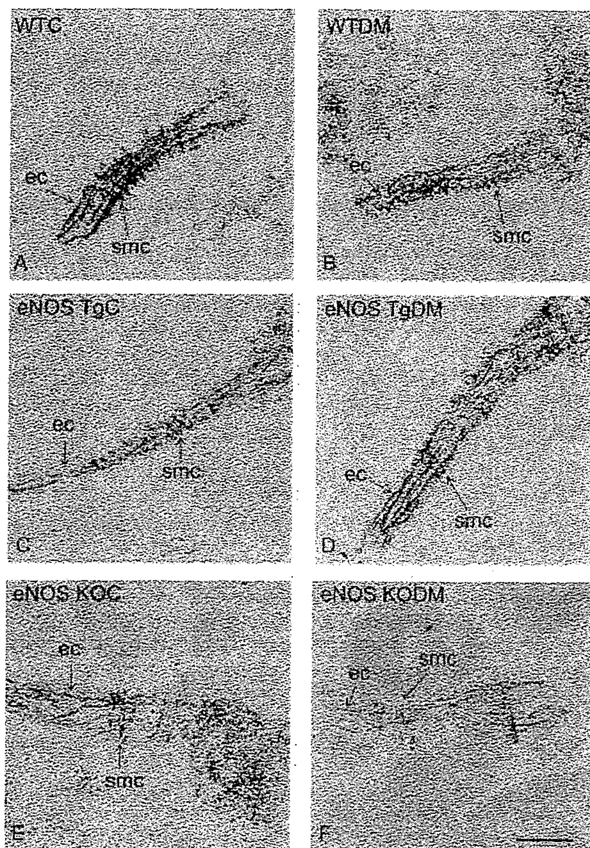

Fig. 1. Expression of Cx37 in afferent arterioles. Cx37 was found in longitudinally aligned endothelial cells (ec) and transversely aligned smooth muscle cells (smc) of afferent arterioles from wild-type (WTC, **A**), eNOS transgenic control (eNOS TgC; **C**), eNOS knockout control mice (eNOS KOC, **E**), and their corresponding diabetic mice (WTDM, **B**; eNOS TgDM, **D**; eNOS KODM, **F**). The vessels are oriented longitudinally from left to right. Scale bar = 20 μm.

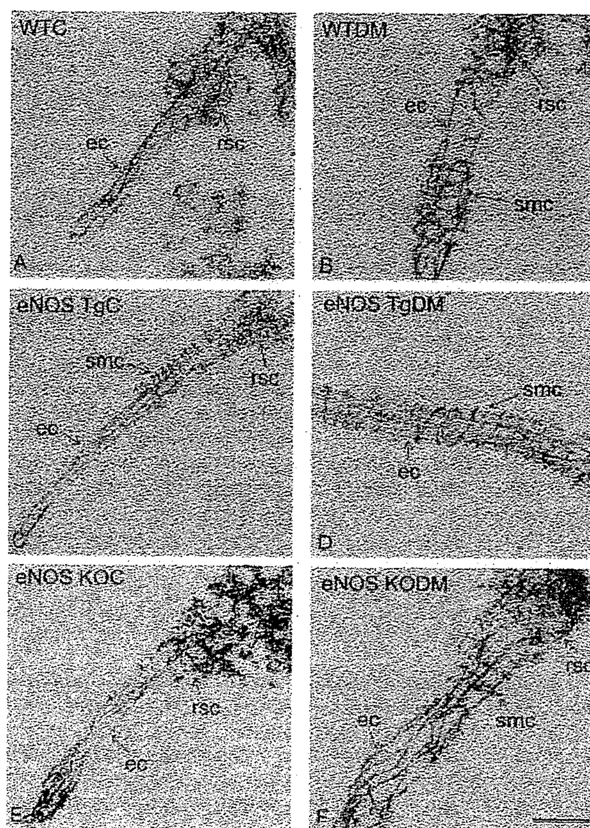

Fig. 2. Expression of Cx40 in afferent arterioles. Cx40 was detected in endothelial cells (ec), renin-secreting cells (rsc), and the immediately adjacent smooth muscle cells (smc) from wild-type control (WTC, **A**), eNOS transgenic (eNOS TgC, **C**), eNOS knockout control (eNOS KOC, **E**), and eNOS knockout diabetic mice (eNOS KODM, **F**). An increased expression of Cx40 in the more distal smooth muscle cells was detected in diabetic mice from wild-type (WTDM, **B**) and eNOS transgenic (eNOS TgDM, **D**) but not in eNOS KO mice. The longitudinal axis of the vessels runs from left to right. Scale bar = 20 μm.

arteries (Harrison-Bernard et al., 2006), and regular, uniform antimyosin staining of smooth muscle cells. In contrast, antimyosin staining in smooth muscle cells of efferent arterioles was irregular and heterogeneous, in part due to the irregular alignment of smooth muscle cells (Evan and Dail, 1977; Gattone et al., 1984; Bankir et al., 1987; Yuan et al., 1990: e.g., Fig. 4A–F). Renin-secreting cells were identified by their anatomical position immediately adjacent to the vascular pole of the glomerulus (Zaki et al., 1982). Extraglomerular mesangial cells were identified by their location in the triangle surrounded by the afferent arteriole, the efferent arteriole, and the distal tubule.

Double labeling with Cx and myosin antibodies confirmed that Cx staining oriented along the vessel axis was associated with the endothelium while that at right angles to the vessel axis lay between smooth muscle cells as we have demonstrated previously (Zhang and Hill, 2005). For all Cx antibodies, there was no Cx staining when the primary antibody was omitted or when the primary antibody was preincubated with the corresponding antigenic peptide. No differences were noted in the immunohistochemical results between the two methods to induce diabetes.

Connexin Expression in Afferent Arterioles

Nondiabetic mice. Cx37 and Cx40 were consistently detected in endothelial cells and renin-secreting cells of afferent arterioles from wild-type, eNOS transgenic, and eNOS knockout mice (Figs. 1A, C, and E and 2A, C, and E).

In wild-type and eNOS transgenic mice, expression of Cx37 was more regular and consistent in smooth muscle cells of the afferent arterioles (Fig. 1A and C) compared to that in eNOS knockout mice (Fig. 1E). However, no statistically significant difference was detected among the three mice groups, although there was a tendency for reduced expression in the eNOS knockout mice (Table 1, control).

TABLE 1. Semi-quantification of Cx37 expression in afferent arterioles

	Control	Diabetes
WT	2.0 ± 0.45 (5)	1.8 ± 0.31 (6)
eNOS Tg	2.2 ± 0.31 (6)	2.5 ± 0.50 (4)
eNOS KO	1.5 ± 0.22 (6)	1.3 ± 0.21 (6)

No significant differences amongst wild type (WT), eNOS transgenic (Tg) and eNOS knockout (KO) mice and their diabetic counterparts ($p > 0.05$). The numbers in parentheses represent the number of animals studied.

TABLE 2. Semi-quantification of Cx40 expression in afferent arterioles

	Control	Diabetes
WT	1.6 ± 0.40 (5)	3.0 ± 0.32 (5)*
eNOS Tg	2.3 ± 0.25 (4)#	4.0 ± 0.00 (4)†
eNOS KO	1.0 ± 0.00 (4)	1.5 ± 0.50 (4)

#$p < 0.05$ compared to eNOS knockout control mice; *$p < 0.05$ compared to wild type control mice.
†$p < 0.05$ compared to eNOS Tg control mice. Numbers in parentheses represent the number of animals studied. KO: knockout. WT: wild type, Tg: transgenic; KO: knockout.

In wild-type mice, expression of Cx40 was more restricted to the smooth muscle cells immediately adjacent to the renin-secreting cells (Fig. 2A), while in the corresponding areas in eNOS knockout mice, Cx40 was either similar to or weaker than that in the wild-type mice (Fig. 2E). In contrast, clear and consistent Cx40 staining in the smooth muscle cells beyond the renin-secreting cells was detected in eNOS transgenic mice (Fig. 2C). Semiquantification of Cx40 staining showed a significant increase in Cx expression in the afferent arterioles of eNOS transgenic mice compared to that in eNOS knockout mice ($P < 0.05$; Table 2, control). No significant difference in Cx staining was observed between wild-type and eNOS transgenic mice or wild-type and eNOS knockout mice.

