pGC) (Winquist *et al*., 1984; Drewett *et al*., 1995). Stimulation of either cyclase results in the conversion of GTP to the intracellular second messenger cGMP, which is responsible for regulating cardiovascular homeostasis.

We have demonstrated previously that the NO–sGC–cGMP system is influenced by the ambient concentration of NO, possibly through a cGMP-dependent process (Hussain *et al*., 1999). Blood vessels that exhibit decreased basal NO, as occurs in mice deficient in endothelial NO synthase (eNOS), show increased sensitivity to NO and NO donors (including greater cGMP production). In contrast, prolonged exposure to high concentrations of NO leads to reduced responses to subsequent application of NO (or NO donors). Moreover, we have also identified an interaction between the NO–sGC–cGMP and the ANP–pGC–cGMP systems, such that the changes in the sensitivity of one pathway are mimicked by the alternate cGMP-generating system (Hussain *et al*., 2001). Thus, aortae from eNOS knockout (KO) mice are more sensitive to ANP than tissues from wild-type (WT) animals. In concert, these results suggest that the sGC–cGMP and pGC–cGMP pathways have a complementary role in maintaining cardiovascular homeostasis. The purpose of the present study was to extend these observations and investigate the profile of natriuretic peptide receptors in conduit and resistance arteries and compare how the function of these receptors might be regulated by NO.

Methods

Thoracic aorta

Male eNOS WT and KO mice (25–35 g; kind gift of Paul Huang, Johns Hopkins University, U.S.A. (Huang *et al*., 1995)) were stunned and killed by cervical dislocation. The thoracic aortae were carefully removed, cleaned of connective tissue and cut into three to four ring segments of approximately 4 mm in length. Aortic rings were mounted in 10 ml organ baths containing Krebs-bicarbonate buffer (composition (mM): Na^+ 143; K^+ 5.9; Ca^{2+} 2.5; Mg^{2+} 1.2; Cl^- 128; HCO_3^- 25; HPO_4^{2-} 1.2; SO_4^{2-} 1.2; D-glucose 11) maintained at 37°C and gassed with 95% O_2/5% CO_2. Tension was initially set at 0.3 g and reset at intervals following an equilibration period of 1 h during which time fresh Krebs-bicarbonate buffer was replaced every 15–20 min. After equilibration, the rings were primed with KCl (4.8×10^{-2} M) before a supramaximal concentration of phenylephrine (PE; 10^{-6} M) was added. Once this response had stabilized, acetylcholine (ACh; 10^{-6} M) was added to the bath to assess the integrity of endothelium of WT vessels. If the contractions to PE were not maintained, or relaxations greater than 50% of the PE-induced tone to ACh were not observed, the tissues were discarded.

Tissues were then washed for 30 min (by addition of fresh Krebs-bicarbonate buffer at 15 min intervals) after which cumulative concentrations of PE (10^{-9}–10^{-6} M) were added to the organ bath. The tissues were then washed over 60 min to restore basal tone before contracting to approximately 80% of the maximum PE-induced response in WT vessels (0.70 ± 0.02 g in WT and 0.76 ± 0.02 g in eNOS KO; $n \geqslant 56$ for both; $P > 0.05$). Once a stable response to PE was achieved, cumulative concentration–response curves to Spermine-NONOate (SPER-NO) (10^{-9}–10^{-5} M), ANP (10^{-9}–10^{-6} M), CNP (10^{-9}–10^{-6} M), the selective NPR-C agonist $cANF^{4-23}$ (10^{-9}–10^{-6} M) and ACh (10^{-9}–10^{-6} M) were constructed.

To determine the NPR subtypes activated by ANP and CNP, concentration–response curves to both natriuretic peptides were constructed in tissues from WT animals in the absence or presence of the NPR-A/NPR-B antagonist, HS-142-1 (1×10^{-5} M; 30 min incubation; kind gift of Dr Y. Matsuda, Kyowa Hakko Kogyo Co. Ltd., Japan).

To investigate the effect of chronic NO deficiency on the sGC–cGMP and pGC–cGMP systems, concentration–response curves to SPER-NO, ANP and CNP were constructed in aortae from eNOS KO animals. To examine the effects of acute NO–cGMP deficiency, concentration–response curves to SPER-NO, ANP and CNP were constructed in tissues from WT animals following 30 min incubation with either the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 3×10^{-4} M) or the sGC inhibitor, 1H-[1,2,4]-oxadiazolol-[4,3-a]quinoxalin-1-one (ODQ; 5×10^{-6} M).

To study the effects of excess NO on the sGC–cGMP and pGC–cGMP systems, vessels from eNOS KO mice were incubated with a supramaximal concentration of glyceryl trinitrate (GTN; 3×10^{-5} M; 30 min incubation followed by washout). To assess the effect of excess pGC/NPR-A activation on SPER-NO, ANP- and CNP-induced responses, vessels from eNOS KO mice were incubated with a supramaximal concentration of ANP (1×10^{-7} M; 30 min incubation followed by washout). In experiments investigating the effect of excess NO and ANP on responses to ACh, essentially identical studies were conducted but in tissues from WT animals.

