British Journal of Pharmacology (1999) 128, 1082–1088
http://www.stockton-press.co.uk/bjp

Autoregulation of nitric oxide-soluble guanylate cyclase-cyclic GMP signalling in mouse thoracic aorta

[1,2]Monira B. Hussain, *[,1]Adrian J. Hobbs & [2]Raymond J. MacAllister

[1]Wolfson Institute for Biomedical Research, University College London, The Rayne Institute, 5 University Street, London WC1E 6JJ and [2]Centre for Clinical Pharmacology, University College London, The Rayne Institute, 5 University Street, London WC1E 6JJ

1 The sensitivity of the soluble guanylate cyclase (sGC)-cyclic guanosine-3′,5′-monophosphate (cyclic GMP) system to nitric oxide (NO) was investigated in mouse aorta from wild type (WT) and NO synthase (NOS) knockout (KO) animals.

2 The NO donor, spermine-NONOate (SPER-NO) was more potent in aortas from eNOS KO mice compared to WT (pEC_{50} 7.30 ± 0.06 and 6.56 ± 0.04, respectively; $n=6$; $P<0.05$). In contrast, the non-NO based sGC activator, YC-1 was equipotent in vessels from eNOS WT and KO mice. The sensitivity of aortas from nNOS and iNOS KO animals to SPER-NO was unchanged. Forskolin (an adenylate cyclase activator), was equipotent in vessels from eNOS WT and KO animals.

3 The cyclic GMP analogue, 8-Br-cGMP was equipotent in eNOS WT and KO mice (pEC_{50} 4.38 ± 0.04 and 4.40 ± 0.05, respectively; $n=5$; $P>0.05$). Zaprinast (10^{-5} M) a phosphodiesterase type V (PDE V) inhibitor, had no effect on the response to SPER-NO in vessels from eNOS WT or KO mice.

4 The NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 3×10^{-4} M) increased the potency of SPER-NO in aortas from WT mice (pEC_{50} 6.64 ± 0.02 and 7.37 ± 0.02 in the absence and presence of L-NAME, respectively; $n=4$; $P<0.05$).

5 In summary, there is increased sensitivity of vessels from eNOS KO animals to NO. Cyclic AMP-mediated dilatation is unchanged, consistent with a specific up-regulation of sGC–cyclic GMP signalling. The functional activity of cyclic GMP-dependent protein kinase (G-kinase) and PDE V was also unchanged, suggesting that sGC is the site of up-regulation. These alterations in the sensitivity of the sGC–cyclic GMP pathway might represent a mechanism for the dynamic regulation of NO bioactivity.

Keywords: eNOS knockout mice; nitric oxide; YC-1; soluble guanylate cyclase; feedback regulation; cyclic GMP

Abbreviations: cyclic GMP, cyclic guanosine-3′,5′-monophosphate; eNOS, endothelial nitric oxide synthase; G-kinase, cyclic GMP-dependent protein kinase; GTP, guanosine-5′-triphosphate; iNOS, inducible nitric oxide synthase; KO, knockout; L-NAME, N^G-nitro-L-arginine methylester; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PE, phenylephrine; sGC, soluble guanylate cyclase; SPER-NO, spermine-NONOate; WT, wild type; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole; ODQ, 1H-[1,2,4]oxadiazolol[4,3,a]quinoxa-lin-1-one

Introduction

Activation of the haemoprotein soluble guanylate cyclase (sGC) by nitric oxide (NO) facilitates the conversion of guanosine-5′-triphosphate (GTP) to the intracellular second messenger cyclic guanosine-3′,5′-monophosphate (cylic GMP). In turn, cyclic GMP modulates cellular function *via* interaction with cyclic GMP-dependent protein kinases, cyclic GMP-binding phosphodiesterases or cyclic GMP-gated ion channels (Hobbs, 1997); as such, the NO-sGC–cyclic GMP system represents an important physiological signalling pathway, involved in regulating platelet reactivity, smooth muscle relaxation and non-adrenergic non-cholinergic neurotransmission. NO is generated endogenously by a family of enzymes termed NO synthases (NOS; Knowles & Moncada, 1994). NO synthesized by NOS present in vascular endothelial cells (eNOS) activates sGC located in the adjacent vascular smooth muscle cells to increase levels of cyclic GMP and induce relaxation; this mechanism is thought to play a major role in the regulation of vascular tone and blood pressure (Knowles & Moncada, 1994).

Most endogenous signalling pathways incorporate regulatory feedback loops which permit adaptation, in both a positive and negative fashion, to changes in the frequency of activation. This is exemplified by sympathetic neurotransmission, where there are pre- and post-synaptic mechanisms to alter activity (Langer, 1980, De Blasi, 1989, Bristow *et al*., 1989). In the case of NO-sGC–cyclic GMP signalling, a mechanism regulating the synthesis of NO has been identified; NO interacts with the haem iron of NOS, reducing its catalytic activity (Griscavage *et al*., 1994). Previous findings have hinted at the possibility that downstream (analogous to post-synaptic) mechanisms for autoregulation of NO–sGC signalling pathway also exist, whereby the sensitivity of the sGC–cyclic GMP system is influenced by the ambient NO concentration. Blood vessels *in vitro* or *in vivo* exhibit greater sensitivity to NO if endogenous synthesis of NO is reduced (by NOS inhibitors or endothelial denudation; Moncada *et al*., 1991; Kovach *et al*., 1992; Dowell *et al*., 1996). In addition, prolonged exposure of vascular smooth muscle to NO-donors *in vitro* and *in vivo* reduces the response of the system to subsequent challenge with NO (Waldman & Murad, 1987; Ljusegren *et al*., 1998). As a whole, these observations suggest that the

*Author for correspondence.

responsiveness of the sGC–cyclic GMP system is altered to compensate for changes in the production of NO. However, the mechanism(s) underlying these adaptations have not been defined. A full understanding of this autoregulatory loop may have important therapeutic implications for conditions in which there is inappropriate NO production and/or sGC activation such as septic shock, atherosclerosis, erectile impotence or tolerance following nitrate therapy.

