Neuronal NOS-dependent dilation to flow in coronary arteries of male eNOS-KO mice

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Huang, An, Dong Sun, Edward G. Shesely, Ellen M. Levee, Akos Koller, and Gabor Kaley. Neuronal NOS-dependent dilation to flow in coronary arteries of male eNOS-KO mice. Am J Physiol Heart Circ Physiol 282: H429–H436, 2002; 10.1152/ajpheart.00501.2001.—Flow-induced dilation was examined in isolated coronary arteries of endothelial nitric oxide (NO) synthase knockout mice (eNOS-KO) and wild-type (WT) mice. The basal tone of arteries (percentage of passive diameter) was significantly greater in eNOS-KO than in WT mice; their flow-induced dilations, however, were similar. Endothelial removal eliminated the dilations in vessels of both strains of mice. In arteries of WT mice, Nω-nitro-l-arginine methyl ester (l-NAME) (10−4 M) or indomethacin (10−5 M) alone, inhibited flow-induced dilation by ~50%, whereas their simultaneous administration abolished the responses. In arteries of eNOS-KO mice, flow-induced dilation was inhibited by ~40% with l-NAME. The residual portion (60%) of the response was eliminated by the additional administration of indomethacin. 7-Nitroindazole (10−4 M) attenuated flow-induced dilation by ~40% in arteries of eNOS-KO mice, but did not affect responses in those of WT mice. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (3 × 10−5 M) inhibited the l-NAME/7-nitroindazole-sensitive portion of the responses in arteries of eNOS-KO mice. Immunohistochemical evidence confirms the presence of neuronal NOS (nNOS) in the arterial endothelium of eNOS-KO mice. In conclusion, nNOS-derived NO, via activation of cGMP, together with prostaglandins, maintains flow-induced dilation in coronary arteries of male eNOS-KO mice.

endothelial nitric oxide; prostaglandins; flow-induced dilation

ALTHOUGH CARDIAC METABOLISM is a major determinant of coronary perfusion (8), local mechanisms activated by changes in hemodynamic forces, such as changes in blood flow (14, 17) and intravascular pressure (25, 33), also participate significantly in the control of coronary vascular resistance via shear stress and myogenic mechanisms. The shear stress-sensitive mechanism is believed to be a potent regulator of coronary vascular tone (29). Shear stress acting on the endothelial lining of blood vessels has been demonstrated to be an important physiological stimulus for the release of endothelial mediators, such as nitric oxide (NO) (28), prostaglandins (15), and endothelium-derived hyperpolarizing factor (EDHF) (9, 23), leading to vessel dilation. In the coronary circulation, NO released by endothelial NO synthase (eNOS) is considered to be a primary intrinsic regulatory factor that controls vascular tone in response to changes in shear stress (17, 28). We reported previously (13, 32) that the mechanisms by which endothelial cells release NO in response to changes in shear stress, elicited by changes in perfusate flow, are altered in certain disease states, such as hypertension and heart failure, as indicated specifically by an impaired flow-induced dilation due to a loss of the NO-mediated portion of the responses. However, we also found that in skeletal muscle arterioles of transgenic mice in which the gene for eNOS was deleted, endothelial responses to flow/shear stress are preserved via NO-independent signal transduction pathways, resulting in the release of prostaglandins (31) and EDHF (9), to maintain flow-induced dilations in male and female mice, respectively. Although the capacity of coronary vessels to compensate for the loss of NO-mediated dilations to agonists has already been reported (2, 7, 18), there are no studies extant that have examined the mechanisms by which coronary arteries are responding to shear stress when the gene for eNOS is deleted. It was of interest, therefore, to elucidate the possible changes in or adaptation of shear stress-sensitive mechanisms in coronary arteries of mice that have a targeted disruption of the gene encoding for eNOS. In this context, in addition to the possible upregulation of prostaglandin and/or EDHF synthesis, we also considered the contribution of a non-eNOS-derived NO. Neuronal NOS (nNOS) localized in intracardiac neurons lining coronary vessels and in specialized cardiac conducting tissue, such as the sinoatrial and atrioventricular nodes, may also contribute to cardiac NO formation (12). Thus the aim of the studies was to identify the endothelial pathways that account for the transduction of the shear stress signal in coronary arteries of eNOS-deficient mice and to contrast the results with those obtained in vessels of wild-type (WT) mice.