Mesenteric artery

Male eNOS WT and KO mice were stunned and killed by cervical dislocation. The mesentery was removed and transferred to Krebs buffer and third-order arteries were stripped of adherent tissues, cut into rings of 3 mm in length and mounted horizontally between two stainless-steel wires (40 μm in diameter) in an automated tension myograph (Danish Myotechnology, Denmark). Vessels were maintained at 37°C in Krebs solutions bubbled with 95% O_2/5% CO_2. After an equilibration period of 45 min, vessels were stretched in a

stepwise manner to determine the relationship between the passive tension and internal circumference according to the Laplace equation; from this relation, the internal diameter was determined (Mulvany & Halpern, 1977). Vessels were then stretched to 90% of the diameter achieved when under a transmural pressure of 100 mmHg. The mean diameter of the vessels used in this study was $167.8 \pm 7.57\,\mu$m for WT ($n=28$) and $167.6 \pm 6.59\,\mu$m for eNOS KO ($n=32$; $P>0.05$) tissues.

Following normalization, each vessel was contracted repeatedly with the thromboxane mimetic 9,11-dideoxy-11$^{\alpha}$,9$^{\alpha}$-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619; 10^{-6} M) until the response was reproducible. The vessels were then washed to restore basal tone before contracting to approximately 50% of the maximum U46619-induced response. Once a stable response to U46619 was achieved, cumulative concentrations of SPER-NO (10^{-9}–10^{-5} M), ANP (10^{-9}–10^{-6} M) and CNP (10^{-9}–10^{-6} M) were added to tissues from eNOS WT and KO mice.

Materials

L-NAME, ACh and ANP (rat) were purchased from Sigma Chemical Co (Dorset, U.K.). Nitronal (preservative-free glyceryl trinitrate) was obtained from Lipha Pharmaceuticals Ltd. (Middlesex, U.K). ODQ was synthesised by the Medical Chemistry Department at the Wolfson Institute for Biomedical Research. CNP and *N*-[2-aminoethyl]-*N*-[2-hydroxy-2-nitroso-hydrazino]-1,2-ethylenediamine (SPER-NO) were purchased from Calbiochem (Nottingham, U.K.). 9, 11-dideoxy-11$^{\alpha}$, 9$^{\alpha}$-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) was purchased from Affiniti (Exeter, U.K.).

Data analysis

Relaxations are expressed as percent reversal of PE-induced tone (mean ± s.e.m. of n animals). Curves were fitted to all the data using nonlinear regression and the –log [M] of each drug giving a half-maximal response (pEC_{50}) were used to compare potency. Curves were analysed using two-way analysis of variance and $P<0.05$ was taken as statistically significant. Statistical analysis was undertaken using Prism (GraphPad software San Diego, CA, U.S.A.).

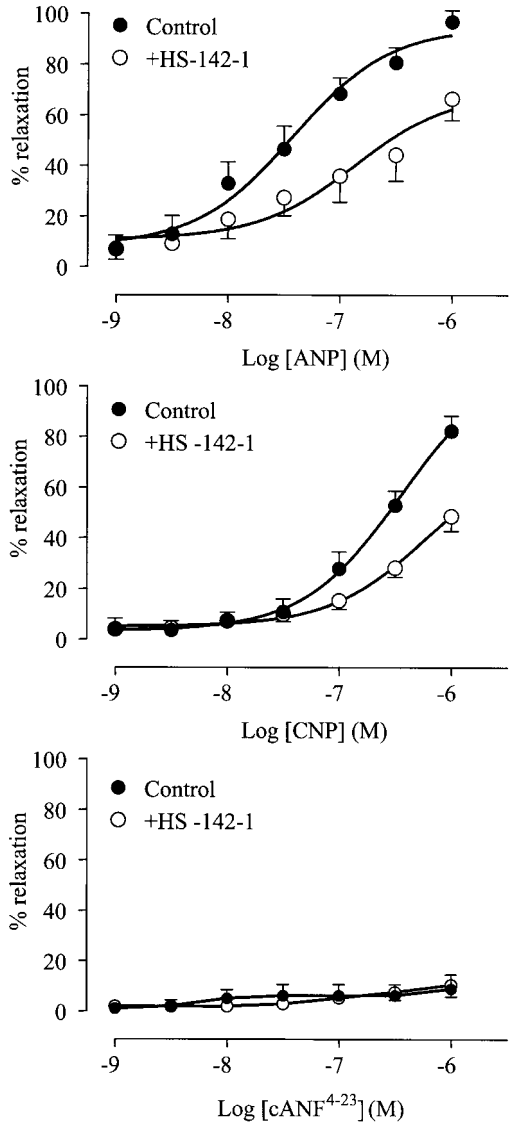

Figure 1 Concentration–response curves to ANP (upper panel), CNP (middle panel) and $cANF^{4-23}$ (lower panel) in aortic rings from WT animals in the presence and absence of the NPR-A/NPR-B antagonist HS-142-1 (10^{-5} M). Relaxation is expressed as mean ± s.e.m. percentage reversal of PE-induced tone. $P<0.05$, control *versus* HS-142-1 for ANP and CNP only ($n=8$).

