Increased blood flow causes coordinated upregulation of arterial eNOS and biosynthesis of tetrahydrobiopterin

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eNOS may be uncoupled when BH$_4$ is insufficiently supplied, thereby leading to cellular production of superoxide anions rather than NO (7, 24, 38).

Our present study was thus designed to test the hypothesis that in vivo increased blood flow causes upregulation of GTPCH-I protein expression and enzymatic activity, thereby increasing local concentration of BH$_4$ in arterial wall and thus contributing to the increase in eNOS observed in this setting.

METHODS

Rat model of aortocaval fistula. The aortocaval (AC) fistula model has been described in detail in previous reports (23, 28). In brief, a fistula was created in the abdominal aorta and adjacent inferior vena cava of Sprague-Dawley rats (~250 g) by puncturing the vessels using an 18-gauge needle. The entry point of the needle into aorta was sealed with a drop of cyanoacrylate glue. The sham-operated rats underwent laparotomy, cross-clamping of the aorta and inferior vena cava for 30 s without puncturing, and the placement of a drop of glue at the abdominal aorta. All procedures were approved and performed in accordance with the guidelines of the Animal Care and Use Committee of the Mayo Clinic and Foundation.

Eight weeks after the creation of AC fistula, systolic blood pressure was measured in conscious rats by an automated tail-cuff method (Harvard Apparatus, Kent, UK). Rats were then anesthetized with ketamine (30 mg/kg im) and xylazine (6 mg/kg im) and subjected to a midline abdominal incision, and the aorta was exposed. Blood flow in the abdominal aorta at the level above renal vessels was measured by an ultrasonic flow probe (Transonic System, Ithaca, NY). Rats were euthanized with injection of pentobarbital sodium (250 mg/kg ip) after the hemodynamic measurements, and the abdominal aorta was harvested.

Western blot analysis. Isolated aortic segments were lysed in buffer containing (in mmol/l) 50 NaCl, 50 NaF, 50 sodium pyrophosphate, 5
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(A) Western blot analysis of Akt and Akt-S473 in aortas from sham-operated and AC fistula rats. *P < 0.05; n = 4–5 different animals for each protein analysis.

B) Measurement of tissue concentrations of cGMP in aorta homogenates of sham and AC fistula rats.

**RESULTS**

Hemodynamic measurements. Eight weeks after surgery, systolic blood pressure was similar between rats that received sham or AC fistula operation (106 ± 5 vs. 103 ± 3 mmHg, sham vs. fistula, respectively; n = 4–5 rats; P = 0.46). When compared with sham-operated animals, rats with the AC fistula had significantly higher aortic blood flow measured at the level of renal vessels (0.12 ± 0.03 vs. 0.22 ± 0.01 ml·min⁻¹·g body wt⁻¹, respectively; n = 3 rats; P = 0.04).

Western blot analysis. When compared with distal aortic segments (lower blood flow), segments proximal to the fistula (higher blood flow) expressed significantly higher levels of eNOS, eNOS-Ser1177, HSP90, and caveolin-1 (Fig. 1, A and B).

Measurement of tissue biopterin and GTPCH-I activity and expression. Biopterin levels were determined after differential oxidation in acid and base conditions by reverse-phase HPLC (8). GTPCH-I activity was assessed as a function of neopterin production under standard conditions with GTP as a substrate (19). The expression of GTPCH-I was also analyzed by Western blot analysis (immunoblotted against rabbit anti-mouse GTPCH-I antibody, 1:50 dilution, custom-made, Invitrogen).

**Antibodies and drugs.** Unless otherwise specified, all the antibodies used in the present study were purchased from BD Transduction Laboratories (eNOS, eNOS-Ser1177, HSP90, and caveolin-1) or Stressgen (Akt and Akt-S473). IBMX, phenylephrine, acetylcholine, DEA-NONOate, and papaverine were obtained from Sigma. All other reagents were of analytical grade.

