British Journal of Pharmacology (2002) 135, 299–302
www.nature.com/bjp

SPECIAL REPORT

Lack of critical involvement of endothelial nitric oxide synthase in vascular nitrate tolerance in mice

[1]Ellen Q. Wang, [1]Woo-In Lee & *,[1]Ho-Leung Fung

[1]Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, SUNY, Buffalo, NY 14260-1200, U.S.A.

We examined the direct involvement of endothelial nitric oxide (eNOS) in nitrate tolerance using eNOS knockout (eNOS (−/−)) and wild-type (eNOS (+/+)) mice. Animals were treated with either nitroglycerin (NTG, 20 mg kg^{-1}s.c. 3 × daily for 3 days) or vehicle (5% dextrose, D5W), and nitrate tolerance was assessed *ex vivo* in isolated aorta by vascular relaxation studies and cyclic GMP accumulation. Western blot was performed to determine NOS expression after NTG treatment. In both the eNOS (−/−) and (+/+) mice, the EC_{50} from NTG concentration-response curve was increased by ~3 fold, and vascular cyclic GMP accumulation was similarly decreased after NTG pretreatment. Vascular tolerance did not lead to changes in eNOS protein expression in eNOS (+/+) mice. These results indicate that vascular nitrate tolerance was similarly induced in eNOS (−/−) and (+/+) mice, suggesting that eNOS may not be critically involved in nitrate tolerance development in mice.
British Journal of Pharmacology (2002) **135,** 299–302

Keywords: Nitrate tolerance; endothelial nitric oxide synthase; eNOS gene knockout; mice aorta; cyclic GMP

Abbreviations: CRC, concentration-response curve; D5W, 5% dextrose; eNOS (−/−), eNOS knockout mice; eNOS (+/+), eNOS wild-type mice; NOS, nitric oxide synthase; NTG, nitroglycerin

Introduction The rapid development of vascular tolerance toward organic nitrates like nitroglycerin (NTG) has been known for over a century. Various mechanisms have been put forth to explain this phenomenon (Fung & Bauer, 1994; Munzel *et al*., 1995). Recently, Munzel *et al*. (2000) found that nitrate tolerance, induced by NTG infusion in rats for 3 days, is accompanied by upregulation of endothelial nitric oxide synthase (eNOS), and uncoupling of this enzyme to produce excess superoxide. The uncoupling of NOS to produce superoxide has been reported for all three isoforms (Pou *et al*., 1992; Xia *et al*., 1998; Xia & Zweier, 1997). Thus, the possibility of NOS contribution to nitrate tolerance *via* superoxide production has been raised.

Recently, Abou-Mohamed *et al*. (2000) also found that *in vitro* exposure of rat aortic rings to NTG for 2 h induced vascular tolerance, which was partially reversed by L-arginine, but not by D-arginine. Although questions have been raised about the cross-consistency of these observations (MacAllister, 2000), the data nevertheless also suggested a role of eNOS in vascular nitrate tolerance.

These findings raised the question about the possible importance of the vascular endothelium in affecting the dilatory responses of NTG, a vasodilator that has been traditionally considered endothelium-independent. Consistent with this latter view, past studies have found that *in vivo* nitrate tolerance produced little, if any, cross-tolerance toward endothelium-dependent vasodilators such as acetylcholine in animals and in humans (Du *et al*., 1992; Namiki *et al*., 1991; Stewart *et al*., 1987). On the other hand, other studies (Caramori *et al*., 1998; Munzel *et al*., 1995) found that vascular nitrate tolerance is accompanied by attenuation of vascular endothelial response. Thus, the evidence for eNOS involvement in nitrate tolerance, either as a cause or as an effect, remains equivocal.

In an attempt to further understand the role of eNOS in nitrate tolerance *in vivo*, we examined the critical necessity for the presence of eNOS in producing this phenomenon, through the comparison of eNOS knockout (eNOS (−/−)) mice *vs* wild-type (eNOS (+/+)) controls by monitoring vascular relaxation and vascular cyclic GMP accumulation in mouse aorta after chronic *in vivo* NTG treatment.