Cx43 was detected in endothelial cells of afferent arterioles from wild-type, eNOS transgenic, and eNOS knockout mice (Fig. 3A, C, and E). Cx43 was not detected in smooth muscle cells of afferent arterioles in any of the three groups.

Diabetic mice. Since the previous sections indicated that overexpression of eNOS mimicked changes of Cxs seen previously in diabetes (Zhang and Hill, 2005), we tested this hypothesis by inducing diabetes in eNOS knockout mice.

Expression of Cx37 and Cx40 in endothelial cells and renin-secreting cells of afferent arterioles was similar among wild-type, eNOS transgenic, and eNOS knockout diabetic mice (Figs. 1B, D, and F and 2B, D, and F).

In the smooth muscle cells, expression of Cx37 was also similar among wild-type, eNOS transgenic, and eNOS knockout diabetic mice (Fig. 1B, D, and F). No statistically significant difference was detected between control mice and their counterpart diabetic mice, although there was a tendency for reduced expression in eNOS knockout mice ($P > 0.05$; Table 1).

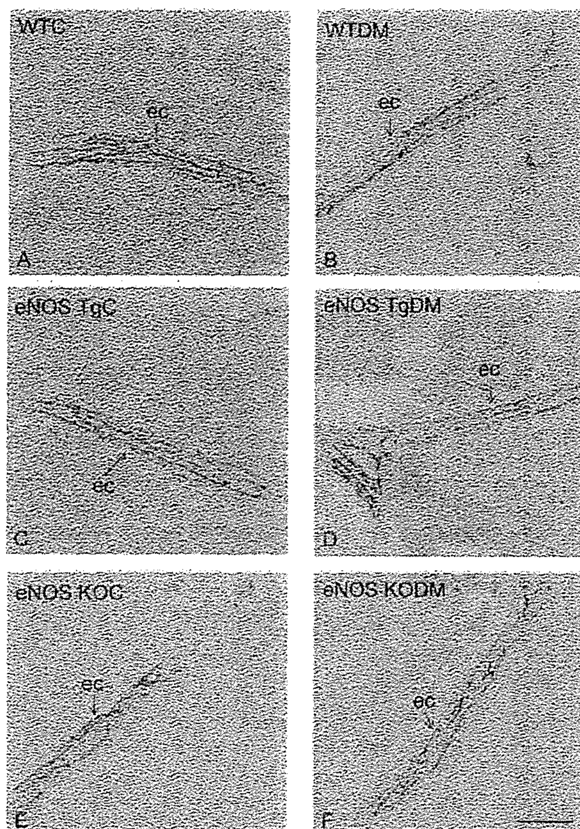

Fig. 3. Expression of Cx43 in afferent arterioles. Cx43 was detected in endothelial cells (ec) in wild-type control (WTC, **A**) and diabetic mice (WTDM, **B**), eNOS transgenic control (eNOS TgC, **C**) and diabetic mice (eNOS TgDM, **D**), eNOS knockout control (eNOS KOC, **E**), and diabetic mice (eNOS KODM, **F**). No staining was detected in the smooth muscle cells. The vessels were oriented longitudinally from left to right. Scale bar = 20 μm.

Expression of Cx40 was increased in the distal smooth muscle cells of afferent arterioles of both wild-type diabetic mice and eNOS transgenic diabetic mice, the staining being more consistent in eNOS transgenic mice than that in wild-type mice (Fig. 2A–D). In eNOS knockout diabetic mice, Cx40 staining was restricted to the smooth muscle cells immediately adjacent renin-secreting cells (Fig. 2F). Semiquantification of Cx40 in smooth muscle cells showed a significant increase in Cx staining in wild-type diabetic mice compared to wild-type control and in eNOS transgenic diabetic mice compared to eNOS transgenic control mice ($P < 0.05$; Table 2). No significant difference was detected in Cx staining between eNOS knockout diabetic mice and eNOS knockout control mice ($P > 0.05$; Table 2). However, expression of Cx40 in afferent arterioles of eNOS knockout diabetic mice was significantly less than that in afferent arterioles of wild-type diabetic or eNOS transgenic diabetic mice ($P < 0.05$).

Cx43 was detected in endothelial cells of afferent arterioles from all diabetic groups in a similar manner to

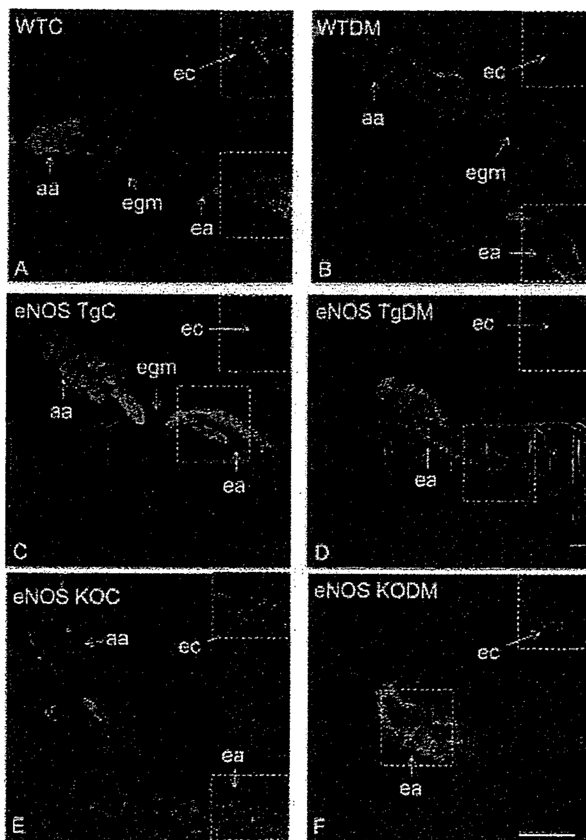

Fig. 4. Expression of Cx43 in efferent arterioles. When arterioles were labeled with antimyosin staining, Cx43 was detected in endothelial cells (ec) of the efferent arterioles (ea) in wild-type (WTC, **A**) and eNOS knockout control mice (eNOS KOC, **E**), but it was not detected in eNOS transgenic control mice (eNOS TgC, **C**). In diabetes, Cx43 staining was reduced in wild-type diabetic (WTDM, **B**), while it was unchanged in eNOS knockout diabetic mice (eNOS KODM, **F**). Cx43 was not detected in eNOS transgenic diabetic mice (eNOS TgDM, **D**). Cx43 was not detected in extraglomerular mesangial cells (egm) in all groups. The insets show Cx43 staining in the same field as the box but without double labeling with antimyosin. Afferent arterioles (aa) were oriented on the left (A, B, C, E). Scale bar = 20 μm.

that in the control groups (Fig. 3). Cx43 was not detected in the smooth muscle cells of any diabetic mice (Fig. 3B, D, and F).

Connexin Expression in Efferent Arterioles

Nondiabetic mice. Only Cx43 was detected in endothelial cells of efferent arterioles from wild-type mice (Fig. 4A), as we have reported previously (Zhang and Hill, 2005). In contrast, Cx43 was not found in endothelial cells of efferent arterioles in eNOS transgenic mice (Fig. 4C) but was detected strongly in endothelial cells of efferent arterioles in eNOS knockout mice (Fig. 4E). Semiquantification of Cx staining showed a significant decrease in Cx expression in endothelial cells of efferent arterioles in eNOS transgenic mice compared to that in

TABLE 3. Semi-quantification of Cx43 in efferent arterioles

	Control	Diabetes
WT	2.2 ± 0.20 (5)	1.4 ± 0.25 (5)*
eNOS Tg	1.0 ± 0.00 (4)†	1.0 ± 0.00 (4)
eNOS KO	2.8 ± 0.25 (4)	2.8 ± 0.25 (4)

†p < 0.05 compared to wild type (WT) control and eNOS knockout (KO) control mice.
*p < 0.05 compared to wild type control mice. The numbers in the parentheses represent the number of animals studied. Tg: transgenic.

wild-type control mice or in eNOS knockout control mice ($P < 0.05$; Table 3, control). However, no difference was detected between wild-type control and eNOS knockout control mice ($P > 0.05$; Table 3, control). Neither Cxs37, 40 (not shown), nor Cx43 was detected in the smooth muscle cells of efferent arterioles in any of the three groups (Fig. 4A, C, and E).