**Statistical analysis.** Results are presented as means ± SE. Mean values comparing sham-operated and AC fistula rats were analyzed by an unpaired t-test. Mean values comparing aortic segments proximal from and distal to fistula were analyzed by a paired t-test. ANOVA was used to compare the concentration-dependent curves between groups. Statistical significance was accepted at a level of P < 0.05.
Expression of these proteins, except HSP90 in aortic segments proximal to the fistula, was also higher than that observed in sham-operated rats (Fig. 1, A and B). Phosphorylation of the Ser473 residue of Akt was increased in vessels exposed to high flow, whereas the total level of Akt remained unchanged (Fig. 2, A and B).

Measurement of cGMP. The amount of NO released from the aorta was quantified by the tissue concentrations of cGMP after incubation in IBMX for 30 min. Concentrations of cGMP were significantly elevated in the aortic segments exposed to higher blood flow compared with sham-operated and low-flow aortic segments, whereas there was no difference between aorta isolated from sham-operated animals and aorta distal to fistula (Fig. 3).

Vascular reactivity. Contractions to KCl (40 mM) and PE (10^-9 to 10^-5 M) were significantly reduced in segments isolated from the proximal aorta, as shown in Table 1 and Fig. 4, A and B. EC50 of PE was increased in the aorta proximal to the fistula compared with the distal and sham-operated aortas, but the differences were not statistically significant (Table 1). Treatment with l-NAME significantly potentiated the contraction responses in the proximal aorta, whereas changes in the distal aorta were less affected (Table 1). Endothelium-dependent relaxations to acetylcholine and DEA-NONOate were not significantly different among sham-operated aortas, as well as proximal and distal aortas (Fig. 4, C and D).

**Table 1. Contractions to KCl and phenylephrine in aortas of sham- and fistula-operated rats in absence or presence of l-NAME**

<table>
<thead>
<tr>
<th></th>
<th>Control Cmax</th>
<th>Control EC50</th>
<th>l-NAME Cmax</th>
<th>l-NAME EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham KCl</td>
<td>1.9±0.25*</td>
<td>2.1±0.22</td>
<td>2.92±0.25*</td>
<td>9.25±0.65</td>
</tr>
<tr>
<td>Proximal KCl</td>
<td>1.3±0.19</td>
<td>1.9±0.18†</td>
<td>1.65±0.33</td>
<td>18.6±6.19</td>
</tr>
<tr>
<td>Distal KCl</td>
<td>2.4±0.26*</td>
<td>2.1±0.19</td>
<td>3.34±0.34*</td>
<td>8.00±1.98</td>
</tr>
<tr>
<td>Sham PE</td>
<td></td>
<td></td>
<td>4.4±0.17†</td>
<td>7.0±1.4</td>
</tr>
<tr>
<td>Proximal PE</td>
<td></td>
<td></td>
<td>2.5±0.50</td>
<td>13±2.9</td>
</tr>
<tr>
<td>Distal PE</td>
<td></td>
<td></td>
<td>3.4±0.40</td>
<td>4.2±0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 different animals for each group. l-NAME, N^6^-nitro-l-arginine methyl ester, a nitric oxide inhibitor (10^-5 M); Cmax, maximal contraction; EC50, mean concentrations of phenylephrine that induced 50% of the Cmax; Proximal, aortic segments proximal to the fistula; Distal, aortic segments distal to the fistula. Contraction and Cmax values are in units of force in g. EC50 values are in units of 10^-8 M. *P < 0.05 compared with proximal aorta. †P < 0.05 compared with control measurement of each group.
different animals in each group. 