Therefore, the present study has attempted to characterize regulatory aspects of the sGC–cyclic GMP system using tissues from wild type (WT) and eNOS knockout (KO) mice, the latter representing a model of chronic NO deficiency. The sensitivity of sGC was assessed using the NO-donor, spermine-NONOate (SPER-NO), and the non-NO based activator, YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole). G-kinase sensitivity in these vessels was assessed using the cell-permeable cyclic GMP analogue, 8-bromo cyclic GMP (8-Br-cyclic GMP). The functional activity of phosphodiesterase type V (PDE V) was determined using the selective inhibitor, zaprinast. To exclude non-specific effects of genetic manipulation, certain studies were repeated using forskolin (to test the sensitivity of cyclic AMP-mediated relaxation), or using vessels from iNOS and nNOS KO animals.

Methods

Male eNOS, nNOS and iNOS WT and KO mice (25–35 g; eNOS and nNOS, F1 hybrid between SV129 and C57BLK/6, Cardiovascular Research Center, Massachusetts General Hospital-East, USA; iNOS, C57BLK/6J, The Jackson Laboratory, Maine, U.S.A.) were stunned and killed by cervical dislocation. The thoracic aorta was dissected out, cleaned of connective tissue and cut into rings (3–4 mm wide). Aortic rings were suspended in 25 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. A resting tension of 0.3 *g* was applied to each tissue and changes in isometric tension measured using a force displacement transducer (FT03) connected to a Rikadenki chart recorder. The tissues were allowed to equilibrate for 60 min prior to experimentation. During this time tissues were washed three times, and the tension re-adjusted to baseline.

Tissues were primed with KCl (4.8×10^{-2} M) before a concentration (10^{-7} M) of phenylephrine (PE) producing a sub-maximal (70–85% of maximum) contraction was added. Once the response had stabilized (5 min) acetylcholine (10^{-6} M) was added to assess the endothelial integrity of WT vessels. If the contractions to PE were not maintained or relaxations >50% of the PE induced tone to acetylcholine were not observed, the tissues were discarded. Endothelial integrity of the aortic rings from eNOS KO mice was not tested functionally. However, during processing of vessels, 96% of all rings from WT animals were endothelium-intact; therefore we have assumed that a similar proportion of rings from KO animals will also have an intact endothelium.

Tissues were washed over 30 min after which a cumulative concentration-response curve to PE was constructed. The tissues were then washed over 60 min to restore basal tone before contracting to approximately 80% of the maximum PE-induced response. Once a stable response to PE was obtained, concentration-response curves to SPER-NO (10^{-9}M–10^{-5} M), 8-Br-cyclic GMP (10^{-6} M–3×10^{-4} M), YC-1 (3×10^{-8} M–3×10^{-5} M) or forskolin (3×10^{-9} M–10^{-6} M) were obtained.

To assess if the responses to SPER-NO and YC-1 were mediated by sGC, concentration-response curves to these agents were carried out in the WT tissues before and in the presence of the sGC inhibitor, ODQ (1H-[1,2,4]oxadiazolol [4,3,a]quinoxalin-1-one; 5×10^{-6} M; 30 min incubation) and compared to a time-matched control. The specificity of ODQ was tested by carrying out concentration-response curve to forskolin in WT tissues in the presence of the same concentration of ODQ.

To investigate the functional activity of PDE V, concentration-response curves to SPER-NO were obtained in the presence of the PDE V inhibitor zaprinast (10^{-5} M; 30 min incubation), and the effects compared with a time-control (carried out in tissues from the same animals). To study the effects of acute NOS inhibition, concentration-response curves to SPER-NO were carried out in WT tissues in the presence of the NOS inhibitor N^G-nitro-L-arginine methylester (L-NAME; 3×10^{-4} M; 30 min incubation) and the effects compared with a time control.

Data analysis

Relaxations are expressed as a percentage reversal of the PE-induced tone. Responses are plotted graphically as means from at least four separate experiments with vertical bars representing standard error of the mean ± s.e. mean. Curves were fitted to all the data by non-linear regression using Prism (GraphPAD software San Diego, California, U.S.A.) to calculate pEC_{50} values (−log of the EC_{50} values). pEC_{50} values were used to compare the relaxant effects of the drugs.

A *t*-test was used to assess the significance of differences between pEC_{50} values. A P value <0.05 was taken to indicate a statistically significant difference. Statistical analysis was performed using Prism software.

Drugs

Phenylephrine hydrochloride, acetylcholine chloride, 8-Br-cyclic GMP, forskolin, L-NAME and zaprinast were obtained from Sigma (Poole, Dorset, U.K.). Spermine-NONOate ([N-(2-Aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine; SPER-NO) was obtained from Calbiochem (Nottingham, U.K.). ODQ and YC-1 were synthesized by the Medicinal Chemistry Department at the Wolfson Institute for Biomedical Research.

Preparation of all stock solutions and their subsequent dilution were made using distilled water. Exceptions to this were zaprinast, ODQ and YC-1 which were dissolved in DMSO. Control responses to DMSO alone were conducted to allow for the possibility of non-specific solvent effects (data not shown).