Diabetic mice. During diabetes, Cxs37 and 40 remained absent from endothelial cells of efferent arterioles in wild-type, eNOS transgenic, and knockout mice. Expression of Cx43 in endothelial cells of efferent arterioles was reduced in wild-type diabetic mice compared to that in wild-type control mice (Fig. 4A and B). No Cx43 was detected in eNOS transgenic diabetic mice (Fig. 4D), while Cx43 was readily detected in endothelial cells of efferent arterioles in eNOS knockout diabetic mice (Fig. 4F). Semiquantification of Cx staining showed a significant decrease in Cx43 in wild-type diabetic mice compared to that in wild-type control mice ($P < 0.05$; Table 3), consistent with our previous results (Zhang and Hill, 2005). No significant difference was detected between eNOS transgenic control and diabetic mice or between eNOS knockout control and diabetic mice ($P > 0.05$; Table 3). Expression of Cx43 was significantly greater in efferent arterioles of eNOS knockout diabetic mice than in efferent arterioles of wild-type diabetic or eNOS transgenic diabetic mice ($P < 0.05$). No Cxs were detected in the smooth muscle cells of efferent arterioles in any of the diabetic groups.

Expression of eNOS Protein in Normal and Diabetic Mice

eNOS was expressed in endothelial cells of large and small arteries in wild-type mice (Fig. 5A). In eNOS transgenic mice, staining for eNOS was markedly stronger but still confined to the endothelium of the vascular wall (Fig. 5B). eNOS expression was absent from the endothelium of the vascular wall in eNOS knockout mice (Fig. 5C).

During diabetes, the intensity of eNOS fluorescence in the endothelium of afferent arterioles was markedly increased in wild-type diabetic mice (Fig. 5D) compared to that in wild-type control mice (Fig. 5A). eNOS expression in eNOS transgenic diabetic mice (Fig. 5E) was similar to or weaker than that in eNOS transgenic control mice (Fig. 5B). eNOS expression was absent from the endothelium of the eNOS knockout diabetic mice.

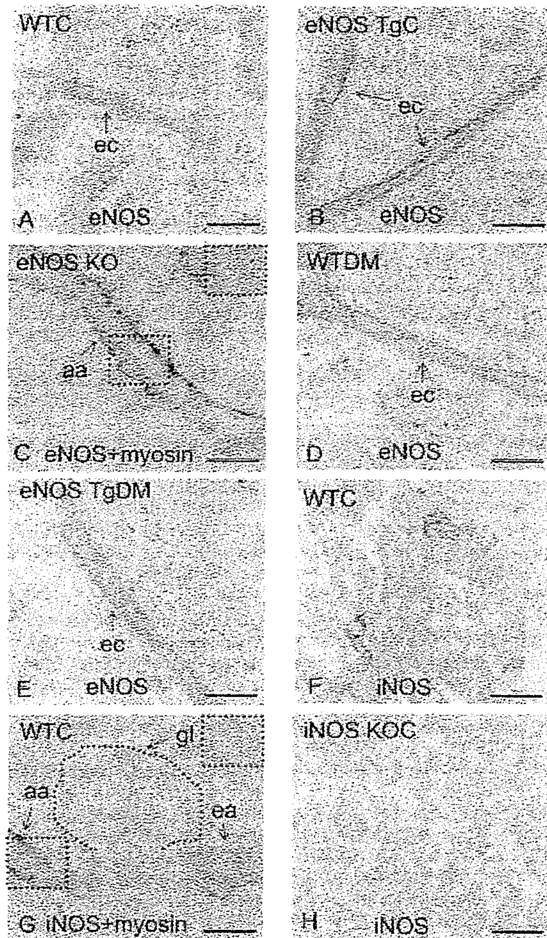

Fig. 5. Expression of eNOS and iNOS protein. eNOS was detected in endothelial cells of afferent arterioles (aa) in wild-type control (WTC, **A**) and diabetic mice (WTDM, **D**), eNOS transgenic control (eNOS TgC, **B**) and diabetic mice (eNOS TgDM, **E**). eNOS protein was not detected in eNOS knockout control mice when double-labeled with antimyosin staining (eNOS KOC, **C**). iNOS expression was not detected in smooth muscle cells of afferent arterioles (aa) or efferent arterioles (ea) in wild-type control mice when double-labeled with antimyosin staining (WTC, **G**), nor was iNOS detected inside the glomerulus (WTC, G). iNOS protein was detected in renal tubules in wild-type control mice (WTC, **F**) but not in iNOS knockout mice (iNOS KOC, **H**). The insets showed anti-eNOS (C) or anti-iNOS (G) staining in the same field as the box but without double labeling with antimyosin. gl, glomerulus. Scale bars = 20 μm in A–D, E and G; 50 μm in F and H.

Expression of iNOS Protein in Juxtaglomerular Apparatus

iNOS protein expression was mainly detected in the tubules located in the medulla and occasionally in some tubules in the cortex of wild-type and eNOS transgenic mice (Fig. 5F). iNOS protein was not expressed in the afferent or efferent arterioles when double-labeled with antimyosin staining (Fig. 5G) in any of the mice groups under normal or diabetic conditions. The specificity of iNOS antibody was confirmed by lack of iNOS staining in tubules within the renal medulla of iNOS knockout mice (Fig. 5H).

DISCUSSION

Our study has shown that in eNOS transgenic mice, Cx40 was well expressed in smooth muscle cells of afferent arterioles, while Cx43 was absent from endothelial cells of efferent arterioles. This pattern is similar to that observed in wild-type mice during diabetes (Zhang and Hill, 2005). In contrast, expression levels of Cxs40 and 43 in afferent and efferent arterioles of eNOS knockout mice were similar to those of wild-type mice. Furthermore, induction of diabetes in eNOS knockout mice failed to produce any changes in Cx expression in either afferent or efferent arterioles, suggesting that changes in Cx expression associated with diabetes correlate with an increase in eNOS expression. Immunohistochemistry confirmed that eNOS was upregulated in endothelial cells of wild-type mice during diabetes and in eNOS transgenic mice but was absent from eNOS knockout mice. The effect on Cx expression was not due to compensatory changes in iNOS since expression of iNOS was not detected in either afferent arterioles or efferent arterioles in any of the mice groups.

Our data suggest that overexpression of eNOS during diabetes produces completely different changes in Cxs in afferent and efferent arterioles. This differential responsiveness is consistent with numerous studies that have shown differences in structure and function of these two arterioles. Thus, the vascular wall of afferent arterioles is thick and composed of uniformly distributed smooth muscle cells, while the vascular wall of efferent arterioles in the superficial cortex is thin, irregular, and composed of irregularly shaped smooth muscle cells (Evan and Dail, 1977; Gattone et al., 1984; Bankir et al., 1987; Yuan et al., 1990). This morphological heterogeneity of renal arterioles was confirmed by antimyosin staining in the present study and was one of the criteria used to differentiate afferent arterioles from efferent arterioles. Furthermore, myosin heavy chain-B isoform is predominantly expressed in afferent arterioles, whereas myosin heavy chain-A isoform is only expressed in the efferent arterioles (Shiraishi et al., 2003). From a functional standpoint, differences also exist. Thus, afferent arterioles express both angiotensin type 1A and type 1B receptors while efferent arterioles exclusively express type 1A receptors (Harrison-Bernard et al., 2006). Furthermore, contractile responses in afferent arterioles are more dominated by voltage-dependent ion channels than those of efferent arterioles (Carmines and Navar, 1989; Loutzenhiser and Loutzenhiser, 2000; Hansen et al., 2001). Differential remodeling of afferent and efferent arterioles during diabetes has also been reported in streptozotocin-treated diabetic rats (Turoni et al., 2005).