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Determination of Animal Genotype

Heterozygous eNOS (+/-) mice were interbred to generate eNOS WT (+/+) and homozygous mutant (-/-) mice. Mice were genotyped by polymerase chain reaction (PCR) analysis. A small piece of mouse tail was digested in 0.4 ml lysis buffer (pH 7.5) containing (in mM) 50 Tris, 100 EDTA, and 100 NaCl and 1% sodium dodecyl sulfate and 0.5 mg/ml proteinase K overnight at 42°C. Isolation of tail DNA was followed by the additional administration of 0.2 ml of saturated NaCl. After the lysis mixture was centrifuged (14,000 rpm/min, for 30 min), 450 μl of the supernatant was transferred to pure ethanol (0.9 ml) to form a cluster of DNA that was then picked up and rehydrated in Tris·EDTA buffer (10 mM Tris·HCl, pH 8.0, and 1 mM EDTA) overnight. The DNA (5 μl) sample was amplified by one sense primer (5’-CTC-CAACCTAGTGGAGGCTCT-3’ for binding to the eNOS DNA) and two antisense primers (5’-ATGTTGCTTCACAGCAT-CTT-3’ for normal sequence and 5’-CTTCTCGTGTCTT-TACCGTA-3’ for knockout targeting sequence, respectively) in PCR. The PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

Animals

Male eNOS knockout (eNOS-KO) and WT mice were used. All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the American Physiological Society’s “Guidelines for the Use and Care of Laboratory Animals.” eNOS-KO and WT mice were bred in the Department of Comparative Medicine at New York Medical College.

Isolation of Arteries

Mice were euthanized by cervical dislocation. After thoracotomy, the heart was rapidly removed and placed in a petri dish containing cold (0-4°C) physiological salt solution (pH 7.4). Left anterior descending coronary arteries (0.7–1 mm in length; ~100 μm in active diameter) were isolated from the midpoint of the left anterior descending artery, distal to its main branches, under a dissecting microscope and then cannulated in a vessel chamber (32). To have a vessel of sufficient length, all branches of the isolated vessel segment were ligated.

Experimental Setup

The isolated artery was cannulated with two glass pipettes in a vessel chamber (1 ml/vol) and suffused (1 ml/min) with physiological salt solution, buffered with NaHCO3 (24.0 mM), and 5% CO2 plus ambient air to maintain the pH at 7.4. Intravascular pressure and temperature were maintained at 60 mmHg and 37°C, respectively. Intraluminal flow was established by changing proximal and distal pressure, controlled by two pressure-servo systems (Living Systems), to an equal degree but in opposite directions without changing intravascular pressure (31). The flow rate was measured by a ball flowmeter (model FL-300, Omega) calibrated to measure flow in a range of 0–100 μl/min. The diameter of vessels was measured with an image-shearing monitor (model 907; IPM) and recorded with a chart recorder (Multicorder model MC6625, Graphitec). When the vessel was pressurized, the pressure-servo system was placed in the manual mode (i.e., no automatic maintenance of pressure) to ascertain that there were no leaks in the system. If no leaks were detected (i.e., perfusion pressure remained constant), the pressure-servo system was then set in the automatic mode during the entire experiment.

Experimental Procedures

Flow-induced dilation. Changes in diameter of coronary arteries in response to increases in perfuse flow were studied (9, 31). The vessels were equilibrated at 60 mmHg of perfusion pressure for ~1 h in a no-flow condition to develop spontaneous tone. In all protocols, only those vessels that developed spontaneous tone to pressure were used. Vessels that did not develop tone were not used for further study. The range of spontaneous tone of eNOS-KO arteries was 55–70% and that from WT vessels was ~60–80%. After equilibration for 1 h, flow-induced responses were assessed. Perfusate flow was increased from 0 to 10 μl/min in 2 μl/min steps.