To-BH2 biopterin ratios were similar among the three vessel demonstrates that increased blood flow causes coordinated (proximal vs. sham) and by paired activity in rat aorta determined as function of neopterin production; 

**DISCUSSION** 

Aortic segments proximal to the fistula (Fig. 5, enzymatic activity of GTPCH I were significantly increased in aortas obtained from sham-operated rats, both expression and 

**BH4 levels.** Total biopterin levels were increased in aortic segments exposed to increased blood flow compared with sham-operated and low-flow aortas (Fig. 6A). This increase was due to the elevated BH4 levels (Fig. 6B). Levels of oxidative products of BH4 (7,8-BH2/biopterin) and the BH4- to-BH2 biopterin ratios were similar among the three vessel segments (Fig. 6, C and D), indicating the absence of increased oxidation of BH4. 

**Fig. 5.** A: representative Western blot analysis demonstrating the expression of GTP cyclohydrolase I (GTPCH I) in aortic segments. Protein levels were quantified by scanning densitometry (Scion Image) and are shown in relative density; *n* = 4–5 different animals in each group. B: levels of GTPCH-I activity in rat aorta determined as function of neopterin production; *n* = 9–12 different animals in each group. *P* < 0.05 analyzed by unpaired *t*-test (proximal vs. sham) and by paired *t*-test (proximal vs. distal). 

with aortas obtained from sham-operated rats, both expression and enzymatic activity of GTPCH I were significantly increased in aortic segments proximal to the fistula (Fig. 5, A and B). 

BH4 levels. Total biopterin levels were increased in aortic segments exposed to increased blood flow compared with sham-operated and low-flow aortas (Fig. 6A). This increase was due to the elevated BH4 levels (Fig. 6B). Levels of oxidative products of BH4 (7,8-BH2/biopterin) and the BH4- to-BH2 biopterin ratios were similar among the three vessel segments (Fig. 6, C and D), indicating the absence of increased oxidation of BH4.

**DISCUSSION** 

With the use of the aortocaval model, the present study demonstrates that increased blood flow causes coordinated upregulation of eNOS and GTPCH I. Elevation of GTPCH-I protein expression is accompanied by increased amounts of BH4, the latter resulting from enhanced enzymatic activity of GTPCH I. We also demonstrate that blood flow does not affect oxidation of BH4, suggesting that increased net biosynthesis of BH4 per se is a major mechanism responsible for increased availability of BH4. 

The aortocaval fistula model in rats was developed about 25 yr ago (18). This model predictably raises increased blood flow and shear stress in the proximal aorta, the latter shown to be increased some threefold within 10 min after the creation of the fistula (11). This model has been successfully utilized in studies (27) designed to determine the in vivo effects of blood flow on endothelial function, demonstrating, for example, the enhanced expression (both mRNA and protein) and activity of eNOS in the rat aorta. Such changes in this model occur without any elevation in mean arterial pressure (11, 35, 39). Consistent findings were observed in our studies utilizing this model at 8 wk: systolic blood pressures were unaltered in this model, whereas blood flow was increased almost twofold in the aorta proximal to the fistula compared with aortic blood flow distal to the fistula. 

To the best of our knowledge, the present study is the first to demonstrate that increased blood flow stimulates arterial biosynthesis of BH4 and that such increased synthesis results from increased protein expression and enzymatic activity of GTPCH I. As such, our study provides novel insights regarding the molecular basis for increased NO synthesis in the setting of increased blood flow. Increased availability of BH4 (the reduced form of biopterin) has been shown to be vascular protective and can prevent endothelial dysfunction induced by hypercholesterolemia, diabetes, hypertension, and smoking (2, 5, 40). In this regard, it is notable that increased blood flow, and presumably increased laminar shear stress, not only induced the synthesis of BH4 but also preserved it in reduced state. Inspection of the nucleotide sequence of the GTPCH-I gene (20, 21) detected the shear-stress response elements (GAGACC and GTGCTC) (30) in the promoter region of the gene (Table 2), indirectly supporting our findings that GTPCH I may be transcriptionally regulated by shear stress, although this hypothesis requires further investigation. Our data provide the first evidence that biosynthesis of BH4 is governed by hemodynamic forces. This finding has important implications for an understanding of flow-dependent regulation of eNOS. Availability of BH4 is critical for enzymatic activity of eNOS, and the ability of blood flow to upregulate GTPCH I and eNOS in coordinated fashion is most likely designed to optimize the production of NO.