The nature of the changes in Cx expression in afferent versus efferent arterioles suggests that eNOS overexpression accentuates the limited coupling between preglomerular vasculature and postglomerular vasculature. This accentuation might further aggravate glomerular hyperfiltration in the early stage of diabetes (Carmines and Fujiwara, 2002). Increased Cx expression within the preglomerular smooth muscle might also enhance the differential neural control of afferent and efferent arterioles, leading to increased pressure drop across the glo-

merulus (Denton et al., 2004), further precipitating glomerular hyperfiltration.

Increased expression of eNOS has been shown previously in the aorta (Asaba et al., 2005) and glomeruli of diabetic rats (Satoh et al., 2005). However, the increased eNOS expression in the latter study was accounted for by monomeric eNOS, rather than the functional dimeric eNOS, which was decreased. Thus, these changes in eNOS resulted in reduced generation of NO (Satoh et al., 2005) and increased production of reactive oxygen species (ROS) due to eNOS uncoupling (Satoh et al., 2005; Bevers et al., 2006). Since ROS and high glucose are also NO scavengers, the bioavailability of NO during diabetes is further reduced, as found in the renal cortex of diabetic rats (Palm et al., 2005). On the other hand, increased eNOS expression in eNOS transgenic mice results in increased production of NO as demonstrated in the aorta (Yamashita et al., 2000). Therefore, although the Cx changes in diabetes are similar to those observed in eNOS transgenic mice, the effect cannot be simply attributed to increased production of endothelial NO. In spite of these differences, changes to one of the downstream effectors of NO appear to be similar in diabetic and eNOS transgenic animals. Thus, although cGMP levels were increased in eNOS transgenic mice, NO-induced vasorelaxation was reduced due to decreased activity of both guanylate cyclase and protein kinase G (Yamashita et al., 2000). A similar decrease in NO-induced vasorelaxation has been reported in aorta of streptozotocin-induced diabetic rats due to decrease in guanylate cyclase and protein kinase G-protein (Zanetti et al., 2005). Interestingly, the activity of guanylate cyclase was increased in eNOS knockout mice (Brandes et al., 2000). Further experimentation is therefore necessary to clarify the involvement of guanylate cyclase and ROS in the mechanism by which elevated eNOS protein can alter Cx expression.

Together, our data demonstrate that an increase in eNOS protein can produce significant changes in expression of Cxs40 and 43 in afferent and efferent arterioles. These changes mimic those occurring in diabetes, during which eNOS protein is similarly increased in these arterioles. The regulation by eNOS of vascular Cx expression differs between the afferent and efferent arterioles in relation to both the cell type and Cx subtype involved, providing further evidence of the appreciable differences in structure and function of these two renal vessels.

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Experimental Physiology

Cardiovascular Control: Relationships between nitric oxide-mediated endothelial function, eNOS coupling and blood pressure revealed by eNOS–GTP cyclohydrolase 1 double transgenic mice

D. Adlam, J. K. Bendall, J. P. De Bono, N. J. Alp, J. Khoo, T. Nicoli, M. Yokoyama, S. Kawashima and K. M. Channon

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Experimental Physiology

Relationships between nitric oxide-mediated endothelial function, eNOS coupling and blood pressure revealed by eNOS–GTP cyclohydrolase 1 double transgenic mice

D. Adlam[1], J. K. Bendall[1], J. P. De Bono[1], N. J. Alp[1], J. Khoo[1], T. Nicoli[1], M. Yokoyama[2], S. Kawashima[2] and K. M. Channon[1]

[1] *Department of Cardiovascular Medicine, University of Oxford, Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Oxford OX3 9DU, UK*
[2] *Kobe University of Medicine, Japan*

Endothelium-dependent relaxation in conduit vessels is mediated largely by nitric oxide (NO), produced by the enzyme endothelial nitric oxide synthase (eNOS) in the presence of the cofactor tetrahydrobiopterin (BH4) and mediated through a cGMP-dependent downstream signalling cascade. Endothelial NOS regulates blood pressure *in vivo*, and impaired endothelial NO bioactivity in vascular disease states may contribute to systemic hypertension. In the absence of sufficient levels of the cofactor BH4, NO becomes uncoupled from arginine oxidation and eNOS produces superoxide rather than NO. The enzymatic uncoupling of eNOS is an important feature of vascular disease states associated with increased oxidative stress. However, whether eNOS coupling, rather than overall eNOS activity, has specific effects on endothelium-dependent vasorelaxation *in vitro*, or on blood pressure regulation *in vivo*, remains unclear. In this study, we evaluate the relationships between blood pressure and endothelial function in models of eNOS uncoupling, using mice with endothelium-targeted transgenic eNOS overexpression (eNOS-Tg), in comparison with littermates in which eNOS coupling was rescued by additional endothelium-targeted overexpression of GTP cyclohydrolase 1 (eNOS/GCH-Tg) to increase endothelial BH4 levels. Despite the previously characterized differences in eNOS-dependent superoxide production between these animals, we find that blood pressure is equally reduced in both genotypes, compared with wild-type animals. Furthermore, both eNOS-Tg and eNOS/GCH-Tg mice exhibit similarly impaired endothelium-dependent vasorelaxation. We show that reduced vasorelaxation responses result from desensitization of cGMP-mediated signalling and are associated with increased NO production rather than changes in superoxide production.

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Corresponding author K. M. Channon: Department of Cardiovascular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, OX3 8DU, UK. Email: keith.channon@cardiov.ox.ac.uk

The endothelium plays a central role in regulating vascular smooth muscle tone and blood pressure through the production of nitric oxide (NO) by the homodimeric enzyme endothelial nitric oxide synthase (eNOS; Furchgott & Zawadzki, 1980; Ignarro, 2002). Nitric oxide levels are a function of NO synthesis by eNOS and NO scavenging by reactive oxygen species (ROS). Reduced NO bioavailability is an important feature of vascular disease states, including hypertension, diabetes and atherosclerosis, in both humans and animal models (Kiff *et al.* 1991; Panza *et al.* 1995; Heitzer *et al.* 2001; Laursen *et al.* 2001; Alp *et al.* 2003; Henry *et al.* 2004). The importance of eNOS-derived NO for blood pressure regulation is supported by evidence of systemic hypertension in the eNOS knockout mouse (Huang *et al.* 1995; Shesely *et al.* 1996; Kojda *et al.* 1999) and hypotension in eNOS transgenic (eNOS-Tg) animals (Ohashi *et al.* 1998; van Haperen *et al.* 2002).

While these observations establish the basis for a relationship between eNOS protein levels, NO-mediated

D. Adlam, J. K. Bendall and J. P. De Bono contributed equally to this work.

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endothelial function and blood pressure, recent evidence suggests that physiological regulation of eNOS is more complex. The pteridine cofactor tetrahydrobiopterin (BH4) is critical for eNOS enzymatic activity, hence NO production. In the absence of adequate levels of BH4, eNOS becomes 'uncoupled' from L-arginine oxidation and instead molecular oxygen is reduced to form superoxide (Vasquez-Vivar *et al.* 1998, 2003; Landmesser *et al.* 2003). Although transgenic mice with endothelium-targeted eNOS overexpression have increased NO production and low blood pressure (Ohashi *et al.* 1998; van Haperen *et al.* 2002), the greatly increased levels of eNOS in these animals leads to eNOS uncoupling that is related to eNOS–BH4 stoichiometry in the endothelium (Bendall *et al.* 2005). Specifically, transgenic mice with endothelial eNOS overexpression have a marked increase in eNOS-derived superoxide production, owing to eNOS uncoupling, that is normalized by augmentiion of the endothelial BH4 levels by crossing with transgenic mice with endothelial expression of GTP cyclohydrolase 1 (GCH), the rate limiting enzyme in BH4 biosythesis (Bendall & Channon, 2005). However, it is not known whether the effects of coupled *versus* uncoupled eNOS on local NO and superoxide production have important effects on haemodynamic regulation. In particular, it remains unclear whether NO production alone, or other functionally related radicals such as superoxide, is the key determinant of vasorelaxation responses in isolated vessels, and how the changes in vasorelaxation remain quantitatively related to changes in blood pressure *in vivo*.