Results

Aortic rings

Effect of the NPR-A/NPR-B antagonist, HS-142-1, on responses to ANP and CNP Following incubation of vessels from WT animals with HS-142-1 (10^{-5} M), there was a significant rightward shift in the concentration–response curves to ANP (pEC_{50}: 7.44 ± 0.16 and 6.80 ± 0.33 in the presence and absence of HS-142-1, respectively; $P<0.05$; $n=8$; Figure 1) and CNP (pEC_{50}: 6.44 ± 0.16 and 6.22 ± 0.23 in the presence and absence of HS-142-1, respectively; $P<0.05$; $n=8$; Figure 1). However, the selective NPR-C agonist, $cANF^{4-23}$ was unable to cause significant relaxation of the tissue in the presence or absence of HS-142-1 (Figure 1).

Effect of eNOS gene deletion on the sensitivity of the sGC-cGMP and pGC-cGMP pathways SPER-NO reversed PE-induced tone with greater potency in aortic rings from eNOS KO mice compared with those from WT animals (pEC_{50} 6.94 ± 0.15 and 6.39 ± 0.08 in KO and WT, respectively; $P<0.05$; $n=8$), as we have previously reported (Hussain *et al.*, 1999). Aortic rings from eNOS KO animals were also more sensitive to ANP (pEC_{50}: 8.85 ± 0.01 and 8.41 ± 0.02 in eNOS KO and WT, respectively; $P<0.05$; $n=6$; Figure 2) and CNP (pEC_{50} 7.50 ± 0.12 and 6.87 ± 0.09 in eNOS KO and WT, respectively; $P<0.05$; $n=8$; Figure 2) than tissues from eNOS WT mice.

Acute effects of NOS and sGC inhibition on responses to SPER-NO, ANP and CNP Incubation of WT vessels with L-NAME (3×10^{-4} M) increased the potency of SPER-NO (pEC_{50}: 6.25 ± 0.07 and 6.76 ± 0.08, in the absence and presence of L-NAME, respectively; $P<0.05$; $n=5$), confirming

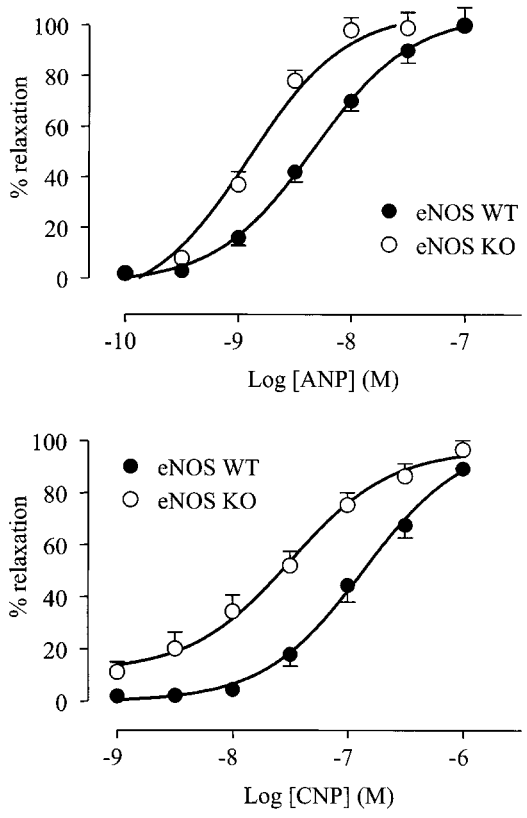

Figure 2 Concentration–response curves to ANP (upper panel) and CNP (lower panel) in aortic rings from WT and eNOS KO animals. Relaxation is expressed as mean ± s.e.m. percentage reversal of PE-induced tone. $P<0.05$, WT *versus* eNOS KO for both ($n=8$).