Results

PE produced concentration-dependent contractions of the mouse thoracic aorta with similar pEC_{50} values in both WT and eNOS KO animals (pEC_{50} 7.12 ± 0.05 and 7.15 ± 0.06 in WT and KO, respectively; 126 observations from $n=60$ animals; $P>0.05$). The maximum contractions produced by PE were greater in tissues from eNOS KO mice (1.00 ± 0.03 g) compared to WT (0.80 ± 0.02 g; 126 observations from $n=60$ animals; $P<0.001$). Acetylcholine relaxed pre-contracted vessels from WT mice by 68.1 ± 0.93% (126 observations from $n=60$ animals).

Effects of NOS gene disruption on the sensitivity of the sGC-cyclic GMP pathway

The NO-donor, SPER-NO produced concentration-dependent relaxations of the PE pre-contracted aortic rings from eNOS WT and KO mice, but was significantly more potent in KO animals (pEC_{50} 6.56 ± 0.04 and 7.30 ± 0.06 in WT and KO, respectively; $n=6$; $P<0.05$; Figure 1a). In contrast, the non NO-based sGC activator, YC-1 was equipotent on aorta from WT and eNOS KO animals (pEC_{50} 6.36 ± 0.04 and 6.23 ± 0.06, in eNOS WT and KO, respectively; $n=5$; $P>0.05$; Figure 1b). Concentration-response curves to both SPER-NO and YC-1 were shifted to the right in the presence of the sGC inhibitor ODQ (SPER-NO pEC_{50} 6.64 ± 0.04 and 5.12 ± 0.07; YC-1 pEC_{50} 6.16 ± 0.04 and 5.29 ± 0.05 in the absence and presence of ODQ, respectively; both $n=6$; $P<0.05$; Figure 2a,b).

SPER-NO was equipotent in aorta from nNOS WT and KO (pEC_{50} 6.87 ± 0.04 and 6.83 ± 0.04, respectively; $n=4$; $P>0.05$; Figure 3a) and iNOS WT and KO animals (pEC_{50} 6.76 ± 0.05 and 6.69 ± 0.04, respectively; $n=6$; $P>0.05$; Figure 3b). Relaxations to the adenylate cyclase activator, forskolin were similar in WT and eNOS KO animals (pEC_{50} 7.24 ± 0.02 and 7.18 ± 0.04 respectively; $n=8$; $P>0.05$; Figure 3c); these responses were not affected by a concentration of ODQ (5×10^{-6} M) which significantly inhibited relaxations to both SPER-NO and YC-1 (pEC_{50} 7.58 ± 0.04 and 7.55 ± 0.02 in the absence and presence of ODQ, respectively; $n=4$; $P>0.05$).

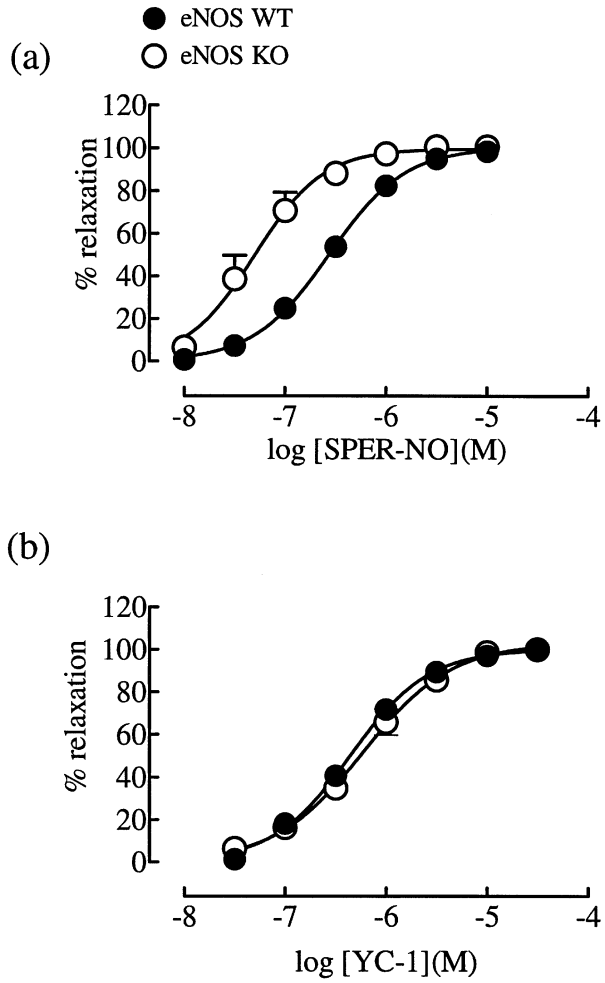

Figure 1 Concentration-response curves to SPER-NO (a) $n=6$ and YC-1 (b) $n=5$ in PE pre-contracted aortic rings from WT and eNOS KO mice. Relaxation is expressed as the mean $\pm$ s.e.mean percentage reversal of the PE induced tone. SPER-NO, but not YC-1, was more potent in vessels from eNOS KO animals.