In this paper, we compare changes in blood pressure, NOS activity and vascular relaxation responses in endothelium-targeted transgenic mice with overexpression of either eNOS or GTP cyclohydrolase 1, and in double transgenic mice with both eNOS and GCH overexpression. We demonstrate that blood pressure is determined principally by NO production rather than eNOS coupling, and that chronic increases in vascular NO production lead to desensitization of downstream cGMP-mediated signalling, independent of eNOS uncoupling.

Methods

Animals

Studies were performed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. Mice were provided with standard chow and water *ad libitum* and housed singly at 24°C in individually ventilated cages (Techniplast Inc., Buguggiate, Italy). All mice were exposed to a regular 12 h–12 h light–dark cycle and were 11–18 weeks old at the time of study.

Mouse lines had previously been fully back-crossed onto C57bL/6 strain. Endothelial nitric oxide synthase transgenic mice (eNOS-Tg) were generated by targeting bovine eNOS overexpression to vascular endothelium under the control of the murine preproendothelin-1 promoter, as previously described (Ohashi *et al.* 1998). These animals have been shown to have an eightfold elevation in eNOS protein levels in lung and aortic tissue, with transgene expression confined to the endothelium (Ohashi *et al.* 1998; Bendall & Channon, 2005). Guanosine tripohosphate cyclohydrolase 1 transgenic mice (GCH-Tg) were generated by endothelium-targeted overexpression of human GCH under the control of the murine Tie-2 promoter, as previously described (Alp *et al.* 2003, 2004). These animals have been shown to have a threefold increase in tissue BH4 levels in lung, heart and aortic tissue (Alp *et al.* 2003; Bendall & Channon, 2005). eNOS-Tg and GCH-Tg heterozygote mice were crossbred to produce eNOS/GCH double Tg, eNOS-Tg, GCH-Tg and wild-type (WT) littermates in a 1:1:1:1 ratio for study. All animals were genotyped by polymerase chain reactions (PCR) on DNA prepared by phenol–chloroform extraction of ear-notch biopsies. Genotypes were double-checked using DNA prepared from lung tissue snap frozen at the time of killing.

Haemodynamic studies

Blood pressure was measured by direct invasive methods under general anaesthesia using the Millar® catheter system. Animals were anaesthetized using inhalational isofluorane vaporised on oxygen and maintained at a temperature of 36.5°C using a warming blanket. Surgical anaesthesia was determined by loss of the pedal withdrawal reflex. A mid-line incision was made in the neck, and the left carotid artery isolated. The cranial end of the artery was tied using 3.0 mersilk, and a stay suture looped around the caudal artery. A 1.5 F Millar® catheter was then introduced and advanced until a good blood pressure waveform trace could be detected. The proximal suture was then tied to provide haemostasis. Experimental anaesthesia was lightened to obtain a respiratory rate of 80 min^{-1}. Following equilibration for 15 min, intra-arterial blood pressure was recorded for 10 min. In some animals, pharmacological studies were performed by intraperitoneal (I.P.) injections of the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 mg kg^{-1}) or the adrenergic agonist phenylephrine (PE, 3 mg kg^{-1}).

Isometric tension vasomotor studies

Aortic vasomotor function was assessed using isometric tension studies in a wire myograph (Multi-Myograph 610M, Danish Myo Technology, Aarhus, Denmark). Mice were killed by cervical dislocation. Freshly harvested and cleaned thoracic aortas ($n = 5$–10 per group) were cut into two 2 mm aortic rings, which

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were mounted in organ bath chambers containing 5 ml of Krebs–Henseleit buffer (KHB [in mmol l^{-1}]: NaCl, 120; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; and glucose, 5.5) at 37°C, gassed with 95% O_2–5% CO_2. All experiments were performed in the presence of 10 μM indomethacin to inhibit endogenous prostaglandin release. Rings were first allowed to equilibrate for 30 min and then gradually stretched to a passive tension of 15 mN. Rings were constricted with 60 mM KCl for 5 min to assess vessel viability. Dose–response contraction curves were performed using cumulative half-log concentrations of phenylephrine (10^{-9}–10^{-5} M). Vessels were then washed repeatedly with fresh KHB for 30 min, and then precontracted to approximately 90% of maximal tension with PE (typically 3×10^{-6} M). Dose–response relaxation curves for increasing cumulative concentrations of acetylcholine (ACh, 10^{-9}–10^{-5} M) were then established to assess endothelium-dependent relaxation mediated by endothelial NO release. Responses were expressed as a percentage of the precontracted tension. Vessels were washed and precontracted again to 90% maximal contraction with PE. N^{ω}-Nitro-L-arginine methyl ester (10^{-4} M; Sigma-Aldrich, UK) was then added to inhibit endogenous NO release from eNOS. Finally, the NO donor sodium nitroprusside (SNP, 10^{-10}–10^{-6} M) was added in increasing cumulative concentrations to test endothelium-independent smooth muscle relaxation in response to exogenous NO.

Measurement of NO production

Nitric oxide production was measured using an L-arginine to citruilline conversion assay in the presence of the specific arginase inhibitor *N*-hydroxy-nor-L-arginine (nor-NOHA), as previously described (de Bono *et al.* 2006). Aortas were opened longitudinally, and incubated in 250 μl of KHB containing 1 μM calcium ionophore and 5 μl of 1.85 MBq ml^{-1} ubiquitously labelled [^{14}C]L-arginine for 90 min at 37°C, prior to removing the supernatant. Endothelium lysis was induced by three cycles of freeze–thawing in 250 μl of water added to this supernatant. Sixty microlitres of 10% trichloroacetic acid was then added to deproteinate the samples, prior to centrifugation. Five hundred microlitres of this supernatant was then collected and added to 360 μl distilled water with 140 μl 10% trichloroacetic acid. Citruilline was resolved from arginine by HPLC, as previously described (de Bono *et al.* 2006), using a 250 mm × 4.6 mm Supelcosil LC-SCX 5 cation-exchange column (Sigma-Aldrich), a DG-980-50 degasser, two PU-2080 Plus pumps, a MX-2080-32 dynamic mixer (all from Jasco Ltd, Great Dunmow, UK) and a Lablogic β-RAM Model 3 continuous flow liquid scintillation detector (Lablogic Systems Ltd, Sheffield, UK). Products of arginine metabolism were eluted over 30 min using the following buffer (rate, 1 ml min^{-1}): 0–5 min 100% distilled water, 5–15 min linear gradient from 100% distilled water to 100% 200 mM sodium citrate (pH 3.0), and 15–30 min 100% sodium citrate (pH 3.0). Scintillant fluid (Lablogic) was mixed in-line at a ratio of 0.5:1.0 after elution from the column, before passage through the liquid scintillation detector. The integrals of citruilline peaks were expressed as a percentage of the total ^{14}C count.

Measurement of cyclic GMP levels

Cyclic GMP levels in aortas were measured as previously described (Ohashi *et al.* 1998). Mice were killed by cervical dislocation. Briefly, aortas ($n = 4$–7 per group) were opened longitudinally and pre-incubated in oxygenated Krebs–Hepes solution with 0.1 mmol l^{-1} 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich) at 37°C for 15 min and then stimulated with 1 μM acetylcholine for 3 min. Vessels were immediately snap-frozen in liquid nitrogen before homogenization in ice-cold 5% trichloroacetic acid containing 0.5 mM IBMX. Homogenates were centrifuged at 2000*g*, and trichloroacetic acid in the supernatant fractions was extracted with water-saturated ether. Cyclic GMP levels were measured in these fractions using a cGMP enzyme immunoassay kit (Cayman Chemical Co., Nottingham, UK), and results expressed as picomoles per milligram of trichloroacetic acid-precipitable protein solubilized with 1 M sodium hydroxide.