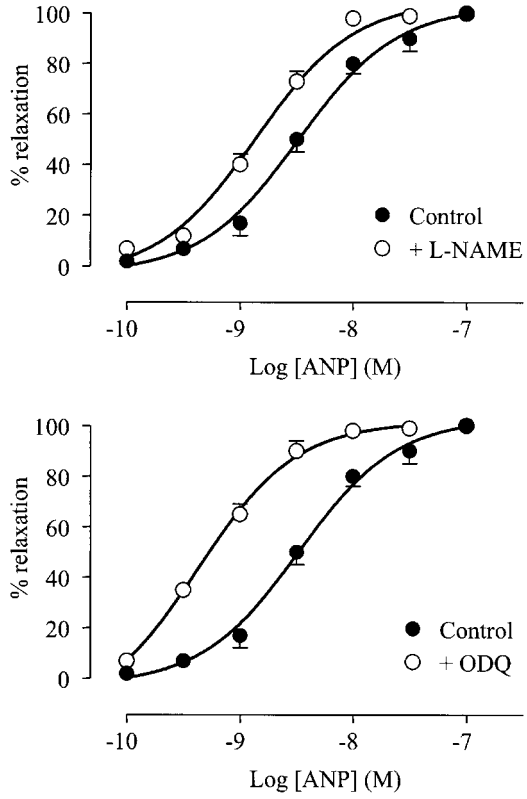

Figure 3 Concentration–response curves to ANP in aortic rings from WT animals in the presence and absence of the NOS inhibitor L-NAME (3×10^{-4} M; upper panel) or sGC inhibitor ODQ (5×10^{-6} M; lower panel). Relaxation is expressed as mean ± s.e.m. percentage reversal of PE-induced tone. $P<0.05$, control *versus* L-NAME or ODQ ($n\geqslant5$).

our previous observations (Hussain *et al*., 1999). In the presence of the sGC inhibitor ODQ (5×10^{-6} M), the response to SPER-NO was inhibited (pEC_{50}: 6.59 ± 0.17 and 4.81 ± 0.29 in the absence and presence of ODQ, respectively; $P<0.05$; $n\geqslant5$), validating that this concentration of ODQ was effectively blocking sGC activation. In contrast, the potency of ANP was significantly increased after pretreatment with L-NAME (pEC_{50}: 8.47 ± 0.07 and 8.86 ± 0.08 in the absence and presence of L-NAME, respectively; $P<0.05$; $n=4$; Figure 3) and ODQ (pEC_{50}: 8.56 ± 0.05 and 9.26 ± 0.03 in the absence and presence of ODQ, respectively; $P<0.05$; $n=4$; Figure 3). Responses to CNP were also significantly enhanced following acute NOS inhibition (pEC_{50}: 6.95 ± 0.13 and 7.18 ± 0.10 in the absence and presence of L-NAME, respectively; $P<0.05$; $n\geqslant5$; Figure 4) or sGC inhibition (pEC_{50}: 6.82 ± 0.10 and 7.17 ± 0.13 in the absence and presence of ODQ, respectively; $P<0.05$; $n\geqslant5$; Figure 4).

Effect of excess NO on responses to SPER-NO, ANP and CNP Incubation of tissues from eNOS KO animals with GTN (3×10^{-5} M) decreased the potency of SPER-NO (pEC_{50}: 7.00 ± 0.11 and 6.53 ± 0.16 before and after GTN, respectively; $P<0.05$; $n=7$). GTN pretreatment also decreased the relaxant potency of ANP (pEC_{50}: 9.24 ± 0.05 and 8.43 ± 0.04 before and after GTN, respectively; $P<0.05$; $n=4$; Figure 5) and CNP (pEC_{50}: 7.50 ± 0.12 and 6.32 ± 0.23 before and after GTN, respectively; $P<0.05$; $n=7$; Figure 5).

Effect of excess NO on responses to ACh To investigate the effect of high-ambient NO concentrations on endogenous NO–sGC–cGMP signalling, responses to ACh (to release endothelium-derived NO) in aortae from WT mice were studied before and after exposure to a supramaximal concentration of GTN. In this case, following treatment of tissues with GTN (3×10^{-5} M), responses to ACh were significantly reduced (pEC_{50}: 7.01 ± 0.03 and 6.81 ± 0.06 before and after GTN, respectively; $P<0.05$; $n\geqslant5$; Figure 6).

Effect of excess ANP on responses to SPER-NO, ANP and CNP Exposure of eNOS KO vessels to a supramaximal concentration of ANP (10^{-7} M) decreased the potency of SPER-NO (pEC_{50}: 7.53 ± 0.08 and 6.90 ± 0.13 before and after ANP, respectively; $P<0.05$; $n=7$; Figure 7), ANP (pEC_{50}: 8.93 ± 0.03 and 8.01 ± 0.17 before and after ANP, respectively; $P<0.05$; $n=4$; Figure 7) and CNP (pEC_{50}: 7.53 ± 0.08 and 6.39 ± 0.27 before and after ANP, respectively; $P<0.05$; $n=7$; Figure 7).