Methods *Materials* NTG was obtained from Schwarz Pharma (Germany). NOS monoclonal antibodies against human eNOS, neuronal NOS (iNOS) and inducible NOS (iNOS) were obtained from Transduction Laboratories (San Diego, CA, U.S.A.). All other materials were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

In vivo *NTG tolerance induction* All procedures were performed according to protocols approved by the SUNY Institutional Animal Care and Use Committee. Adult wild-type C57BL/6 mice (eNOS (+/+)), weighing 20–30 g were obtained from Harlan (Indianapolis, IN, U.S.A.) and homozygous mutant mice lacking eNOS (eNOS (−/−)), were bred at the University at Buffalo from breeding pairs kindly provided by Dr P.L. Huang of the Massachusetts General Hospital. Animals received s.c. injections of either NTG (20 mg kg^{-1}) or vehicle (5% dextrose, D5W) 3 × daily for 3 days. Eight hours following the last injection, animals were sacrificed and thoracic aorta was collected for vascular relaxation studies (n=4–5), cyclic GMP measurements (n=4–7), and Western blotting (n=3).

*Author for correspondence; E-mail: hlfung@acsu.buffalo.edu

In vitro *vascular relaxation experiment* Procedures for tissue bath relaxation studies were carried according to Russell & Watts (2000), but 4 μM phenylephrine was used and the ring tension was adjusted to 1 g. The EC_{50} and the slope of the NTG concentration-response curve (CRC) were obtained using Graph-Pad Prism (Version 1.03).

In vitro *cyclic GMP measurement* Cyclic GMP accumulation was examined as previously described (Hasegawa *et al*., 1999), but in the presence of the endothelium. Following a 30 min equilibration, aortas were challenged with 1 μM NTG at 37°C for 30 s and cyclic GMP content in aortic sample was determined using a radioimmunoassay kit (Biomedical Technologies Inc., MA, U.S.A.).

Western blotting analysis Western blot analyses for all three isoforms of NOS were carried out as previously described (Kielbasa & Fung, 2000). The intensity of the eNOS bands was quantified by the Scion Image software from NIH.

Statistical analysis Data are presented as mean$\pm$s.e.mean. The differences in NTG CRC's were analysed using two-way ANOVA, and unpaired Student's *t*-test was used to determine the differences in the EC_{50} and the slope of the NTG CRC's. Statistical significance was declared at $P<0.05$.

Results *Effects of* in vivo *NTG treatment on vascular relaxation* Figure 1 shows that in both groups of animals, NTG pretreatment for 3 days exhibited a modest but similar rightward shift in NTG CRC. Although two-way ANOVA revealed that the NTG CRC's were not statistically different between D5W and NTG treatment ($P>0.05$), the EC_{50}'s from the NTG-treated groups were found to be significantly higher than the corresponding D5W controls in both the eNOS (+/+) and (−/−) mice. The EC_{50}'s of D5W- vs NTG-treated eNOS (+/+) mice were 10.4$\pm$2.1 and 25.4$\pm$2.3 nM, respectively ($P<0.05$), a shift of about 2.5 fold. In comparison, the EC_{50} values in the eNOS (−/−) mice were 1.37$\pm$0.21 and 4.33$\pm$1.14 nM for D5W- and NTG-treated animals, respectively ($P<0.01$), representing a 3.2 fold rightward shift in NTG CRC. The lack of significant difference in NTG CRC by two-way ANOVA was likely due to the modest shift in the CRC, and the inclusion of plateau regions of these curves in the analysis. In both the NTG and D5W pretreated animals, a leftward shift in NTG concentration-response curves (CRC) was observed in the eNOS (−/−) mice compared to the eNOS (+/+) controls. In addition, the slopes of the NTG CRC's from eNOS (−/−) mice were significantly steeper than the corresponding eNOS (+/+) mice for both NTG- and D5W-pretreated animals (NTG-pretreated: 1.92$\pm$0.15 vs 0.77$\pm$0.05, $P<0.01$; D5W-pretreated: 1.88$\pm$0.24 vs 0.89$\pm$0.16, $P<0.01$, for eNOS (−/−) and (+/+) mice, respectively). The steeper slopes of the CRC's observed with eNOS (−/−) mouse aortas are consistent with their higher sensitivity toward NTG-induced relaxation.