Statistics

Mean data for haemodynamic parameters, NO production and cGMP formation were analysed by one-way ANOVA with Bonferroni correction for repeated measures. For vasomotor studies, the mean responses of two arotic rings from each animal were combined to produce an *n* of 1. Dose–response curves from each group were compared using ANOVA for repeated measures followed by Bonferroni *post hoc* correction.

Results

Haemodynamic regulation in eNOS, GCH and eNOS/GCH transgenic mice

The haemodynamic findings by direct intra-arterial Millar® catheter measurements revealed no differences in systolic, mean or diastolic blood pressure between GCH-Tg and WT littermate mice. There was a significant reduction in systolic blood pressure in eNOS-Tg compared with their WT littermates ($P < 0.001$), with a non-significant trend towards a reduction in mean and diastolic pressure. This was reflected by a significant reduction

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in pulse pressure in mice carrying the eNOS transgene ($P < 0.001$). However, the addition of the GCH transgene in eNOS/GCH double transgenic mice did not confer any additional effect on blood pressure when compared to eNOS-Tg alone. There were no significant differences in heart rate between the transgenic mice and their WT littermates (Fig. 1). This baseline haemodynamic phenotype was also confirmed by prior tail-cuff assessment in the same cohort (data not shown).

Non-selective systemic NOS inhibition by administration of 100 mg kg^{-1} intraperitoneal L-NAME caused a significant rise in blood pressure and a fall in heart rate in all animals (Fig. 2). The genotype-specific differences in systolic blood pressure at baseline were abolished by L-NAME, with a significantly greater rise in blood pressure in both eNOS-Tg and eNOS/GCH-Tg mice compared with WT littermates (for eNOS-Tg, systolic $P = 0.06$, mean $P < 0.05$, diastolic $P < 0.005$; for eNOS/GCH-Tg, systolic, mean and diastolic $P < 0.005$). There were no genotype-specific differences in the heart rate response to L-NAME (Fig. 2).

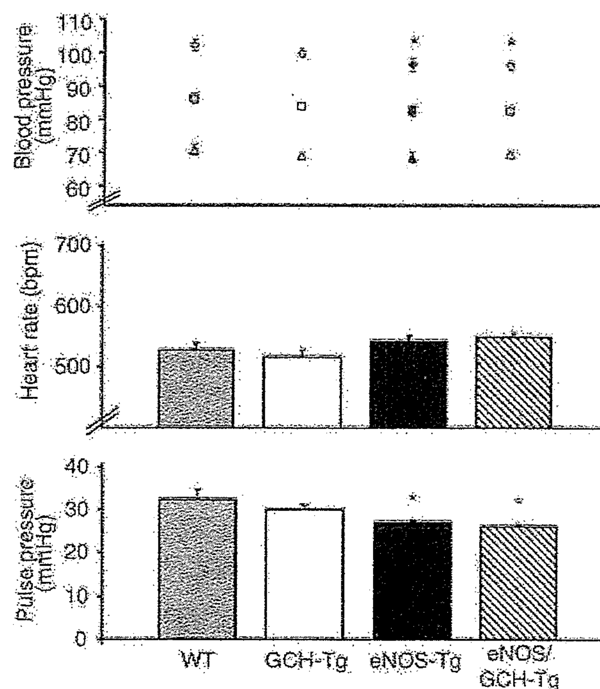

Figure 1. Invasive Millar® catheter haemodynamic measurements (systolic, diamonds; mean, squares; and diastolic, triangles) in $n = 7$–17 mice
There is a significant reduction in baseline systolic blood pressure in eNOS-Tg and eNOS/GCH-Tg animals compared with WT littermates (*$P < 0.01$). No significant difference in mean or diastolic blood pressure is shown by this technique. The proportionally greater effect on systolic blood pressure is reflected by a fall in pulse pressure in eNOS-Tg and eNOS/GCH-Tg animals (*$P < 0.01$) compared with WT littermates (means ± S.E.M.).

Adrenergic stimulation by administration of 3 mg kg^{-1} I.P. phenylephrine after NOS inhibition with L-NAME produced a significant rise in blood pressure and heart rate in WT and GCH-Tg mice, with no differences in the responses between these two groups (Fig. 2). Both eNOS-Tg and eNOS/GCH-Tg mice had a markedly reduced blood pressure response to phenylephrine compared to WT littermates ($P < 0.0005$). There were no differences in the heart rate response to I.P. phenylephrine between the genotype groups (Fig. 2).

Vasomotor function studies

We determined the functional significance of altering eNOS protein and BH4 bioavailability *in vitro* using isometric tension studies on a wire myograph (Fig. 3). The contractile responses to PE were unchanged in GCH-Tg mice, but were significantly reduced in aortic rings from both eNOS-Tg and eNOS/GCH-Tg mice compared with wild-type littermates. Endothelium-dependent relaxations in reponse to the receptor-mediated eNOS agonist ACh were unchanged in GCH-Tg mice but were significantly attenuated in eNOS-Tg mice compared with wild-type littermates. However, the presence of the GCH transgene on the eNOS-Tg background in eNOS/GCH-Tg animals had no effect, since relaxations in response to ACh were not different between eNOS-Tg and eNOS/GCH-Tg mice. Endothelium-independent relaxations in response to the NO donor SNP, unchanged in GCH-Tg mice, were significantly attenuated in both eNOS-Tg and eNOS/GCH-Tg mice, indicating a reduction

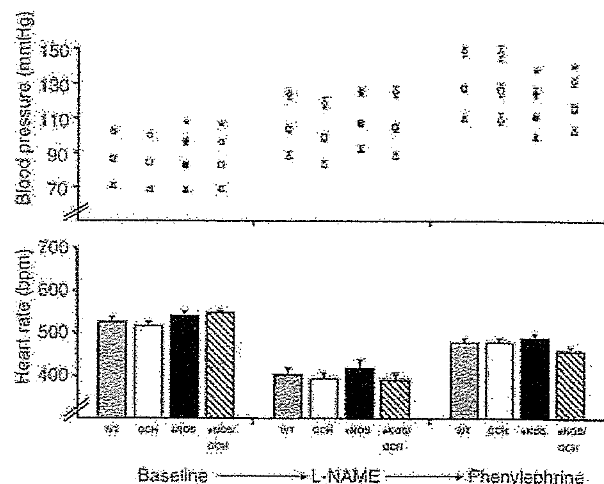

Figure 2. Intraperitoneal L-NAME (100 mg kg^{-1}) causes a rise in blood pressure and a fall in heart rate in all animals
This treatment abolished the baseline difference in blood pressure between genotype groups. Serial administration of 3 mg kg^{-1} I.P. phenylephrine following L-NAME caused a rise in heart rate in all animals. A rise in blood pressure was seen in WT and GCH-Tg animals but not in eNOS-Tg or eNOS/GCH-Tg littermates (*$P < 0.0001$; means ± S.E.M.).

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in vascular smooth muscle responsiveness to NO (Fig. 3).

Vascular NO production

We examined the effect of endothelial eNOS and GCH overexpression on NO production from aortas by measurement of L-arginine to citruilline conversion in the presence of the specific arginase inhibitor *N*-hydroxy-nor-L-arginine (nor-NOHA). Citruilline production was markedly increased in the aortas of both eNOS-Tg and eNOS/GCH-Tg animals ($P < 0.0001$) compared with wild-type littermates. However, there was no further significant increase in NO production in eNOS/GCH-Tg aortas compared with eNOS-Tg alone (Fig. 4*B*).

Aortic cGMP formation

Since aortic relaxations in response to exogenous, as well as endothelium-derived, NO were significantly attenuated in eNOS-Tg and eNOS/GCH-Tg mice, we hypothesized that this may result from a desensitization in their downstream NO–cGMP pathway as a result of chronically enhanced NO production. In accordance with the vasomotor studies, aortic ACh-stimulated cGMP formation was found to be significantly reduced in both eNOS-Tg and eNOS/GCH-Tg mice compared with wild-type littermates (Fig. 4*A*).