Effect of excess ANP on responses to ACh To investigate the effect of high-ambient ANP concentrations on endogenous NO–sGC–cGMP signalling, responses to ACh (to release endothelium-derived NO) in aortae from WT mice were studied before and after exposure to a supramaximal concentration of ANP. In this case, following treatment of tissues with ANP (10^{-7} M), responses to ACh were significantly

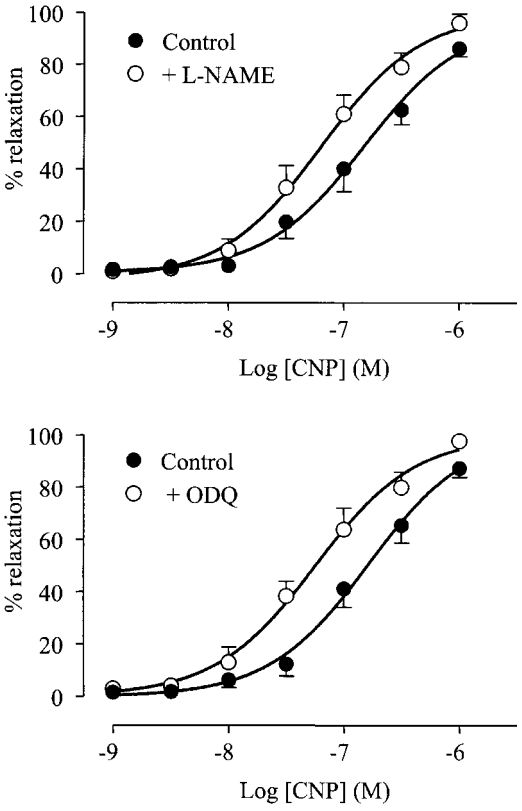

Figure 4 Concentration–response curves to CNP in aortic rings from WT animals in the presence and absence of the NOS inhibitor L-NAME (3×10^{-4} M; upper panel) or sGC inhibitor ODQ (5×10^{-6} M; lower panel). Relaxation is expressed as mean ± s.e.m. percentage reversal of PE-induced tone. $P<0.05$, control *versus* L-NAME or ODQ ($n \geqslant 5$).

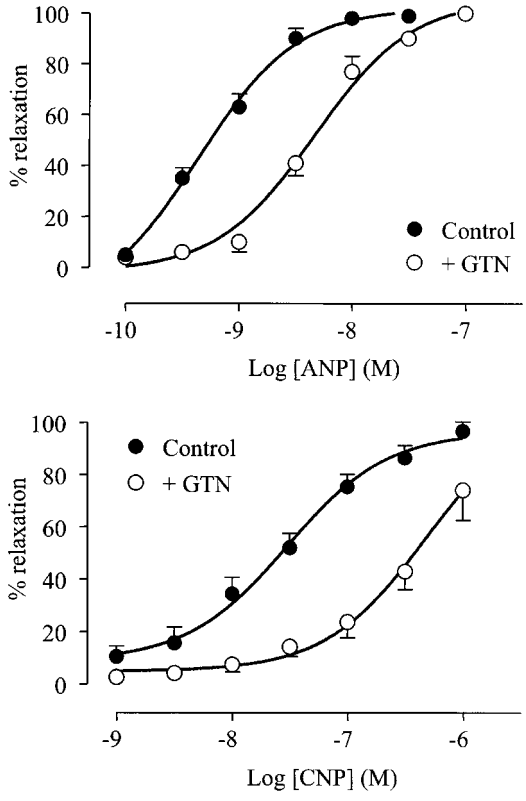

Figure 5 Concentration–response curves to ANP (upper panel) and CNP (lower panel) in aortic rings from eNOS KO animals in the presence and absence of GTN pretreatment (3×10^{-5} M; 30 min). Relaxation is expressed as mean ± s.e.m. percentage reversal of PE-induced tone. $P<0.05$, control *versus* GTN pretreatment for both ($n \geqslant 7$).

reduced (pEC_{50}: 7.03 ± 0.08 and 6.98 ± 0.01 before and after GTN, respectively; $P<0.05$; $n \geqslant 5$; Figure 6).

Mesenteric arteries

Interactions between the sGC–cGMP and pGC–cGMP pathways in mesenteric arteries SPER-NO produced concentration-dependent relaxations of U46619 precontracted mesenteric arteries from eNOS WT and KO animals, but was significantly more potent in tissues from KO mice (pEC_{50}: 5.61 ± 0.21 and 6.73 ± 0.12 for WT and eNOS KO, respectively; $P<0.05$; $n \geqslant 5$; Figure 8). CNP was also more potent in mesenteric arteries from eNOS KO mice compared with vessels from WT animals (pEC_{50}: 6.21 ± 1.08 and 7.12 ± 0.34 for WT and eNOS KO, respectively; $P<0.05$; $n \geqslant 4$; Figure 8). In contrast, ANP did not relax vessels from WT or eNOS KO animals to any significant extent (Figure 8).