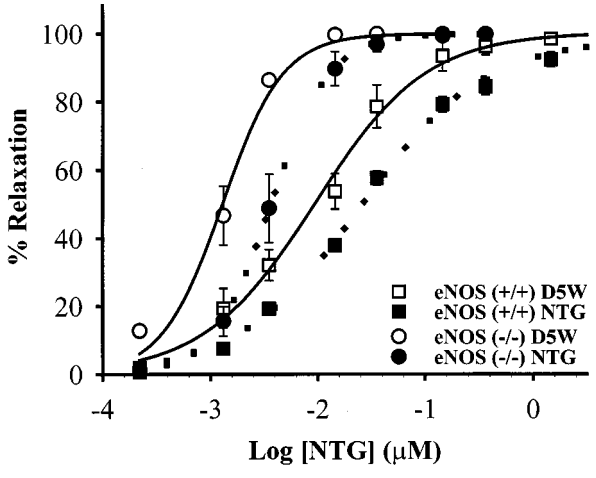

Figure 1 *Ex vivo* concentration vs response curves of isolated mouse aorta toward NTG in eNOS (+/+) and eNOS (−/−) mice after *in vivo* NTG (20 mg kg^{-1} 3$\times$daily for 3 days) or D5W control treatment. The dotted lines showed a rightward shift of the NTG-relaxation curves vs their respective controls, consistent with vascular tolerance development. Data are expressed as mean$\pm$s.e.mean, $n=4-5$ animals.

Effect of in vivo *NTG treatment on cyclic GMP accumulation in mouse aorta* In preliminary experiments using eNOS (+/+) mice, exposure of isolated aorta to 1 μM NTG for 30 s induced $\sim$ a 2 fold increase in *in vitro* cyclic GMP accumulation (25.9 pmol mg^{-1} protein and 46.4 pmol mg^{-1} protein for D5W- and NTG-challenged, respectively). In addition, this challenge dose has been shown to produce similar vascular cGMP accumulation in both eNOS (+/+) and (−/−) mice (eNOS (+/+): 51.0$\pm$12.1 vs eNOS (−/−): 59.2$\pm$7.1 pmol mg^{-1} protein, $P>0.05$). Thus, at this challenge regimen, there was no apparent difference in the total accumulation of cyclic GMP for both animal groups, in the absence of any tolerance induction. This challenge regimen was therefore used to determine the effects of *in vivo* NTG vs D5W treatment on vascular cyclic GMP accumulation *in vitro*. Figure 2 compares the *in vitro* cyclic GMP responses of aorta in the four groups of animals under examination. Cyclic GMP accumulation from mouse aorta after D5W treatment was not significantly difference between eNOS (+/+) vs eNOS (−/−) animals (bars marked A). In the NTG treated mice (bars marked B), NTG-induced vascular cyclic GMP accumulation was significantly attenuated from their respective treatment controls. The eNOS (−/−) mice exhibited a 59.2$\pm$16.5%

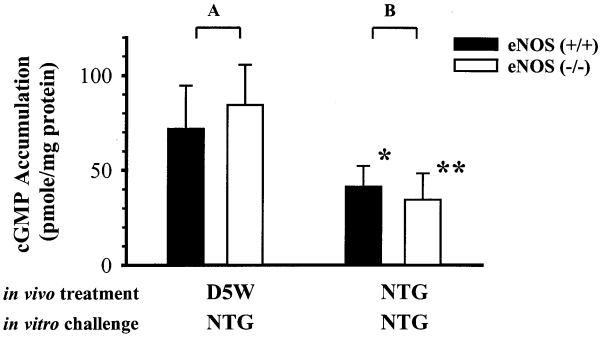

Figure 2 Effects of *in vivo* NTG treatment on *in vitro* cGMP accumulation in mouse aorta upon 1 μM NTG challenge in eNOS (+/+) and eNOS (−/−) animals. A Bars: D5W-treated, B Bars: NTG-treated. *$P<0.05$, **$P<0.01$ vs corresponding D5W treatment. data are expressed as mean$\pm$s.e.mean, $n=4-7$ animals.

