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COX-2 contributes to the maintenance of flow-induced dilation in arterioles of eNOS-knockout mice

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Abstract

Our previous studies demonstrated that, in gracilis muscle arterioles of male mice deficient in the gene for endothelial nitric oxide synthase (eNOS), flow-induced dilation (FID) is mediated by endothelial PGs. Thus the present study aimed to identify the specific isoform of cyclooxygenase (COX) responsible for the compensatory mediation of FID in arterioles of eNOS-knockout (KO) mice. Experiments were conducted on gracilis muscle arterioles of male eNOS-KO and wild-type (WT) mice. Basal tone and magnitude of FID of arterioles were comparable in the two strains of mice. A role for COX isoforms in the mediation of the responses was assessed by use of valeryl salicylate (3 mM) and NS-398 (10 μ M), inhibitors of COX-1 and COX-2, respectively. In eNOS-KO arterioles, valeryl salicylate or NS-398 alone inhibited FID (at maximal flow rate) by ~51% and ~58%, respectively. Administration of both inhibitors eliminated the dilation. In WT arterioles, inhibition of COX-2 did not significantly affect FID, whereas inhibition of COX-1 decreased the dilation by ~57%. The residual portion of the response was abolished by additional administration of *N*^o-nitro-L-arginine methyl ester. Western blot analysis indicated a comparable content of COX-1 protein in arterioles of WT and eNOS-KO mice. COX-2 protein, which was not detectable in arterioles of WT mice, was strongly expressed in arterioles of eNOS-KO mice, together with an upregulation of COX-2 gene expression. Immunohistochemical staining confirmed the presence of COX-2 in the endothelium of eNOS-KO arterioles. In conclusion, COX-2-derived PGs are the mediators responsible for maintenance of FID in arterioles of eNOS-deficient mice.

Keywords

nitric oxide; endothelium

Our previous studies demonstrated that endothelial nitric oxide (NO) and prostaglandins (PGs) participate in the mediation of flow- and shear stress-dependent dilator responses in skeletal muscle arterioles of male mice and rats (19, 31, 34). In the presence of NO deficiency elicited by knockout (KO) of the endothelial NO synthase (eNOS) gene of mice or chronic treatment of rats with an NO synthase (NOS) inhibitor, flow-induced dilation of

arterioles was well maintained but was entirely mediated by PGs (31, 34). This suggests an upregulation of PG synthesis in compensation for NO deficiency to preserve shear stress-sensitive mechanisms in skeletal muscle microvessels.

Shear stress stimulates the release of PGs, as well as NO, through multiple pathways, one of which involves upregulation of cyclooxygenase (COX) and eNOS gene expression, respectively (5, 7, 9, 25). The PG biosynthetic pathway is initiated by activation of phospholipase A₂, which is responsible for shear stress-induced, as well as agonist-induced, arachidonic acid release from membrane phospholipids (21, 25). COX-1 and COX-2 act to convert arachidonic acid to PGH₂, which is subsequently converted to a variety of eicosanoids, depending on the downstream enzymatic machinery present in a particular cell type.

Whether there is “cross talk” between the NOS and COX pathways remains open to question. The evidence supporting a negative relation between NO and PG synthesis is provided by studies that indicate an enhanced production of PGs as a consequence of inhibition of NO synthesis (3, 25, 26). In contrast, other studies demonstrated that NO stimulates PG synthesis in endothelial cells and macrophages via a mechanism involving a reaction with the heme component that binds to the active site of COX enzymes (20, 27). Our own data, from study of shear stress-mediated regulation of arteriolar tone, seem to support a compensatory upregulation of PG synthesis when NO-mediated regulation is impaired (31, 34). In the present study, we aimed to identify the specific isoform of COX that is responsible for the maintenance of flow- and shear stress-induced dilation in skeletal muscle arterioles of eNOS-KO mice. In addition, we attempted to clarify the molecular basis of the upregulation of COX-2, as well as the site of its formation.

METHODS

Animals and Isolation of Arterioles

Heterozygous eNOS (+/−) mice, originally developed by Shesely et al. (28), were interbred in the Department of Comparative Medicine, New York Medical College, to generate eNOS wild-type (WT, +/+) and homozygous mutant (eNOS-KO, −/−) mice. The mice were killed by inhalation of 100% CO₂ at 22–24 wk of age. First-order gracilis muscle arterioles were isolated and cannulated in a vessel chamber (13, 31). All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conform to the National Institutes of Health and American Physiological Society guidelines for the use and care of laboratory animals.

Experimental Procedures

Flow-induced dilation—Changes in arteriolar diameter response to increases in perfusate flow (0–10 μl/min in 2 μl/min steps) were studied at 80 mmHg perfusion pressure.

In the first protocol, the role of specific isoforms of COX (COX-1 and COX-2) in the mediation of flow-induced dilation was studied in arterioles of both strains of mice. After flow-diameter curves were obtained in the control condition, vessels were subjected to

valeryl salicylate (VS, 3 mM) or NS-398 (10 μ M), specific inhibitors of COX-1 and COX-2 (1, 35), respectively, for 30 min, and then flow-induced dilation was reassessed.