Discussion

The present study demonstrates that both the NPR-A- and NPR-B-linked pGC pathways are modulated by NO in mouse conduit (aorta) and resistance (mesenteric) arteries; moreover, the ability of ODQ to alter the sensitivity of NPR-A/NPR-B implicates cGMP in this phenomenon, rather than a direct affect of NO. The altered responsiveness of NPR-A/NPR-B during acute NO deficiency, as produced by L-NAME, is similar to that observed in eNOS KO mice, a model of chronic NO shortage, suggesting that the change in the sensitivity of these pGCs is biochemical rather than expressional. Furthermore, we have shown that crosstalk between NPR-A and NPR-B, which has been indicated by biochemical studies (Potter & Garbers, 1992; Potter & Hunter, 1998), occurs at a functional level and may therefore have important physiological implications. Finally, this study has defined an important feedback pathway regulating the biological activity of endothelium-derived NO, which is altered in response to changes in sGC and pGC activation, which may have important functional consequences in pathological conditions characterised by excessive production of NO and/or natriuretic peptides (e.g. sepsis, heart failure).

To identify the NPR subtypes activated by ANP and CNP to mediate vasorelaxation in the aorta, the selective NPR-A/NPR-B antagonist HS-142-1 (a polysaccharide isolated from *Aureobasidium*) (Morishita *et al*., 1991) was employed. In the presence of HS-142-1, the responses of WT vessels to both ANP and CNP were significantly reduced. These observations indicated that ANP and CNP activated the pGC-linked NPR-A and NPR-B, respectively, to mediate vasorelaxation. This thesis was supported by the inability of $cANF^{4-23}$, a selective NPR-C agonist (Maack *et al*., 1987), to cause smooth muscle relaxation in this tissue. Thus, we assumed that ANP and CNP could be used as pharmacological tools in the mouse

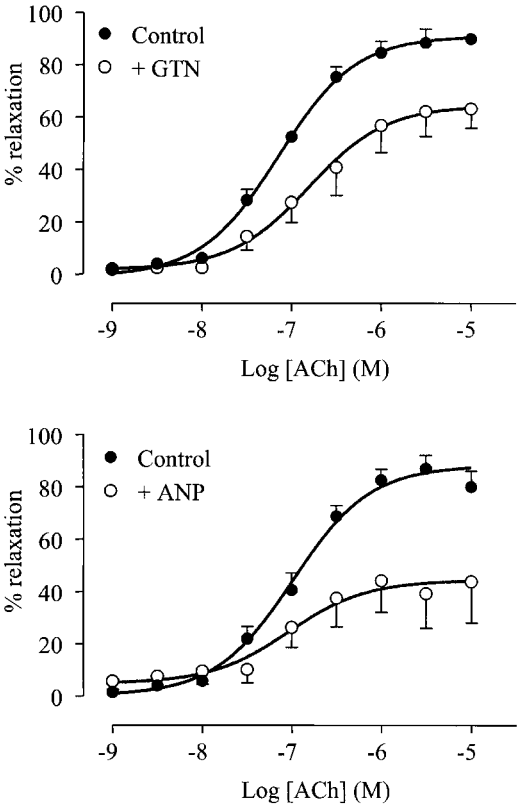

Figure 6 Concentration–response curves to ACh in aortic rings from WT animals in the presence and absence of GTN (3×10^{-5} M; 30 min; upper panel) or ANP (10^{-7} M; 30 min; lower panel) pretreatment. Relaxation is expressed as mean ± s.e.m. percentage reversal of PE-induced tone. $P<0.05$, control *versus* GTN or ANP pretreatment ($n \geqslant 5$).