In the vessels with increased blood flow, the expression of eNOS and its phosphorylated form (eNOS-Ser1177) were both significantly enhanced. These findings support the concept that both Ca2+/calmodulin-dependent and -independent activation of eNOS is upregulated by prolonged exposure to high flow. High blood flow also increased expression of phosphorylated Akt (Akt-Ser473), whereas the level of total Akt remained unchanged. The upregulation of Akt-Ser473 is consistent with the reported activation of phosphatidylinositol 3-OH kinase/Akt pathway leading to the phosphorylation of eNOS (10, 13). Activation of Akt may also cause the dissociation of eNOS from caveolin-1 and therefore increase the activity of eNOS (26).

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Increased level of cGMP, the second messenger of NO, in the aortic segments with increased blood flow, was consistent with a detected increase in the expression of eNOS and phosphorylated eNOS. With the use of Western blot analysis, the expression of heme oxygenase-1, which may also activate soluble guanylyl cyclase via the release of carbon monoxide, was not increased in the aortic segments with increased blood flow (data not shown). Furthermore, contractions to KCl and α1-adrenergic receptor activation were significantly reduced in the aorta proximal to the fistula. Inhibition of NOS potentiates the contractions to KCl and phenylephrine in the proximal aorta but not in the distal, low-flow aorta, suggesting that high local concentrations of NO and subsequent elevation of cGMP contribute to the reduced reactivity to vasoconstrictors in aorta exposed to high blood flow. These results are consistent with the findings reported by Rudic et al. (34), in which contractions of mouse carotid artery were reduced 7 days after exposure to high blood flow.

In the present study, endothelium-dependent and -independent relaxation responses of aorta exposed to high flow were not significantly different from those in sham-operated aorta or aorta exposed to low flow. Our data are in line with the results reported by Rudic et al. (34) but at variance with the results obtained on arteriovenous fistula in canine femoral vasculature (25). Augmented endothelium-dependent relaxations to acetylcholine were found in the femoral artery exposed to high blood flow for 6 wk (25). Several major differences, including experimental design (6 vs. 8 wk of high flow), different anatomical location of fistula, as well as species differences, may account for differential reactivity to acetylcholine. Furthermore, high local concentrations of NO associated with a significant elevation of cGMP may incur adaptive reduction in the vasodilator effect of NO released from endothelium in response to acetylcholine. This desensitization phenomenon is clearly demonstrated in transgenic mouse overexpressing eNOS wherein endothelium-dependent relaxations to acetylcholine are impaired despite significant elevation of arterial cGMP. Thus increased generation of NO in the vasculature does not necessarily predict enhanced endothelium-dependent and -independent relaxation responses. However, we wish to point out that the major conclusion of our study is in full agreement with prior in vivo studies (25, 27, 36); namely, high arterial blood flow stimulates production of endothelial NO.

With the use of the aortocaval fistula in rats, we demonstrated that a long-term increase in arterial laminar blood flow upregulates the expression of eNOS and its GTPCH I. Activation of Akt/eNOS-Ser1177 pathway is also an important mechanism responsible for stimulation of eNOS enzymatic activity. The results of the present study are the first to show that increased blood flow stimulates vascular biosynthesis of BH4 and to delineate the basis for such increased BH4 synthesis, namely, increased GTPCH-I protein expression and enzymatic
activity of GTPCH I. Blood flow does not affect oxidation of BH4, thereby reinforcing the conclusion that increased biosynthesis of BH4 is a major mechanism responsible for increased availability of BH4. Elevations of intracellular BH4 concentration appears to be required for optimal production of NO in arterial endothelium and vasodilatation of arteries exposed to high blood flow.

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GRANTS

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