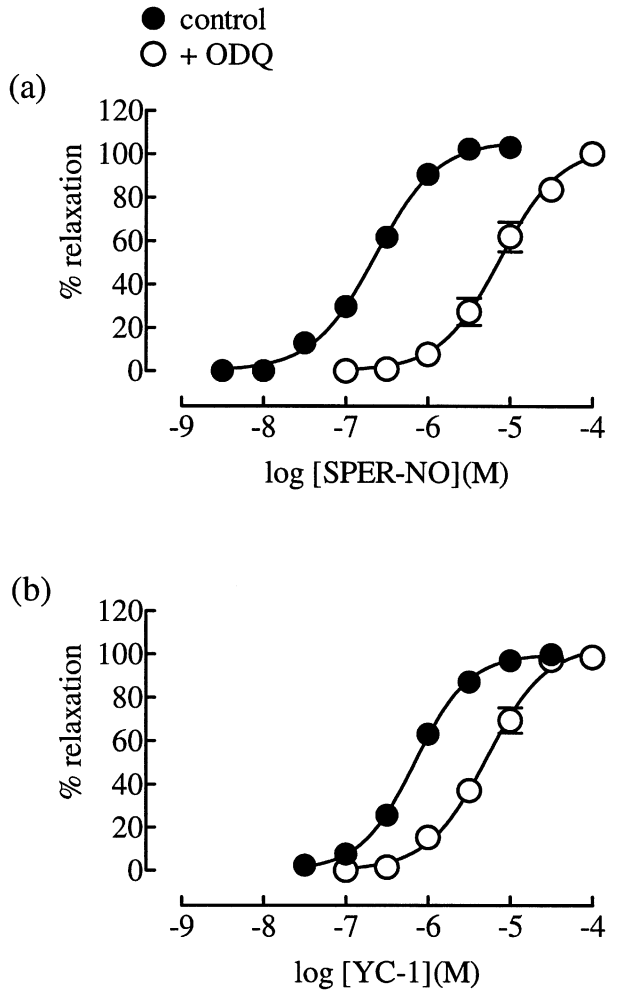

Figure 2 Concentration-response curves to SPER-NO (a) $n=6$ and YC-1 (b) $n=6$ in PE pre-contracted aortic rings from WT mice in the absence and presence of ODQ (5×10^{-6} M). Relaxation is expressed as the mean $\pm$ s.e.mean percentage reversal of the PE induced tone. ODQ inhibited the responses to both SPER-NO and YC-1.

Assessment of G-kinase and PDE V activity

The cell-permeable cyclic GMP analogue, 8-Br-cyclic GMP was equipotent in aorta from WT and eNOS KO mice (pEC_{50} 4.38 ± 0.04 and 4.40 ± 0.05, respectively; $n=5$; $P>0.05$; Figure 4). Incubation of vessels with zaprinast (10^{-5} M) had no effect on the responses to SPER-NO. In the absence and presence of zaprinast, the pEC_{50} values were 6.74 ± 04 and 6.75 ± 0.07, respectively in WT ($n=5$; $P>0.05$) and 7.31 ± 0.04 and 7.28 ± 0.03, respectively in KO mice ($n=5$; $P>0.05$; Figure 5).

Acute effect of eNOS inhibition in vessels from WT animals

Incubation of tissues from WT animals with L-NAME (3×10^{-4} M; 30 min) produced a 5 fold leftward shift of the SPER-NO concentration-response curve. The pEC_{50} values were 6.64 ± 0.02 before and 7.37 ± 0.02 in the presence of L-NAME ($n=4$; $P<0.05$; Figure 6). The potency of SPER-NO in aortas from eNOS KO animals was similar to that in WT animals in the presence of L-NAME.

Discussion

The present study demonstrates that vascular smooth muscle from eNOS knockout mice exhibits increased sensitivity to the NO-donor SPER-NO. This is consistent with increased sensitivity of the sGC-cyclic GMP pathway in chronic vascular NO deficiency. YC-1, a non NO-based activator of sGC that causes cyclic GMP-dependent vascular smooth muscle relaxation, was equipotent in vessels from WT and KO animals. This finding suggests that there is a specific increase in the responsiveness of sGC to NO. However, changes in the sensitivity of other aspects of this signalling cascade do not appear to occur. 8-Br-cyclic GMP was equipotent in vessels from WT and KO mice, suggesting equivalent responsiveness of G-kinase to cyclic GMP. In addition, the lack of effect of zaprinast on the response of these vessels to SPER-NO implicates low levels of PDE V activity. Incubation of vessels from WT animals with the NOS inhibitor, L-NAME mimicked the increase in sensitivity of vessels to SPER-NO that occurred in eNOS KO animals. This observation suggests that up-regulation of sGC–cyclic GMP signalling can occur rapidly (within minutes) in response to a reduction in NO levels.

Previous studies have suggested that the sensitivity of blood vessels to NO and NO donors can be modified by the prevailing NO concentration. Vessels that are denuded of the endothelium or exposed to NOS inhibitors are more sensitive to exogenous NO (Moncada *et al.*, 1991; Dowell *et al.*, 1996); conversely, exposure of vessels to NO donors reduces subsequent responsiveness to exogenous NO (Waldman & Murad, 1987; Ljusegren *et al.*, 1988). Under such conditions, changes in the sensitivity of the sGC–cyclic GMP system might compensate for altered concentrations of NO. This might be achieved by several mechanisms including modification of the sensitivity of sGC to NO (Papapetropoulos *et al.*, 1996a), altered expression of sGC (Papapetropoulos *et al.*, 1996b;) or G-kinase (Soff *et al.*, 1997), or changes in the activity of PDE V (Holzmann *et al.*, 1996). The current investigation has attempted to identify which aspects of sGC–cyclic GMP signalling might adjust to bring about these changes in the sensitivity of the pathway as a whole.