decrease, compared to a 43.5±15.4% decrease exhibited by eNOS (+/+) mice. In separate experiments, baseline vascular cyclic GMP levels were also measured in the eNOS (−/−) mice. Consistent with the lack of endogenous NO production in the eNOS (−/−) mice, baseline cyclic GMP was significantly lower in the aorta of the eNOS (−/−) (1.9±0.3 pmol mg^{-1} protein) vs eNOS (+/+) mice (25.9±1.5 pmol mg^{-1} protein, $P<0.0001$).

Effect of in vivo *NTG treatment on NOS protein expression* Western blot analysis demonstrated that eNOS protein was present in the various tissues of the eNOS (+/+) mice, but absent in the eNOS (−/−) mice (data not shown). *In vivo* NTG treatment did not induce any apparent changes in eNOS protein expression in the eNOS (+/+) mice. Densitometric analysis of eNOS bands observed in the eNOS (+/+) mice indicated that the NTG-treated group did not differ in eNOS protein expression from D5W controls (89±15% of control, $P>0.05$, $n=3$ animals). No detectable nNOS and iNOS bands were observed in the aortas of both groups of animals treated with either D5W or NTG (data not shown).

Discussion Our present study reported for the first time the development of nitrate tolerance in eNOS (−/−) mice after chronic *in vivo* NTG treatment. Vascular relaxation studies showed similar rightward shifts in NTG CRC with similar increases in EC_{50} after *in vivo* NTG treatment in both the eNOS (−/−) and (+/+) mice. Consistent with this observation, NTG-stimulated cyclic GMP accumulation was attenuated to a comparable extent after *in vivo* NTG treatment. Prior NTG exposure caused 59.2 and 43.5% decreases in cyclic GMP production upon subsequent NTG challenge in the eNOS (−/−) and (+/+) mice, respectively. These results suggest that the extent of NTG tolerance development was similar in the eNOS (−/−) and (+/+) mice. In addition, our results showed that chronic NTG treatment at a tolerance-inducing dose did not lead to any apparent change in eNOS protein expression in the eNOS (+/+) mouse aorta.

Our results therefore differed from the findings of two recent reports (Abou-Mohamed *et al*., 2000; Munzel *et al*., 2000) which indicated the possible involvement of eNOS in the development of nitrate tolerance. There are a number of important differences in methodology between these investigations. Our studies utilized a different animal species (mice) vs rats in both of these studies, and it is uncertain whether a species difference may exist in the phenomenon of nitrate tolerance. Our s.c. dose of 60 mg kg^{-1} day^{-1} was slightly higher than the 0.5 mg h^{-1} (52 mg kg^{-1} day^{-1} for a 230 g rat) used by Munzel *et al*. (2000). We used both cyclic GMP accumulation and vascular relaxation as indices of vascular sensitivity toward NTG, while both Munzel *et al*. (2000) and Abou-Mohamed *et al*. (2000) only employed vascular relaxation as an index of tolerance. Induction of vascular tolerance was carried out *in vivo* in our study and that of Munzel *et al*. (2000), while Abou-Mohamed *et al*. (2000) induced vascular nitrate tolerance *in vitro*.

Consistent with literature reports of increased sensitivity of eNOS (−/−) mice to nitric oxide donors (Brandes *et al*., 2000; Hussain *et al*., 1999; Kojda *et al*., 1999), the eNOS (−/−) mice showed a ∼10 fold lower EC_{50} in aorta relaxation when compared to their wild-type counterparts (Figure 1). Kodja *et al*. (1999) also reported a ∼7 fold decrease in the EC_{50} of NTG-induced vascular relaxation in the eNOS (−/−) mice. One possible mechanism for this enhanced sensitivity to NO donors in the eNOS (−/−) mice may involve increased sensitivity of soluble guanylyl cyclase (sGC). Indeed, Brandes *et al*. (2000) had reported a greater sGC activity in the eNOS (−/−) mouse aorta after sodium nitroprusside (SNP, 100 μM) stimulation, and a ∼2 fold higher cyclic GMP production after SNP (300 nM) stimulation in the eNOS (−/−) mice vs (+/+) mice. However, in our present study, the total amounts of vascular cyclic GMP accumulation after 1 μM NTG challenge were not different in the eNOS (−/−) vs (+/+) mice (A bars in Figure 2). The apparent lack of difference in total cyclic GMP accumulation after NTG challenge is in contrast to the increased sensitivity of the eNOS (−/−) to NTG observed in the vascular relaxation study. This may in part be due to the high concentration of NTG used in the *in vitro* cyclic GMP challenge. Indeed, it has been reported that the maximal increase in cyclic GMP production following a high dose of sodium nitroprusside stimulation was similar in the eNOS (−/−) and (+/+) mice (Brandes *et al*., 2000).