Discussion

In this paper, we confirm that increased endothelial NO production in the eNOS-Tg mouse lowers blood pressure and paradoxically reduces vascular reactivity. We report for the first time the haemoydnamic phenotype of the GCH-Tg, a mouse model of endothelial BH4 augmentation, and the haemodynamic effect of increasing endothelial BH4 availability in the eNOS-Tg mouse by generating the eNOS/GCH double transgenic mouse. We show that the previously described changes in superoxide production between eNOS-Tg and eNOS/GCH-Tg mice, related to eNOS uncoupling, do not induce changes in blood pressure, suggesting that blood pressure regulation in these models may be more dependent on NO production than on superoxide production. Furthermore, we show that NO production rather than eNOS coupling by BH4 also appears to be the principal determinant of the cGMP pathway.

The importance of BH4 for the coupling of eNOS enzymatic activity to arginine oxidation and NO production is now well recognized (Vasquez-Vivar *et al.* 1998, 2003, 2004). In situations where BH4 is limiting, eNOS uncoupling results in increased superoxide production. This results in reduced bioavailability of NO both as a result of a reduction in NO production and as a result of increased NO scavenging by reactive oxygen species, leading to the production of peroxynitrite. We have previously shown that eNOS-Tg mice have increased aortic NO but also increased L-NAME-inhibitable superoxide production owing to uncoupling of eNOS as a result of a discordance between eNOS protein and its essential cofactor, BH4 (Bendall & Channon, 2005). This increase in aortic superoxide production was reversed by increasing vascular BH4 production in eNOS/GCH-Tg mice, which overexpress both endothelial eNOS and GTP cyclohydrolase 1 (the rate-limiting enzyme in endothelial BH4 synthesis; Bendall & Channon, 2005). In this study, we describe the effect of endothelial upregulation of eNOS and its cofactor BH4 on haemodynamic regulation in mice *in vivo* and compare it to measurements of vasomotor function *in vitro*.

Endothelium-specific eNOS-Tg mice were significantly hypotensive compared with WT littermates, a difference abolished by the NOS inhibitor L-NAME. This conforms with previously reported data from the same animal

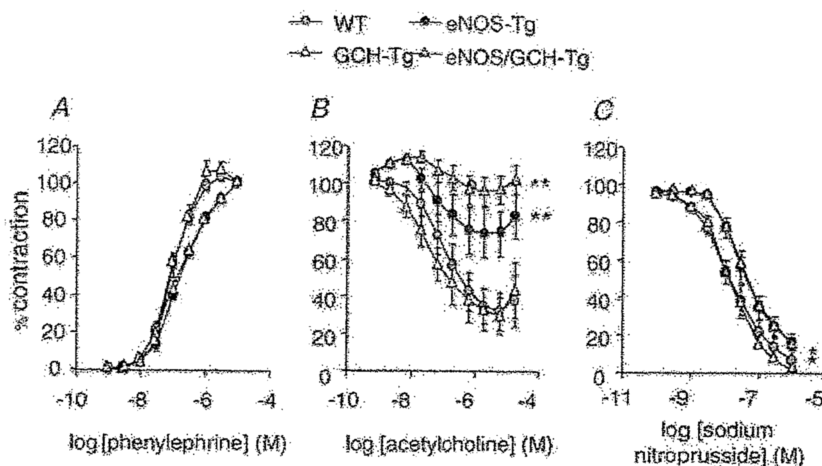

Figure 3. Isometric tension studies in aortic rings from WT, GCH-Tg, eNOS-Tg and eNOS/GCH-Tg mice ($n = 5$–10 animals per group) measured using a wire myograph
Dose–response curves for cumulative half-log concentrations of the α-adrenergic receptor agonist phenylephrine (*A*), the endothelium-dependent NO agonist acetylcholine (*B*) and the exogenous NO donor sodium nitroprusside (*C*). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with wild-type measured using repeated measures ANOVA (means ± S.E.M.).

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model (Ohashi *et al.* 1998). This finding is consistent with the expected effect of endothelial eNOS overexpression on enhanced endothelial NO production, leading to a reduction in smooth muscle tone and reduced blood pressure. It might be expected that this model would be associated with improved vascular responsiveness to activators of endothelial NO release, such as acetylcholine. However, we observed discordance between *in vivo* hypotension *versus* a significant impairment of endothelium-dependant relaxations *in vitro*, measured using wire myography. Furthermore, *in vitro*, aortic contraction in response to phenylephrine and relaxation in response to an exogenous NO donor (SNP) were found to

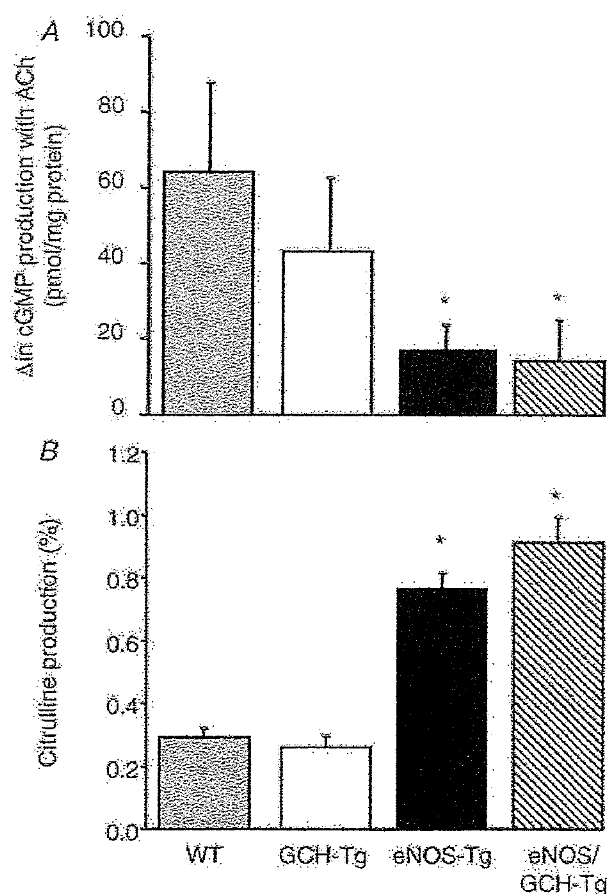

Figure 4. Cyclic GMP production and arginine to citruilline conversion in aortas
A, cyclic GMP levels after stimulation with 1 μmol l^{-1} acetylcholine for 3 min in the presence of 0.1 mmol l^{-1} IBMX at 37°C in aortas from wild-type, GCH-Tg, eNOS-Tg and eNOS/GCH-Tg mice ($n = 4$–7 animals per group). $^{*}P < 0.05$ compared with wild-type (means ± S.E.M.). *B*, NO production measured as L-arginine to citruilline conversion in $n = 8$–11 mice. There is a significant increase ($^{*}P < 0.0001$) in NO production in eNOS-Tg and eNOS/GCH-Tg animals compared with WT or GCH-Tg mice. There is no difference in aortic NO production between eNOS-Tg and eNOS/GCH-Tg animals (means ± S.E.M.).

be significantly impaired in eNOS-Tg and eNOS/GCH-Tg animals. This was reflected by an impaired pressor response to a phenylephrine bolus *in vivo*. These findings are consistent with those reported in the eNOS knockout mouse model, in which the opposite effect has been reported (Kojda *et al.* 2001), and suggest that chronic alterations in endothelial free radical signalling also have secondary effects on endothelium-independent vascular smooth muscle reactivity.