Role of the endothelium. In the first series of experiments, flow-induced dilation was assessed before and after endothelial removal. Endothelial denudation was accomplished by injection of air into the vessel lumen, as described previously (31). The efficacy of removal of endothelium and intact function of smooth muscle were assessed by loss of artery dilation to acetylcholine (10−8 M) and maintained dilation to sodium nitroprusside (10−7 M).

Role of NO and prostaglandins. In these experiments, the role of NO or prostaglandins in the mediation of flow-induced dilation was assessed by incubation of vessels with Nω-nitro-L-arginine methyl ester (l-NAME, 10−4 M) for 20 min or indomethacin (Indo; 10−5 M) for 30 min, inhibitors of NOS and cyclooxygenase, respectively. After control flow-diameter curves were obtained, an inhibitor was administered either alone or in combination with the other before the flow-diameter relationships were once more assessed.

Role of nNOS and cGMP. In this series of experiments, the contribution of nNOS to flow-induced responses was evaluated by performing the experiments before and after administration of 7-nitroindazole (7-Ni; 10−4 M), reported to be a specific inhibitor of nNOS (21), for 30 min. The target for NO in the mediation of flow-induced dilation of arteries from eNOS-KO mice was identified by administrating 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 3 × 10−5 M), an inhibitor of guanylate cyclase, into the suffusate for 30 min.

Passive diameter. At the conclusion of each experiment, the suffusion solution was changed to a Ca2+-free solution containing 1 mM EGTA. Vessels were incubated for 10 min to reach maximal diameter at 60 mmHg of perfusion pressure.

Immunohistochemistry. For analysis of the presence of nNOS within the endothelium of coronary artery in both strains of mice, vessels were perfused with freshly prepared 4% paraformaldehyde in a cannulated and pressurized condition for 10 min. Vessels were then taken off the cannulas, washed in PBS, and embedded in OCT compound. Frozen sections (10 μm thick) of the vessels were cut at ~20°C with the use of a cryostat (Lab-Tek; Westmont, IL). Sections were incubated with 0.1% Triton X-100 in PBS, 10% normal goat serum, and 5% bovine serum albumin overnight at 4°C. The sections were then incubated with rabbit polyclonal antibody to nNOS (1:800) for 24 h at 4°C. Control staining was performed by incubating the anti-nNOS antibody with anti-nNOS blocking peptide (1:4) at 4°C for 1 h, based on the procedure suggested by the manufacturer (Calbiochem; San Diego, CA). The mixture was then centrifuged and the supernatant was incubated with vessel sections for 24 h at 4°C. After 3 × 10-min washes in PBS, vessel sections were incubated at room temperature with Cy3-conjugated goat anti-
rabbits (1:200) for 1 h. Background staining was assessed by directly incubating the sections with Cy3-conjugated goat anti-rabbit IgG for 1 h. The reaction was completed by washing with PBS and mounting the coverslips in Vectashield (Vector Laboratory; Burlingame, CA). The fluorescent image was captured with an Olympus BX60 microscope (×100 oil objective) and a charge-coupled device camera (CoolSNAP, RS Photometrics).

**Chemicals.** Anti-nNOS and anti-nNOS blocking peptide were purchased from Calbiochem. Cy3 was obtained from Jackson Immunoresearch Laboratories (West Grove, PA). All other chemicals were obtained from Sigma (St. Louis, MO) and were prepared on the day of the experiments.