To investigate these mechanisms in vascular smooth muscle the present study employed aortas from eNOS KO as a model for chronic vascular NO deficiency. SPER-NO, which releases NO spontaneously in aqueous solution (Morley *et al.*, 1993), was a more potent relaxant of pre-contracted aortic rings from eNOS KO mice compared with their WT controls. This was specific for vessels with reduced eNOS-generated NO because SPER-NO was equipotent on vessels from WT and iNOS or

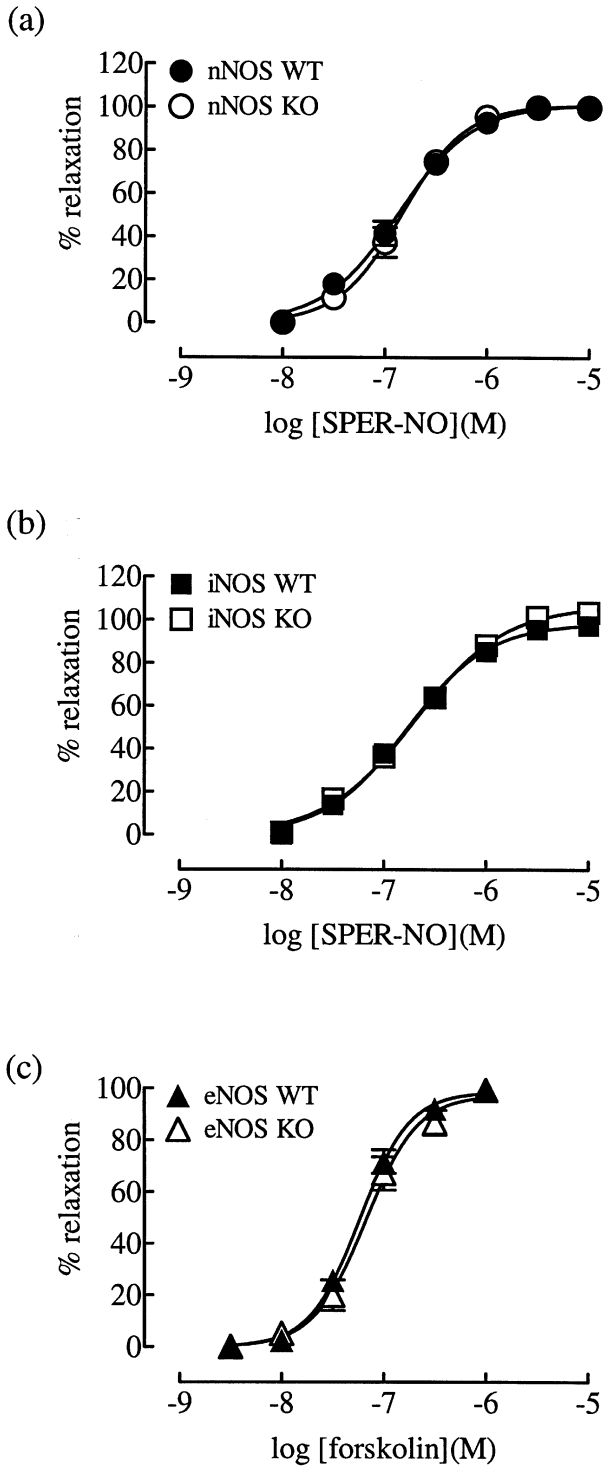

Figure 3 (a,b) Concentration-response curves to SPER-NO in PE pre-contracted aortic rings from (a) nNOS WT and KO mice (both $n=4$) and (b) iNOS WT and KO mice (both $n=6$). (c) Concentration-response curves to forskolin ($n=8$) in PE pre-contracted aortic rings from eNOS WT and KO mice. Relaxation is expressed as the mean ± s.e.mean percentage reversal of the PE induced tone. The responses to the relaxants were similar in vessels from WT and KO animals.

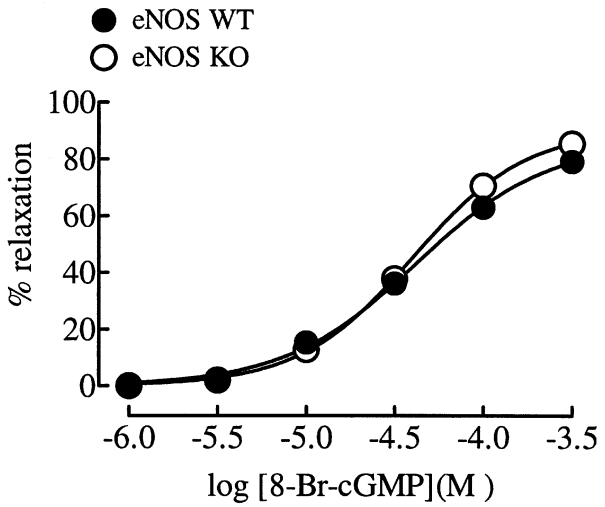

Figure 4 Concentration-response curves to 8-Br–cyclic GMP ($n=5$) in PE pre-contracted aortic rings from WT and eNOS KO mice. Relaxation is expressed as the mean ± s.e.mean percentage reversal of the PE induced tone. 8-Br–cyclic GMP was equipotent in vessels from eNOS WT and KO animals.