In the second series of experiments, flow-induced dilation was obtained before and after exposure of vessels of both strains of mice to the simultaneous presence of COX-1 and COX-2 inhibitors. In arterioles of WT mice, flow-induced dilation was also assessed in the control condition and after incubation of the vessels with 100 μ M *N*^ω-nitro-L-arginine methyl ester (L-NAME) for 30 min.

In separate experiments, flow-induced dilation (10 μ l/min) was assessed in the control condition and after inhibition of the PGI₂ receptor with 10⁻⁶ M CAY-10441. The efficacy of the inhibition was assessed by vasodilator responses to iloprost (10⁻⁷ M), a stable analog of PGI₂, before and after administration of CAY-10441. Dilation to 10⁻⁶ M adenosine was used to assess smooth muscle-dependent responses before and after administration of the inhibitors.

Passive diameter—At the conclusion of each experiment, the suffusion solution was changed to a Ca²⁺-free solution containing 1 mM EGTA. The vessels were incubated for 10 min to reach maximal diameter (passive diameter) at 80 mmHg perfusion pressure.

Western blotting—Gracilis arteries (~3 mm long) were solubilized in Laemmli buffer, sonicated twice for 1 min each, and boiled for 1 min to denature the proteins. Samples were separated on 10% SDS-polyacrylamide gels. Membranes were probed with polyclonal antibodies of COX-1 and COX-2 (Cayman Chemical) overnight at 4°C. The secondary antibody was conjugated to horseradish peroxidase according to the Amersham ECL-Plus protocol. β -Actin was used to normalize for loading variations.

Real-time RT-PCR—Gracilis muscle arterioles were pulverized in liquid nitrogen, and total RNA was extracted using TRI Reagent (Sigma). RNA (1 μ g) was reverse transcribed with random primers (Promega) and SuperScript III (Invitrogen). cDNA was utilized for quantitative real-time RT-PCR using the LightCycler with the Fast-Start DNA Master SYBR Green I kit (Roche Diagnostics). Primers for COX-1 and COX-2 (Sigma Genosys) were designed as follows: 5'-TGGCCAAGGTCTACCCCG-3' (sense) and 5'-CTCTGTACCCAAAGACTGCC-3' (antisense) for COX-1 and 5'-AGTCTCTCAATGAGTACCGGAAA-3' (sense) and 5'-AAGTTCTTCAAATGATGTGTACGG-3' (antisense) for COX-2. A relative quantification method (2^{-C_t} , where C_t is cycle threshold) (22) was used to evaluate the expression of each gene in vessels of KO and WT mice. RT-PCR of GAPDH was used as an internal control and for normalization of all data.

Immunohistochemistry—Gracilis muscle was perfused (80 mmHg) and fixed with freshly prepared 4% paraformaldehyde. Paraffin sections (5 μ m) were probed with anti-COX-1 and anti-COX-2 antibodies (Cayman Chemical) and further processed with the Vectastain Elite ABC staining method using 3,3'-diaminobenzidine as a peroxidase substrate, according to the manufacturer's protocol (Vector), and counterstained with hematoxylin. Control staining was performed by incubation of COX-1 or COX-2 blocking

peptides with corresponding antibodies for 1 h before application to the slides or by elimination of the primary antibodies from the staining process.

Chemicals

VS, NS-398, iloprost, and CAY-10441 were obtained from Cayman Chemical. VS was dissolved in physiological salt solution directly with a brief sonication. NS-398 was dissolved in DMSO at 50 mM and further diluted with physiological salt solution. All other chemicals were obtained from Sigma (St. Louis, MO).

Calculations and Statistics

Flow-induced dilation was normalized by the passive diameter and expressed as percentage of passive diameter or as change in diameter as percentage of passive diameter [(diameter obtained after flow-induced dilation – basal diameter)/passive diameter · 100%] to eliminate the influence of the difference in vessel size on data comparison between the two groups. The difference in flow-diameter curves was compared by repeated-measures ANOVA, followed by the Tukey-Kramer multiple-comparison test. The ratio of band densities of Western blots of COX-1 or COX-2 to β -actin was compared by Student's *t*-test. $P < 0.05$ was considered significant. Values are means $\pm$ SE; *n* indicates the number of mice.

RESULTS

Flow-Induced Dilation

Arteriolar active and passive diameters of eNOS-KO mice (69 ± 3 and 114 ± 4 μ m, respectively) were significantly smaller than those of WT mice (79 ± 4 and 127 ± 6 μ m, respectively). As a result, the basal tone of arterioles, expressed as percentage of passive diameter, was similar in both strains of mice (60 ± 2 and 62 ± 1 %). Arteriolar dilations to increases in perfusate flow were not significantly different in eNOS-KO and WT (at maximal flow rate) mice (77 ± 2 and 78 ± 1 % of passive diameter, respectively). Also, calculated shear stress at maximal flow rate was similar (13 – 16 dyn/cm²) in vessels of the two strains of mice. The specific role of COX-1 and COX-2 in the mediation of PG-dependent, flow-induced dilations of arterioles of eNOS-KO mice was tested with VS and NS-398, respectively. Neither VS nor NS-398 affected significantly the basal tone and adenosine-induced dilations (not shown) in arterioles of either strain of mice. Each inhibitor significantly inhibited the dilation by ~ 50 % (Fig. 1, *A* and *B*); combination of both inhibitors essentially eliminated the responses (Fig. 1*C*).