**Calculations and Statistics**

Both changes in diameter and normalized diameter, expressed as a percentage of the passive diameter in response to increases in flow/shear stress, were evaluated. Wall shear stress (WSS) was calculated as $WSS = 4\eta Q/r^3$, where $\eta$ is viscosity, $Q$ is flow rate, and $r$ is the radius of the vessel. Statistical significance was calculated by repeated-measures analysis of variance, followed by Tukey-Kramer multiple-comparison test. Data are presented as the means ± SE. $N$ and $n$ are the numbers of mice and vessels, respectively. When two or more vessels were studied from one animal, their responses were averaged. Student’s t-test was also used as appropriate. Significance level was taken at $P < 0.05$.

**RESULTS**

The characteristics of coronary arteries of male WT ($N = 15$) and eNOS-KO ($N = 20$) mice are shown in Table 1. Active diameter was significantly smaller in coronary arteries of eNOS-KO than in those of WT mice, whereas their passive diameters were comparable. Consequently, the basal tone of arteries, expressed as percentage of their passive diameter, was significantly greater in eNOS-KO compared with WT mice.

**Role of Endothelium in Flow-induced Dilation**

Increasing perfusate flow from 0 to 10 $\mu$l/min elicited significant increases in diameter of arteries from both WT and eNOS-KO mice. The maximal changes in diameter of arteries were similar in the two strains of mice ($27.2 ± 1.6$ and $27.7 ± 1.1 \mu$m at 10 $\mu$l/min, respectively) despite the presence of greater basal tone in eNOS-KO mice (Fig. 1A and B). That the dilation of arteries from eNOS-KO and WT mice are essentially identical can be seen in Fig. 1A, in which the normalized diameter as a function of perfusate flow is depicted. Also, increases in flow elicited increases in calculated wall shear stress that were identical in the two types of vessels (Fig. 1C). Removal of the endothelium, which did not affect basal diameter, completely eliminated flow-induced dilation in vessels of both WT and eNOS-KO mice (Fig. 2).

**Role of NO and Prostaglandins**

In the next series of experiments, the endothelial factors mediating flow-induced dilation of arteries were investigated by using l-NAME and Indo. These inhibitors had no significant effect on the basal tone of vessels in either strain of mice. In arteries of WT mice (Fig. 3A), l-NAME or Indo alone inhibited flow-induced dilation by ~50%, indicating that NO and prostaglandins participate equally in the mediation of flow-induced dilation. Combined administration of both inhibitors abolished the response. In arteries of eNOS-KO mice (Fig. 3B), inhibition of flow-induced dilation with Indo or l-NAME alone was significantly different (~60

### Table 1. Characteristics of coronary arteries of mice

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>WT ($N = 15$)</th>
<th>eNOS-KO ($N = 20$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, wk</td>
<td>28.8 ± 2.0</td>
<td>27.1 ± 2.0</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>31.0 ± 0.8</td>
<td>28.6 ± 1.0</td>
</tr>
<tr>
<td>Basal diameter, $\mu$m</td>
<td>98.1 ± 4.9</td>
<td>88.8 ± 2.2*</td>
</tr>
<tr>
<td>Passive diameter, $\mu$m</td>
<td>140.7 ± 5.3</td>
<td>137.9 ± 3.3</td>
</tr>
<tr>
<td>Range: max–min</td>
<td>(176–79)</td>
<td>(169–86)</td>
</tr>
<tr>
<td>Basal tone, %passive diameter</td>
<td>69.8 ± 1.3</td>
<td>64.6 ± 1.1*</td>
</tr>
<tr>
<td>Range: max–min</td>
<td>(48.1–79.7)</td>
<td>(50.9–75.0)</td>
</tr>
</tbody>
</table>

Values are means ± SE. $N$, no. of mice. WT, wild-type mice; eNOS-KO, endothelial nitric oxide synthase knockout mice. Values in parentheses are the maximum to minimum range. *$P < 0.05$, significant difference from WT mice.
Role of nNOS and cGMP