Although our study indicates that *in vivo* vascular tolerance toward NTG in mice did not appear to require the participation of eNOS, it did not rule out that eNOS may indeed be involved in some manner in the development of nitrate tolerance in eNOS (+/+) animals. It is possible that in the eNOS (−/−) mice, compensatory pathways may exist to overcome the lack of eNOS participation in, for example, enhanced superoxide generation. Our studies did indicate, however, that these compensatory pathways are unlikely to involve other NOS isoforms, such as nNOS and iNOS, since expression of these proteins was not detected. However, we did not measure mRNA levels for these NOS proteins, nor did we determine nNOS and iNOS activities in the aorta. Further studies involving these measurements will provide a better understanding on how other isoforms of NOS may compensate for the loss of eNOS in eNOS-deficient animals.

Although our findings indicate the lack of critical involvement of eNOS as a cause of vascular tolerance, they do not rule out that eNOS dysfunction could well be an effect of tolerance. The presence of oxidative stress during the development of vascular nitrate tolerance may indeed affect eNOS activity and expression (Vaziri & Ding, 2001). In addition to alteration of NOS expression, activation of NAD(P)H-dependent oxidases has been shown to be another source of superoxide generation during vascular nitrate tolerance (Rajagopalan *et al*., 1996). The contribution of NAD(P)H-dependent oxidases to nitrate tolerance in mice was not examined in the present study.

It is noted that the degree of tolerance, as measured by vascular relaxation responses, was modest. The EC_{50} was right-shifted only ∼3 fold, although statistical significance indicating tolerance development was unambiguously observed. The decrease in cyclic GMP accumulation as a result of vascular tolerance was more marked, producing ∼ 2 fold difference upon challenge of mouse aorta with NTG. It is possible that higher doses of NTG, or a different route of administration (e.g., continuous intravenous infusion *via* an osmotic pump), would produce a higher degree of vascular nitrate tolerance in the mouse. However, because of the internal consistency of our data (through the use of two

indices of vascular nitrate tolerance), we believe that our conclusion about the lack of criticality of eNOS in causing vascular nitrate tolerance is well supported.

This work was partially supported by funds from the University at Buffalo Foundation. We thank Ms Sun Mi Fung and Mr David M. Soda for their extensive technical support.

References

ABOU-MOHAMED, G., KAESEMEYER, W.H., CALDWELL, R.B. & CALDWELL, R.W. (2000). Role of L-arginine in the vascular actions and development of tolerance to nitroglycerin. *Br. J. Pharmacol.,* **130,** 211–218.

BRANDES, R.P., KIM, D., SCHMITZ-WINNENTHAL, F.H., AMIDI, M., GODECKE, A., MULSCH, A. & BUSSE, R. (2000). Increased nitrovasodilator sensitivity in endothelial nitric oxide synthase knockout mice: role of soluble guanylyl cyclase. *Hypertension,* **35,** 231–236.

CARAMORI, P.R., ADELMAN, A.G., AZEBEDO, E.R., NEWTON, G.E., PARKER, A.B. & PARKER, J.D. (1998). Therapy with nitroglycerin increases coronary vasoconstriction in response to acetylcholine. *J. Am. Coll. Cardiol.,* **32,** 1969–1974.