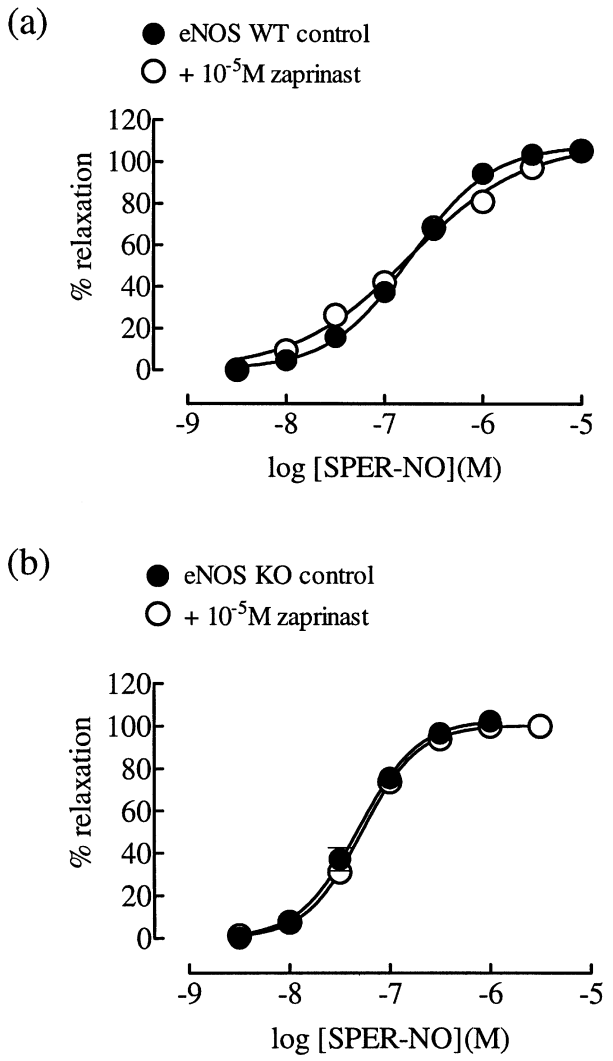

Figure 5 Concentration-response curves to SPER-NO in PE pre-contracted aortic rings from (a) eNOS WT ($n=5$) and (b) eNOS KO ($n=5$) in the absence and presence of zaprinast (10^{-5} M). Relaxation is expressed as the mean ± s.e.mean percentage reversal of the PE induced tone. Incubation with zaprinast for 30 min had no effect on the responses to SPER-NO.

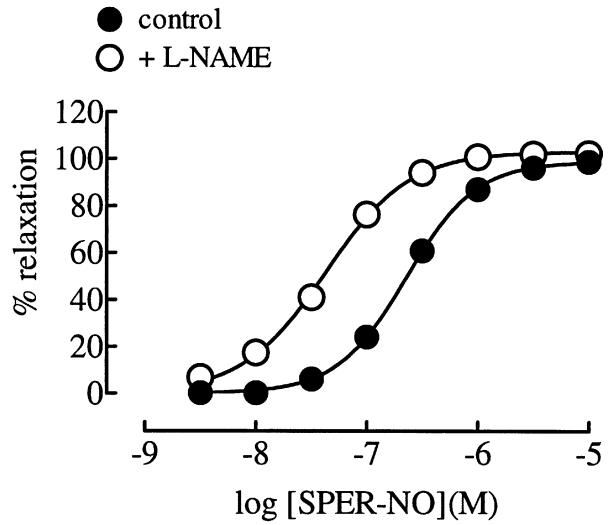

Figure 6 Concentration-response curves to SPER-NO in WT mouse aortic rings in the absence and presence (both $n=4$) of L-NAME (3×10^{-4} M). Relaxation is expressed as the mean ± s.e.mean percentage reversal of the PE induced tone. Following incubation with L-NAME for 30 min, there was an increase in the potency of SPER-NO.

nNOS KO mice (thereby also confirming that genetic manipulation was not responsible for the differences). Moreover, the increase in sensitivity was specific to the sGC–cyclic GMP pathway because forskolin, which causes vascular smooth muscle relaxation through activation of adenylate cyclase, was equipotent on vessels from WT and eNOS KO mice. These observations established the validity of this model in which to explore the mechanism of altered responsiveness to NO.

To explore the site(s) in the sGC–cyclic GMP pathway that might be responsible for the observed increase in sensitivity to NO, the responses of vessels from WT and eNOS KO mice to YC-1 were compared. YC-1 activates sGC by an unknown mechanism, which may involve stabilization of the active conformer of the enzyme, but causes smooth muscle relaxation *via* the generation of cyclic GMP in a similar fashion to NO (Mulsch *et al*., 1997; Friebe & Koesling, 1998) In the present study, the sGC inhibitor ODQ reduced the potency of YC-1 and SPER-NO, suggesting that relaxations caused by both agents were mediated, at least in part, *via* activation of sGC. However, in contrast to SPER-NO, YC-1 was equipotent in vessels from WT and eNOS KO animals. Assuming that the pathways downstream from sGC respond similarly to rises in cyclic GMP irrespective of the mechanism of activation of sGC (i.e. by NO or YC-1), these data indicate that the activities of G-kinase or PDE V are unlikely to account for the increased responsiveness of vessels from eNOS KO mice to NO.

Previous studies have indicated that there is reduced activity and expression of G-kinase associated with increased stimulation of the sGC–cyclic GMP pathway in vascular and non-vascular smooth muscle cells (Soff *et al*., 1997). To investigate specifically the involvement of G-kinase in regulating the sensitivity of sGC–cyclic GMP signalling, the activity of this enzyme was examined functionally in vessels from WT and eNOS KO mice using 8-Br-cyclic GMP (a cell permeable analogue of cyclic GMP that is resistant to hydrolysis by PDE V but which activates G-kinase) to cause vasorelaxation. 8-Br-cyclic GMP was equipotent on vessels from both WT and eNOS KO animals, consistent with equivalent activity of G-kinase in both types of animals.

A second determinant of cyclic GMP-mediated relaxation is the rate of hydrolysis of cyclic GMP to inactive 5′-GMP. PDE V is thought to be the most important cyclic nucleotide phosphodiesterase in regulating cyclic GMP levels in vascular smooth muscle (Rabe *et al*., 1994). This isozyme can be inhibited in a relatively selective manner by zaprinast (Liu *et al*., 1992, Harris *et al*., 1989, Thusu *et al*., 1995). In the present investigation, zaprinast had no effect on the sensitivity of vessels to SPER-NO in both the WT and eNOS KO mice. The inability of zaprinast to alter responses to SPER-NO is consistent with low PDE V activity in the mouse aorta or that alternative PDE isoforms are important in modifying cyclic GMP responses in this tissue (e.g. PDE III; Rabe *et al*., 1994). It is also possible that the concentration of zaprinast was insufficient to cause significant blockade of PDE V. However, the concentration we used (10^{-5} M) has been shown previously to inhibit PDE V in other vessel types. In conjunction with the YC-1 data, this suggests that PDE V activity is not likely to account for the differences in the sensitivity of vessels from WT and eNOS KO mice to NO donors.