To characterize the nature of the L-NAME-sensitive portion of flow-induced dilation in eNOS-KO coronary arteries, 7-Ni was administered in the control condition. Figure 4A shows that 7-Ni significantly inhibited coronary artery dilation to flow by ~40%, an inhibition not different from that caused by L-NAME, indicating the specific role of nNOS in the mediation of the responses. The remaining portion (~60%) of the dilation was essentially eliminated by additional administration of Indo. ODQ was then used to test the participation of the L-arginine-NO-cGMP pathway in the mediation of flow-induced responses (Fig. 4B). ODQ had an inhibitory effect identical to L-NAME (Fig. 3B) and 7-Ni (Fig. 4A), indicating that nNOS-derived NO participates significantly in the mediation of flow-induced dilation, via activation of guanylate cyclase, in arteries of eNOS-KO mice. Figure 5 demonstrates that 7-Ni does not affect flow-induced dilation in arteries of WT mice, a response that is, however, sensitive to L-NAME.

Immunohistochemistry

A specific antibody for nNOS was used to probe cross sections of mouse coronary arteries for the presence of immunoreactive protein. With undetectable background staining, diffuse staining for nNOS was observed in vascular tissue from both WT and eNOS-KO mice (Fig. 6, a and b). However, Fig. 6a shows no staining for nNOS in the endothelium of coronary arteries from WT mice, whereas Fig. 6b demonstrates positive staining of arterial endothelium from eNOS-KO mice, which was abolished by incubation of the vessels with anti-nNOS blocking peptide (Fig. 6c).

DISCUSSION

This study is the first to demonstrate a unique role for nNOS and enhanced prostaglandin synthesis in the dilation to increases in flow/shear stress in coronary arteries of mice lacking the eNOS gene, providing for the preserved flow-induced dilation in eNOS-KO coronary arteries. Subsequent immunohistochemical analysis confirmed the presence of nNOS protein in the endothelium of coronary arteries of eNOS-KO mice, providing further evidence for the contribution of nNOS-derived NO to the mediation of flow-induced responses in the absence of eNOS. In contrast, eNOS-derived NO and prostaglandins participate equally in the mediation of flow-induced dilation in coronary arteries of WT mice.

It has been demonstrated that in the heart, eNOS is expressed primarily in the coronary and endocardial
endothelium (12). Endothelium-derived NO in the mediation of coronary arterial dilation to shear stress contributes importantly to the regulation of coronary flow (17, 28). Our previous studies (13) showed that in skeletal muscle arterioles of hypertensive rats and coronary arterioles of dogs with pacing-induced heart failure (32), the release of endothelial NO is reduced in response to shear stress, resulting in impaired flow-induced dilation. On the other hand, we, as well as others, have reported (7, 9, 31) a preserved flow-induced dilation in skeletal muscle arterioles and normal coronary hemodynamics, indicated by unchanged basal coronary flow and maximal reactive hyperemic flow in eNOS-KO mice. These responses in eNOS mutant mice are most likely related to compensatory mechanisms activated by the gene deletion, unlike in disease states, such as hypertension or heart failure, conditions in which endothelium-dependent responses are impaired.

A role for nNOS in the dilation to ACh has been demonstrated previously in pial arterioles of mice with eNOS gene disruption (21, 22). These studies showed that ACh-induced vasodilation was mediated by an nNOS-cGMP-dependent pathway. More recently, a role for nNOS-derived NO in the mediation of ACh-induced dilation of coronary arteries of eNOS-KO mice has also been demonstrated (18). The question then arises as to whether and to what extent the compensatory mechanisms via nNOS, evoked by the absence of eNOS, would function in response to shear stress in coronary arteries. To answer this question, experiments were conducted on isolated coronary arteries from male eNOS-KO and WT mice to compare the arterial responses to flow/shear stress in the two strains of mice and furthermore to elucidate the endothelial factors mediating the responses.