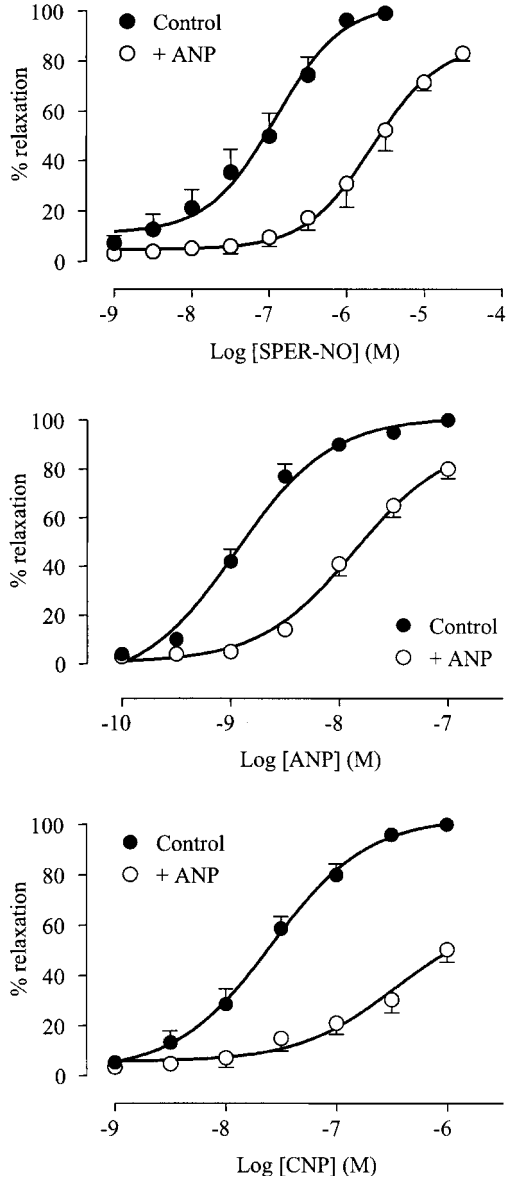

Figure 7 Concentration–response curves to SPER-NO (upper panel), ANP (middle panel) and CNP (lower panel) in aortic rings from eNOS KO animals in the presence and absence of ANP pretreatment (10^{-7} M; 30 min). Relaxation is expressed as mean ± s.e.m. percentage reversal of PE-induced tone. $P<0.05$, control *versus* ANP pretreatment for each ($n \geqslant 7$).

aorta to represent selective NPR-A and NPR-B agonists, respectively.

Having established the site of action of ANP and CNP to relax the mouse aorta, the effects of chronic *versus* acute NO deficiency on both sGC and pGC signalling were investigated. In vessels from eNOS KO mice, responses to NPR-A and NPR-B activation were both enhanced, as were relaxations to NO (as we have shown previously; Hussain *et al*., 1999). In a similar fashion, following acute inhibition of NO synthesis with L-NAME, responses to SPER-NO, ANP and CNP were accentuated. These data suggest that loss of endothelial-derived NO results in upregulation of both the NPR-A and NPR-B pGC signalling pathways. The similar increase in activity following chronic and acute NO deficiency also implies that the mechanism of feedback regulation is a rapid, biochemical change as opposed to a more prolonged, expressional alteration.

The changes observed in responses to ANP and CNP during NO deficiency could be mediated *via* direct actions of NO or be dependent on sGC activation and cGMP production. To differentiate between these putative mechanisms, ODQ was utilised to selectively inhibit sGC without affecting the ambient NO concentration or the pGC systems. Following incubation of WT tissues with ODQ, sensitivity to ANP and CNP was increased, implicating cGMP, at least in part, in the feedback process.

In addition to upregulation of NPR-A- and NPR-B-mediated responses during chronic NO deficiency, we tested the hypothesis that these pathways would be downregulated under conditions of NO excess. We have reported previously that exposure of eNOS KO aorta to GTN decreases the sensitivity of the NO–sGC–cGMP system (Hussain *et al*., 1999). In the present study, following incubation with GTN, we observed a similar decrease of sensitivity in response to both ANP and CNP. These results suggest that the presence of increased NO also affects the vascular activity of ANP and CNP.

Biochemical studies have revealed that phosphorylation/dephosphorylation of NPR-A and NPR-B is important in self-regulating receptor activity (Potter & Garbers, 1992; Potter &

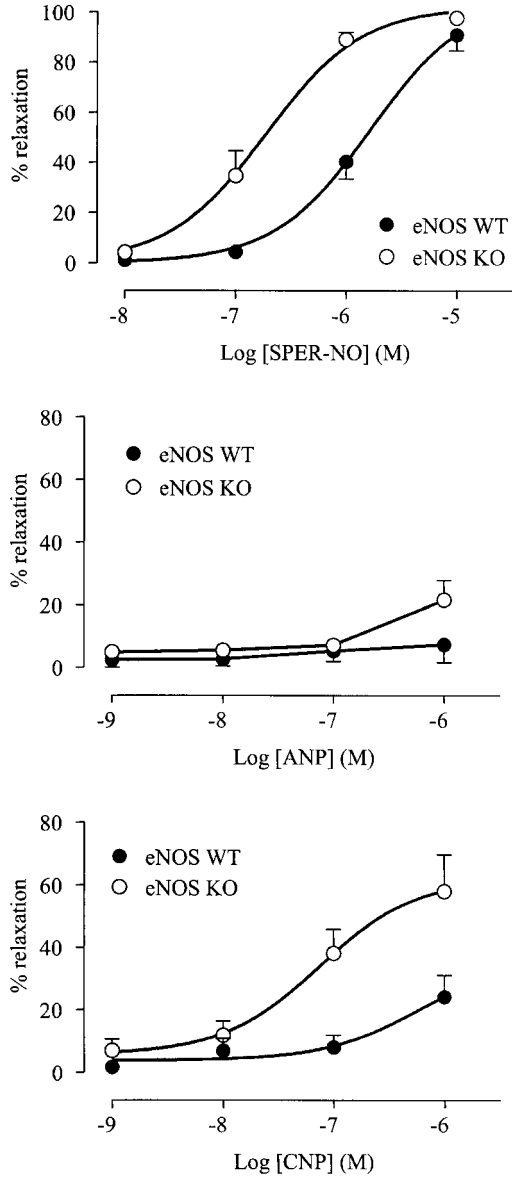

Figure 8 Concentration–response curves to SPER-NO (upper panel), ANP (middle panel) and CNP (lower panel) in mesenteric resistance arteries from WT and eNOS KO animals. Relaxation is expressed as mean ± s.e.m. percentage reversal of U46619-induced tone. $P<0.05$, WT *versus* eNOS KO for SPER-NO and CNP ($n\geqslant 4$).