To compare the effects of chronic versus acute NO deficiency on the responsiveness of the sGC–cyclic GMP system to NO vessels from WT animals were incubated with the NOS inhibitor, L-NAME (Rees *et al*., 1990). Following a 30 min incubation, WT vessels developed equivalent super-

sensitivity to NO as was observed in eNOS KO animals. These observations are consistent with increased activity of constitutive sGC rather than induction of sGC protein in NO-deficient vessels, a conclusion that is also supported by the lack of increased potency of YC-1 in vessels from eNOS KO animals. However, there is evidence from studies on smooth muscle cells in culture that sGC expression is down-regulated by NO donors (Fillippov *et al*., 1997; Scott & Nakayama, 1998). To determine if analogous mechanisms might contribute to the altered sensitivity of the sGC–cyclic GMP pathway to NO observed in the present study, it will be necessary to study sGC mRNA and protein expression in these tissues.

The effect of ODQ on the relaxation to SPER-NO is consistent with a major role for sGC–cyclic GMP signal transduction in response to NO in mouse aorta. However, involvement of other mechanisms in the change of sensitivity of the smooth muscle to NO cannot be excluded. Cyclic GMP-independent mechanisms of relaxation to NO donors have been implicated in rabbit (Weisbrod *et al*., 1998) and rat (Van der Zypp & Majewski, 1998) vessels, perhaps through activation of potassium channels (Wu *et al*., 1999). Whether such mechanisms also contribute to NO-mediated relaxation of mouse aorta remains to be determined.

In summary, there is increased sensitivity of blood vessels from eNOS KO mice to NO donors. Up-regulation seems to occur at the level of sGC because neither functional activity of G-kinase or PDE was changed in aortas from eNOS KO mice. The lack of any increase in the sensitivity to YC-1, and the time-course of up-regulation following acute NOS inhibition, suggests that there is increased sensitivity of constitutive sGC to NO. These alterations in the sensitivity of the sGC–cyclic GMP pathway might represent an important physiological mechanism for the dynamic regulation of NO-mediated responses in the vasculature or other systems. In addition, in diseases where there is inappropriate release NO, autoregulation of sGC–cyclic GMP signalling might be a compensatory homeostatic mechanism; e.g. in vascular sepsis this mechanism might limit excessive vasodilatation without interfering with non-cyclic GMP-mediated effects of NO (such as killing of pathogens; see Vallance & Moncada, 1993). Moreover, failure of autoregulation of the sGC–cyclic GMP system might contribute to the pathophysiology of disease states associated with abnormal NO production. Autoregulation of sGC–cyclic GMP signalling might also have therapeutic implications, by contributing to the development of tolerance to NO-donors or rebound vasoconstriction after cessation of NO-replacement therapy (Warren & Higenbottam, 1996). The results of the present study suggest that tolerance of sGC might be specific for NO, and might not occur with non-NO based activators of sGC. This class of compound might have therapeutic advantages over currently available NO donors.

A.J. Hobbs is a recipient of a Wellcome Trust Career Development Fellowship. This work was supported by a project grant from the British Heart Foundation.

References

BRISTOW, M.R., PORT, J.D., HERSHBERGER, R.E., GILBERT, E.M. & FELDMAN, A.M. (1989). The beta-adrenergic receptor-adenylate cyclase complex as a target for therapeutic intervention in heart failure. *Eur. Heart J.,* **10,** 45–54.

DE BLASI, A. (1989). Advances on beta-adrenergic receptors. Molecular structure and functional regulation. *Am. J. Hypertens.,* **2,** S252–S256.

DOWELL, F.J., HENRION, D., DURIEZ, M. & MICHEL, J-B. (1996). Vascular reactivity in mesenteric arteries following chronic nitric oxide synthase inhibition in Wistar rats. *Br. J. Pharmacol.,* **117,** 341–346.

FILIPPOV, G., BLOCH., D.B. & BLOCH, K.D. (1997). Nitric oxide decreases stability of mRNAs encoding soluble guanylate cyclase subunits in rat pulmonary artery smooth muscle cells. *J. Clin. Invest.,* **100,** 942–948.

FRIEBE, A. & KOESLING, D. (1998). Mechanism of YC-1-induced activation of soluble guanylyl cyclase. *Mol. Pharmacol.,* **53,** 123–127.

GRISCAVAGE, J.M., FUKUTO, J.M., KOMORI, Y. & IGNARRO, L.J. (1994). Nitric oxide inhibits neuronal nitric oxide synthase by interaction with the heme prosthetic group. Role of tetrahydrobiopterin in modulating the inhibitory action of nitric oxide. *J. Biol. Chem.,* **269,** 21644–21649.

HARRIS, A.L., LEMP, B.M., BENTLEY, R.G., PERRONE, M.H., HAMEL, L.T. & SILVER, P.J. (1989). Phosphodiesterase isoenzyme inhibition and the potentiation by zaprinast of endothelium derived relaxing factor and guanylate cyclase stimulating agents in vascular smooth muscle. *J. Pharmacol. Exp. Ther.,* **249,** 394–400.

HOBBS, A.J. (1997). Soluble guanylate cyclase: the forgotten sibling. *Trends Pharmacol. Sci.,* **18,** 484–491.