Enhanced Basal Tone of Coronary Arteries in eNOS-KO Mice

Vessels were carefully selected, based primarily on their anatomic location and branch pattern. The basal diameter of arteries was significantly smaller in eNOS-KO than that in WT mice, but their passive diameter was not different, resulting in a greater basal tone in vessels of eNOS-KO mice (Table 1 and Fig. 1A).
The enhanced basal tone in eNOS-KO arteries was not affected by removal of the endothelium or inhibition of endothelial mediators by L-NAME or Indo. This result is in keeping with previous findings (1) showing that the resting membrane potential of smooth muscle cells from isolated coronary arteries is significantly less negative in eNOS-KO than in those of WT mice. Also, this depolarization is unlikely to be due to the absence of endothelial NO per se, because in both eNOS-KO and WT mice, treatment of the arteries with either L-NNa or Indo or endothelial removal did not affect the resting membrane potential, indicating that basal release of NO or prostaglandins does not modulate membrane potential and/or myogenic tone of vascular smooth muscle of these mice (1, 27, 31). It has been demonstrated that eNOS-KO mice are hypertensive (27); a condition characterized by depolarization of the arterial smooth muscle cells and enhanced myogenic tone with endothelial dysfunction (4, 10, 34). In this context, the enhanced basal tone resulting from depolarization of smooth muscle cells observed in eNOS-KO coronary arteries may be linked to the physical effect of high pressure on the vessel wall or the vascular remodeling in response to the lack of NO. Alternatively, the absence of NO may elicit activation of other enzyme systems that are originally suppressed by NO, such as cytochrome P-450 hydroxylase, the enzyme responsible for the conversion of arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE). Indeed, 20-HETE has been demonstrated, in various vascular beds, to be an integral component of arteriolar response to elevations in transmural pressure (6, 11).

Flow-induced Dilation in Coronary Arteries of WT and eNOS-KO Mice

In response to increases in perfusate flow, coronary arteries of both strains of mice exhibited substantial dilations. The absolute and normalized changes in diameter and the level of shear stress were not different in arteries of WT and eNOS-KO mice (Fig. 1). These findings suggest that despite the absence of NO synthesis by eNOS and the presence of a greater basal tone in eNOS-KO mice, arterial dilation to increases in shear stress is normal. The overlap of the two flow-shear stress curves (Fig. 1C) is indicative of a well-preserved endothelial sensitivity to shear stress in arteries of eNOS-KO mice. We found that flow-induced dilation in eNOS-KO mice seems not to be affected by the greater basal tone that would counteract vasodilator responses (16, 30). One possible explanation could be that the simultaneous presence of greater shear stress elicited by the same flow rate in a vessel of smaller diameter could, in turn, potentiate flow-induced dilator responses. Thus the balance between these two opposite forces makes the maintenance of flow/shear stress-sensitive mechanism in eNOS-KO coronary arteries manifest. We also found that the flow-induced dilation of coronary arteries was entirely endothelium dependent, as indicated by the fact that removal of endothelium eliminated the response in vessels of both groups of mice (Fig. 2), suggesting that the factors mediating flow-dependent dilation are released from endothelial cells.

Endothelial Mediators Contributing to Flow-Induced Dilation in Arteries of WT Mice

In coronary arteries of WT mice, inhibition of either NO or prostaglandin synthesis alone significantly (by ~50%) reduced the dilation to increases in perfusate flow. Moreover, inhibition of the synthesis of both mediators eliminated flow-induced dilation (Fig. 3A). These results correspond to our previous findings observed in dog (32) and human (unpublished data) coronary arterioles, showing that NO and prostaglandins are coreleased in response to increases in flow and are responsible for the ensuing vasodilation. These results are, however, different from those showing a solely NO mediated response to increases in flow in porcine and canine coronary arterioles (17, 28, 29) and Ca^{2+}-activated K⁺ channel inhibitor-sensitive dilation to flow in human coronary arterioles (23).

Endothelial Mediators Contributing to Flow-induced Dilation in Arteries of eNOS-KO Mice

Flow-induced dilation in coronary arteries of eNOS-KO mice (Fig. 3B) was also sensitive to L-NAME although the inhibitory effect of L-NAME was moderately, but significantly, attenuated compared with that in vessels from WT mice (~40% vs. 50%, respectively; Fig. 3A). Indo alone inhibited flow-induced dilation by 60%, suggesting an augmented release of prostaglandins in the absence of eNOS. Previous studies (7, 18) demonstrated a similar compensatory role for prostaglandins in the mediation of agonist- and flow-induced responses in coronary arteries as well as in other vascular beds (1, 31) in eNOS-KO mice.