The GCH-Tg mouse was normotensive and demonstrated no change in vasomotor function at myography compared with WT littermates. We have previously shown that the GCH-Tg preserves vasomotor function in the context of a disease model such as diabetes where it is otherwise impaired (Alp *et al.* 2003). The addition of the GCH transgene to eNOS-Tg mice in the eNOS/GCH-Tg animal had no additional effect on blood pressure compared with eNOS-Tg littermates. In accordance, vasorelaxations in response to endothelium-derived and exogenous NO were no different between eNOS/GCH-Tg and their eNOS-Tg littermates. This suggests that the known difference in superoxide production between the aortas of eNOS-Tg and eNOS/GCH-Tg animals is not an important determinant of blood pressure regulation or vascular tone. Rather, both blood pressure and vasomotor function appear to be more closely related to NO production, which is increased equally in both eNOS-Tg and eNOS/GCH-Tg mice.

Since aortic responses to exogenous as well as endothelium-dependent NO were attenuated in eNOS-Tg and eNOS/GCH-Tg mice, we hypothesized that this may result from downstream desensitization of their NO–cGMP signalling pathway as a result of chronic elevation of NO levels rather than owing to endothelial dysfunction. Acetylcholine-stimulated cGMP production was found to be significantly reduced in both eNOS-Tg and eNOS/GCH-Tg aortas, the two groups with attenuated vasorelaxations to ACh and SNP. It remains possible, however, that a component of the desensitization of the NO–cGMP pathway may result from a reduction in endothelial NO production in response to exogenous stimulation with ACh. Hence, whilst maximal NO production measured by arginine to citruilline conversion (in the presence of calcium ionophore) is increased in eNOS-Tg and eNOS/GCH-Tg aortas, it cannot be said with certainty that NO production in response to ACh is likewise increased.

There is therefore an apparent discordance between hypotension in mice expressing the eNOS transgene and vascular hyporeactivity, although both appear to be mediated directly or indirectly by increased NO production and are independent of superoxide. One explanation might be that the mechanisms which regulate the set-point of vascular tone and therefore blood pressure

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are independent of those which regulate vascular reactivity. It would then be possible to have lower vascular resistance in the context of impaired vascular responsiveness. Thus, whilst we have shown that vascular reactivity is greatly influenced by the NO–cGMP pathway, perhaps vascular tone is mediated by a different NO-dependent pathway. This could be the case if the mechanism of action of NO were different in resistance arterioles from that in conduit vessels. This is suggested by the redundancy of vasoreactive mechanisms previously described in resistance vessels which are not mediators in conduit vessels, including endothelium-derived hyperpolarization factor, products of cyclo-oxygenase and neuronal NOS-derived NO (Popp *et al.* 1998; Chataigneau *et al.* 1999; Sun *et al.* 1999; Ding *et al.* 2000; Huang *et al.* 2001; Scotland *et al.* 2001). An alternative explanation is that endothelial NO production may mediate effects on chronic blood pressure independantly of vascular smooth muscle by effecting renal regulation of endovascular salt and volume.

The results of this study have implications for our understanding of the role of NO signalling in vascular function and haemodynamic regulation. Our data show discordance between systemic blood pressure and vasomotor function by aortic wire myography. This suggests that vasorelaxation responses in models of altered NO signalling should not be interpreted as direct measures of NOS enzymatic activity. Our data support the hypothesis that endothelial NO signalling is an important regulator of systemic blood pressure but suggest that therapeutic strategies targeting upregulation of eNOS should also consider the potential impact of chronic elevation of NO on desensitization of downstream signalling pathways. Furthermore, even correction of eNOS uncoupling and BH4 supplementation *in vivo* seems unlikely to alter desensitization of downstream NO signalling. This may have clinical implications for patients receiving therapeutic exogenous nitrate donors, since chronic administration in the context of disease states associated with increased oxidative stress in the vascular wall might lead to desensitization of the NO–cGMP pathway and exacerbate impairment of vascular reactivity and endothelial function.

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Cardiovascular Control: Relationships between nitric oxide-mediated endothelial function, eNOS coupling and blood pressure revealed by eNOS–GTP cyclohydrolase 1 double transgenic mice
D. Adlam, J. K. Bendall, J. P. De Bono, N. J. Alp, J. Khoo, T. Nicoli, M. Yokoyama, S. Kawashima and K. M. Channon

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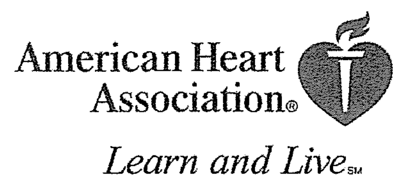

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A specific role for eNOS-derived reactive oxygen species in atherosclerosis progression

Tomofumi Takaya MD, PhD [1], Ken-ichi Hirata MD, PhD [1],
Tomoya Yamashita MD, PhD [1], Masakazu Shinohara MD, PhD [1],
Naoto Sasaki MD [1], Nobutaka Inoue MD, PhD [1],
Toyotaka Yada MD, PhD [2], Masami Goto MD, PhD [2],
Akiko Fukatsu PhD [3], Toshio Hayashi MD, PhD [3],
Nicholas J Alp MD, PhD [4], Keith M Channon MD, PhD [4],
Mitsuhiro Yokoyama MD, PhD [1], Seinosuke Kawashima MD, PhD [1, 5]

Division of Cardiovascular and Respiratory Medicine,
Department of Internal Medicine,
Kobe University Graduate School of Medicine, Kobe, Japan [1]

Department of Medical Engineering and Systems Cardiology,
Kawasaki Medical School, Kurashiki, Japan [2]

Department of Geriatrics,
Nagoya University Graduate School of Medicine, Nagoya, Japan [3]

Department of Cardiovascular Medicine,
University of Oxford, John Radcliffe Hospital, Oxford, United Kingdom [4]

Department of General Medicine,
Saiseikai Nakatsu Hospital, Osaka, Japan [5]

Address for Correspondence:
Seinosuke Kawashima MD, PhD
Department of General Medicine,
Saiseikai Nakatsu Hospital
2-10-39, Shibata, Kita-ku, Osaka, 530-0012, Japan.
Telephone number; 81-6-6372-0333, FAX number; 81-6-6372-0339
E-mail: kawashima1008@nakatsu.saiseikai.or.jp

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Abstract

Objective: When the availability of tetrahydrobiopterin (BH4) is deficient, endothelial nitric oxide synthase (eNOS) produces superoxide rather than NO (uncoupled eNOS). We have shown that the atherosclerotic lesion size was augmented in apolipopritein E-deficient (ApoE-KO) mice overexpressing eNOS due to the enhanced superoxide production. In this study, we addressed the specific importance of uncoupled eNOS in atherosclerosis, and the potential mechanistic role for specific versus non-specific anti-oxidant strategies in restoring eNOS coupling.

Methods and Results: We crossed mice over-expressing eNOS in the endothelium (eNOS-Tg) with mice over-expressing GTP-cyclohydrolase I (GCH), the rate-limiting enzyme in BH4 synthesis, to generate ApoE-KO/eNOS-Tg/GCH-Tg mice. As a comparison, ApoE-KO/eNOS-Tg mice were treated with vitamin C. Atherosclerotic lesion formation was increased in ApoE-KO/eNOS-Tg mice compared with ApoE-KO mice. GCH over-expression in ApoE-KO/eNOS-Tg/GCH-Tg mice increased vascular BH4 levels and reduced plaque area. This reduction was associated with decreased superoxide production from uncoupled eNOS. Vitamin C treatment failed to reduce atherosclerotic lesion size in ApoE-KO/eNOS-Tg mice, despite reducing overall vascular superoxide production.

Conclusion: In contrast to vitamin C treatment, augmenting BH4 levels in the endothelium by GCH over-expression reduced the accelerated atherosclerotic lesion formation in ApoE-KO/eNOS-Tg mice, associated with a reduction of superoxide production from uncoupled eNOS.

Key words: eNOS uncoupling, Tetrahydrobiopterin, Vitamin C, Atherosclerosis, Apolipoprotein E-deficient mice

Condensed abstract

In apolipoprotein E-deficient mice over-expressing eNOS in the endothelium, augmenting BH4 levels in the endothelium by GTP-cyclohydrolase I over-expression was more efficient to reduce the accelerated atherosclerotic lesion formation and superoxide production from uncoupled eNOS compared with chronic vitamin C treatment.

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