HOLZMANN, A., BLOCH, K.D., SANCHEZ, L.S., FILIPPOV, G. & ZAPOL, W.A. (1996). Hyporesponsiveness to inhaled nitric oxide in isolated perfused lungs from endotoxin challenged rats. *Am. J. Physiol.,* **271,** L981–L986.

KNOWLES, R.G. & MONCADA, S. (1994). Nitric oxide synthases in mammals. *Biochem. J.,* **298,** 249–258.

KOVACH, A.G., SZABO, C., BENYO, Z., CSAKI, C., GREENBERG, J.H. & REIVICH, M. (1992). Effects of N^G-nitro-L-arginine and L-arginine on regional cerebral blood flow in the cat. *J Physiol.,* **449,** 183–196.

LANGER, S.Z. (1980). Presynaptic regulation of the release of catecholamines. *Pharmacol. Rev.,* **32,** 337–362.

LIU, S.F., CRAWLEY, D.E., ROHDE, J.A., EVANS, T.W. & BARNES, P.J. (1992). Role of nitric oxide and guanosine 3′,5′-cyclic monophosphate in mediating nonadrenergic, noncholinergic relaxation in guinea-pig pulmonary arteries. *Br. J. Pharmacol.,* **107,** 861–866.

LJUSEGREN, M.E., AHLNER, J. & AXELSSON, K.L. (1988). Studies on vascular smooth muscle tolerance to different cGMP-mediated vasodilators and cross-tolerance to glyceryl trinitate. *Pharmacol. Toxicol.,* **62,** 302–307.

MONCADA, S., REES, D.D., SCHULTZ, R. & PALMER, R.M.J. (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.

MORLEY, D., MARAGOS, C.M., ZHANG, X.Y., BOIGNON, M., WINK, D.A. & KEEFER, L.K. (1993). Mechanism of vascular relaxation induced by the nitric oxide (NO)/nucleophile complexes, a new class of NO-based vasodilators. *J. Cardiovasc. Pharmacol.,* **21,** 670–676.

MULSCH, A., BAUERSACHS, J., SCHAFER, A., STASCH, J.P., KAST, R. & BUSSE, R. (1997). Effect of YC-1, an NO-independent, superoxide-sensitive stimulator of soluble guanylyl cyclase, on smooth muscle responsiveness to nitrovasodilators. *Br. J. Pharmacol.,* **120,** 681–689.

PAPAPETROPOULOS, A., ABOU-MOHAMED, G., MARCZIN, N., MURAD, F., CALDWELL, R.W. & CATRAVAS, J.D. (1996a). Down-regulation of nitrovasodilator-induced cyclic GMP accumulation in cell exposed to endotoxin or interleukin-1β. *Br. J. Pharmacol.,* **118,** 1359–1366.

PAPAPETROPOULOS, A., GO, C.Y., MURAD, F. & CATRAVAS, J.D. (1996b). Mechanism of tolerance to sodium nitroprusside in rat cultured aortic smooth muscle cells. *Br. J. Pharmacol.,* **117,** 147–155.

RABE, K.F., TENOR, H., DENT, G., SCHUDT, C., NAKASHIMA, M. & MAGNUSSEN, H. (1994). Identification of PDE isozymes in human pulmonary artery and effect of selective PDE inhibitors. *Am. J. Physiol.,* **266,** L536 – L543.

REES, D.D., PALMER, R.M., SCHULTZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.,* **101,** 746 – 752.

SCOTT, W.S. & NAKAYAMA, D.K. (1998). Sustained nitric oxide exposure decreases soluble guanylate cyclase mRNA and enzyme activity in pulmonary artery smooth muscle. *J. Surg. Res.,* **79,** 66 – 70.

SOFF, G.A., CORNWELL, T.L., CUNDIFF, D.L., GATELY, S. & LINCOLN, T.M. (1997). Smooth muscle cell expression of type I cyclic GMP dependent Protein Kinase is suppressed by continuous exposure to nitrovasdilators, theophylline, cyclic GMP and cyclic AMP. *J. Clin. Invest.,* **100,** 2580 – 2587.

THUSU, K.G., MORIN, F.C. III, RUSSELL, J.A. & STEINHORN, R.H. (1995). The cGMP phosphodiesterase inhibitor zaprinast enhances the effect of nitric oxide. *Am. J. Respir. Crit. Care Med.,* **152,** 1605 – 1610.

VALLANCE, P. & MONCADA, S. (1993). The role of endogenous nitric oxide in septic shock. *New Horizons,* **1,** 77 – 86.

VAN DER ZYPP, A. & MAJEWSKI, H. (1998). Effect of cGMP inhibitors on the actions of nitrovasdilators in rat aorta. *Clin. Exp. Pharmacol. Physiol.,* **25,** 38 – 43.

WALDMAN, S.A. & MURAD, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.,* **39,** 163 – 196.

WARREN, J.B. & HIGENBOTTAM, T. (1996). Caution with use of inhaled nitric oxide. *Lancet,* **348,** 629 – 630.

WEISBROD, R.M., GRISWOLD, M.C., YAGHOUBI, M., KOMALAVILAS, P., LINCOLN, T.M. & COHEN, R.A. (1998). Evidence that additional mechanisms to cyclic GMP mediate the decrease in intracellular calcium and relaxation of rabbit aortic smooth muscle to nitric oxide. *Br. J. Pharmacol.,* **125,** 1695 – 1707.

WU, C.C., CHEN, S.J. & YEN, M.H. (1999). Cyclic GMP regulates cromakalim-induced relaxation in the rat aortic smooth muscle: role of cyclic GMP in K(ATP)-channel. *Life Sci.,* **64,** 2471 – 2478.

(Received April 26, 1999
Revised August 8, 1999
Accepted August 11, 1999)