The nature of the L-NAME-sensitive portion of flow-induced dilation was characterized by using 7-Ni, a selective blocker of nNOS, in consideration of the fact that nNOS may be the only constitutively expressed isoform in eNOS mutant mice (22). We found that 7-Ni inhibited flow-induced dilation by ~40% (Fig. 4A), an inhibition similar to that obtained with L-NAME (Fig. 3B), indicating a NO-mediated response via activation of nNOS. Moreover, 7-Ni did not affect the L-NAME-sensitive portion of the responses in WT arteries (Fig. 5), providing further evidence that the upregulation of nNOS is a compensation elicited specifically in response to the lack of eNOS. In the absence of eNOS, nNOS-mediated responses were observed by others as well. It was shown recently that 7-Ni or 1-(2-trifluoromethylphenyl)-imidazole (another potentially selective inhibitor of nNOS) inhibited NO-mediated vasodilation to ACh in eNOS-KO mice without interfering with NO-mediated responses in WT mice (18, 21, 22). Because we found that flow-induced dilation was an endothelium-dependent response in vessels from eNOS-KO mice, we surmised that nNOS is present in the endothelium. Indeed, immunohistochemical evidence indicates the presence of nNOS in the endothelium.
lium of coronary arteries of eNOS-KO mice (Fig. 6B) but not in that of WT vessels (Fig. 6A), providing further support to our hypothesis. Others also reported the existence of nNOS in endothelial cells of coronary, pulmonary arteries (20) and aorta (19) as well as capillary endothelium (26). Our findings, as well as those of others (18, 21) advance the concept that NO released by nNOS mediates flow- and agonist-induced dilations when eNOS is absent. Moreover, the fact that inhibition of flow-dependent dilation by ODQ (Fig. 4B) is identical to that of l-NAME (Fig. 3B) or 7-Ni (Fig. 4A) further confirms our hypothesis that the portion of the dilator response sensitive to l-NAME-7-Ni is specifically dependent on nNOS-derived NO, because the soluble guanylate cyclase-cGMP pathway is a major target for NO in vascular smooth muscle (3, 9, 24).

The adaptation to the lack of eNOS may be explained by the ontogenetic role of NOS isoforms. There is an abundant presence of nNOS in the endothelium of coronary and pulmonary arteries from neonatal rats (20), but in adults, nNOS is replaced by the predominant presence of eNOS. Thus this possibly negative feedback relationship between the two NOS isoforms may explain why endothelial nNOS becomes functional only when eNOS activity is compromised in arteries of eNOS-KO mice. Although the coupling between the stimulation by shear stress and activation of nNOS is unknown, the presence of a putative Akt phosphorylation motif in nNOS, which is responsible for the activation of the enzyme by Akt in response to agonists (and perhaps to shear stress as well), has recently been demonstrated (5). Moreover, the addition of an N-myristoylation site to nNOS to potentiate the interaction of the enzyme with biological membranes elicits the release of NO by Akt stimulation in a manner analogous to that observed with eNOS (5).

In conclusion, the present study is the first to demonstrate that shear stress, elicited by increases in flow, can activate endothelial nNOS to release NO compensating for the absence of eNOS-derived NO in the mediation of flow-induced dilation of coronary arteries of eNOS-KO mice. In male WT mice, flow-induced dilation in coronary arteries is mediated equally by both endothelial NO and prostaglandins, whereas in male eNOS-KO mice, it is nNOS-derived NO, together with an enhanced contribution by prostaglandins, that provides for the maintenance of flow-induced dilation. The results reveal a novel compensatory interaction between the two NOS isoforms and may well form the basis of the mechanism responsible for the capacity of the coronary circulation to compensate for eNOS deficiency.

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