Hunter, 1998). In order to investigate if these biochemical modifications have important functional consequences, we examined if high ambient ANP levels would affect subsequent challenge by NO, CNP and ANP itself (as we have demonstrated previously; Hussain *et al*., 2001). In this case, we found that following exposure to excess ANP, responses to NO, CNP and ANP were reduced. These observations suggest that in a reciprocal fashion, changes in NPR receptor activation modulate NO signalling. Moreover, NPR cross-desensitisation is likely to represent an important physiological phenomenon, coordinating the vascular actions of circulating ANP/BNP with the local effects of CNP. The mechanisms underlying this latter interaction remain unclear, but since phosphatase 2A appears important in mediating desensitisation of both NPR-A and NPR-B (Potter & Garbers, 1992; Potter, 1998) (at least at a biochemical level), activation of this phosphatase may be involved in the crossdesensitisation identified functionally in this study. However, phosphatase 2A has not been shown to alter the phosphorylation state of sGC, and alternative mechanisms (perhaps involving protein kinases A, C or G, which have been shown to phosphorylate sGC; Zwiller *et al*., 1981; 1985; Ferrero *et al*., 2000) are likely to be involved.

In concert, the above observations establish an important crosstalk between sGC, NPR-A and NPR-B in the mammalian vasculature. However, these findings were established for interaction between exogenously administered agents. To investigate this phenomenon from a more physiological standpoint, we examined whether exposure of vessels to high ambient concentrations of NO and/or ANP would result in a downregulation of responses to endogenous (endothelium-derived) NO. To achieve this, we assessed the potency of ACh in WT vessels exposed to supramaximal concentrations of NO (i.e. GTN) and ANP. Interestingly, the potency of endothelium-dependent relaxations elicited by ACh was significantly reduced following both interventions. Thus, under conditions associated with high ambient NO and/or ANP concentrations, as occurs in diseases such as sepsis and heart failure, responses to endothelial-derived NO are abrogated. This important observation is likely to have significant implications for the local regulation of vascular tone in pathologies characterised by overproduction of NO and/or ANP.

To determine if NO and ANP interact to regulate tone in other vascular beds, and in resistance vessels in addition to conduit arteries (i.e. aorta), we assessed the sensitivity of the sGC and pGC pathways in murine mesenteric small arteries. In this series of experiments, responses to NO, ANP and CNP were compared in mesenteric resistance vessels from WT and eNOS KO animals. SPER-NO and CNP elicited concentration-dependent relaxations that were significantly more potent in the vessels from eNOS KO animals, suggesting that the upregulation of sGC and pGC sensitivity occurs in response to chronic NO deficiency in the resistance vasculature. However, ANP was unable to cause relaxation of the murine mesenteric vessels in either the WT or KO tissues, suggesting that NPR-A receptors are not present in these vessels or that their activation is not linked to vasorelaxation. Nonetheless, cross-sensitisation between NO and natriuretic peptides (principally CNP) appears to be important and, since both these mediators are derived from the endothelium, regulation of blood flow in the resistance vasculature is likely to be a localised process (i.e. paracrine) rather than *via* the action of hormones (i.e. endocrine).

In conclusion, the current study demonstrates that both NPR-A- and NPR-B-linked pGC pathways are modulated by NO/cGMP in mouse aorta and that crossdesensitisation also occurs between NPR subtypes. Moreover, the biological activity of endothelium-derived NO is also influenced by the ambient concentration of NO and natriuretic peptides, providing further evidence that this heterologous feedback loop regulating the guanylate cyclase family of proteins is important in determining vascular tone and local blood flow. Such an autoregulatory pathway may represent an important physiological homeostatic mechanism and link the paracrine activity of NO and CNP with the endocrine functions of ANP and BNP in the regulation of vascular tone and blood pressure. Furthermore, this feedback regulation might com-

pensate for dysfunction of an alternate signalling pathway. For example, in cardiovascular diseases associated with endothelial dysfunction and deficiencies in NO production (i.e. hypertension and atherosclerosis), ANP/CNP-cGMP pathways could supplement the reduced activity of the NO–cGMP pathway. Conversely, in disease states associated with excessive circulating natriuretic peptide levels, for instance, during heart failure, a consequent downregulation of NO–sGC–cGMP signalling may help offset the systemic hypotension; such a process may also underlie the tachyphylaxis observed in natriuretic peptides in such disorders (in addition to NPR dephosphorylation). This mechanism may also contribute to the development of tolerance to organic nitrates, and be responsible for some of the classical symptoms of nitrate tolerance such as sodium retention and hypervolaemia as a result of diminished ANP activity.

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