DU, Z.Y., BUXTON, B.F. & WOODMAN, O.L. (1992). Tolerance to glyceryl trinitrate in isolated human internal mammary arteries. *J. Thorac. Cardiovasc. Surg.,* **104,** 1280–1284.

FUNG, H.L. & BAUER, J.A. (1994). Mechanisms of nitrate tolerance. *Cardiovasc. Drugs Ther.,* **8,** 489–499.

HASEGAWA, K., TANIGUCHI, T., TAKAKURA, K., GOTO, Y. & MURAMATSU, I. (1999). Possible involvement of nitroglycerin converting step in nitroglycerin tolerance. *Life Sci.,* **64,** 2199–2206.

HUSSAIN, M.B., HOBBS, A.J. & MACALLISTER, R.J. (1999). Autoregulation of nitric oxide-soluble guanylate cyclase-cyclic GMP signalling in mouse thoracic aorta. *Br. J. Pharmacol.,* **128,** 1082–1088.

KIELBASA, W. & FUNG, H.L. (2000). Nitrite inhalation in rats elevates tissue NOS III expression and alters tyrosine nitration and phosphorylation. *Biochem. Biophys. Res. Commun.,* **275,** 335–342.

KOJDA, G., LAURSEN, J.B., RAMASAMY, S., KENT, J.D., KURZ, S., BURCHFIELD, J., SHESELY, E.G. & HARRISON, D.G. (1999). Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial cell nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control. *Cardiovasc. Res.,* **42,** 206–213.

MACALLISTER, R.J. (2000). Arginine and nitrate tolerance. *Br. J. Pharmacol.,* **130,** 209–210.

MUNZEL, T., LI, H., MOLLNAU, H., HINK, U., MATHEIS, E., HARTMANN, M., OELZE, M., SKATCHKOV, M., WARNHOLTZ, A., DUNCKER, L., MEINERTZ, T. & FORSTERMANN, U. (2000). Effects of long-term nitroglycerin treatment on endothelial nitric oxide synthase (NOS III) gene expression, NOS III-mediated superoxide production, and vascular NO bioavailability. *Circ. Res.,* **86,** E7–E12.

MUNZEL, T., SAYEGH, H., FREEMAN, B.A., TARPEY, M.M. & HARRISON, D.G. (1995). Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanisms underlying tolerance and cross-tolerance. *J. Clin. Invest.,* **95,** 187–194.

NAMIKI, A., AIKAWA, J., MOROI, M., MACHII, K. & AKATSUKA, N. (1991). Acetylcholine-induced endothelium-dependent vascular smooth muscle relaxation in nitroglycerin-tolerant isolated rat aorta. *Heart Vessels,* **6,** 175–180.

POU, S., POU, W.S., BREDT, D.S., SNYDER, S.H. & ROSEN, G.M. (1992). Generation of superoxide by purified brain nitric oxide synthase. *J. Biol. Chem.,* **267,** 24173–24176.

RAJAGOPALAN, S., KURZ, S., MUNZEL, T., TARPEY, M., FREEMAN, B.A., GRIENDLING, K.K. & HARRISON, D.G. (1996). Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J. Clin. Invest.,* **97,** 1916–1923.

RUSSELL, A. & WATTS, S. (2000). Vascular reactivity of isolated thoracic aorta of the C57BL/6J mouse. *J. Pharmacol. Exp. Ther.,* **294,** 598–604.

STEWART, D.J., HOLTZ, J. & BASSENGE, E. (1987). Long-term nitroglycerin treatment: effect on direct and endothelium-mediated large coronary artery dilation in conscious dogs. *Circulation,* **75,** 847–856.

VAZIRI, N.D. & DING, Y. (2001). Effect of lead on nitric oxide synthase expression in coronary endothelial cells: role of superoxide. *Hypertension,* **37,** 223–226.

XIA, Y., TSAI, A.L., BERKA, V. & ZWEIER, J.L. (1998). Superoxide generation from endothelial nitric-oxide synthase. A Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J. Biol. Chem.,* **273,** 25804–25808.

XIA, Y. & ZWEIER, J.L. (1997). Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 6954–6958.

(Received September 17, 2001
Revised November 22, 2001
Accepted November 29, 2001)