The endothelial mediators responsible for flow-induced dilations of arterioles of WT mice are illustrated in Fig. 2. In contrast to its effect on eNOS-KO arterioles, NS-398 did not affect the response of WT arterioles (Fig. 2*B*), whereas VS alone or NS-398 + VS significantly inhibited the dilation by ~ 50 % (Fig. 2, *A* and *B*). Flow-induced dilation was abolished by additional administration of L-NAME (Fig. 2*C*).

To confirm that flow-induced dilation in arterioles of eNOS-KO mice is mediated by COX-dependent metabolites, flow-induced dilation was compared in arterioles of WT and eNOS-KO mice before and after administration of CAY-10441, a specific antagonist of the PGI₂

receptor. CAY-10441, which abolished iloprost-induced dilation, inhibited flow-induced dilation in arterioles of WT and eNOS-KO by ~40% and 70%, respectively (Fig. 3).

COX-1 and COX-2 Protein and Gene Expressions

Evidence provided by Western blotting (Fig. 4) indicates that COX-1 protein content in gracilis muscle arterioles of WT and eNOS-KO mice was comparable. However, COX-2 protein, which was basically undetectable in arterioles of WT mice, was strongly expressed in eNOS-KO vessels. The densitometric ratio of COX-1 to β -actin (Fig. 4B) was not different in vessels of the two groups, but the densitometric ratio of COX-2 to β -actin (Fig. 4C) increased significantly, by ~250-fold, in vessels of eNOS-KO mice compared with WT mice. Normalized gene expression of COX-2 increased by 16-fold in arterioles of eNOS-KO mice compared with WT mice (Fig. 5). COX-1 mRNA was not different in the vessels of WT and eNOS-KO mice.

The distribution of COX-1 and COX-2 in the vascular wall of gracilis muscle arterioles was examined by immunostaining of cross sections of gracilis muscle. Similar to the findings from Western blot analysis, the presence of COX-1 was diffuse and comparable in endothelial and smooth muscle layers of arterioles of WT and eNOS-KO mice (Fig. 6, *a* and *e*). However, COX-2 was more intensively stained in the endothelium than in smooth muscle cells (Fig. 6*g*) in vessels of eNOS-KO mice, whereas there was no significant staining of COX-2 in the endothelium of WT vessels (Fig. 6*c*). The specificity of COX-1 and COX-2 stains was demonstrated by complete blocking of the positive stains by preincubation of the antibodies with COX-1 or COX-2 blocking peptides (Fig. 6, *b* and *f*, and *d* and *h*, respectively). When primary antibodies were eliminated from the staining procedure, no positive staining was observed.

DISCUSSION

This study extends our previous findings in which a compensatory increase in arteriolar PG synthesis in response to shear stress, as a consequence of eNOS deficiency, was evidenced by inhibition of flow-induced dilation with indomethacin (31). In the present study, a specific role of COX-2 in the preservation of shear stress-sensitive mechanisms in eNOS-KO arterioles was identified. This was indicated by the finding that NS-398 significantly inhibited a component of flow-induced dilation, which otherwise is sensitive to L-NAME in arterioles of WT mice. Additionally, VS eliminated the residual portion of the dilation in eNOS-KO vessels. This NS-398-sensitive, PG-mediated dilation to shear stress was associated with a significantly increased COX-2 and protein expression in the endothelium of eNOS-KO vessels. This study is, to the best of our knowledge, the first to provide evidence for the upregulation of COX-2 expression in arterioles of eNOS-KO mice.

As demonstrated previously, active and passive diameters of arterioles isolated from eNOS-KO mice were smaller than those from WT mice (31). We presumed that this was caused by a vascular remodeling attributable to the elevated blood pressure in eNOS-null mice, but a causal relation has not been established. On the other hand, the basal tone of arterioles in eNOS-KO mice was not significantly different from that in WT mice; moreover, the capacity of the arteriolar endothelium to sense shear stress, followed by vasodilation, was

not significantly altered in eNOS-KO arterioles. This indicates that endothelial cells adapt to the chronic lack of NO synthesis and maintain a normal or close-to-normal regulation of vascular tone via upregulation of an NO-independent mechanism.

Similar to our previous findings (31), corelease of NO and PGs mediates flow-induced dilation of skeletal muscle arterioles of WT mice, as shown by our findings that VS attenuated the dilation by ~50% and abolished the response when administered in combination with L-NAME (Fig. 2). Also, the PG-mediated responses were not affected by NS-398 (Fig. 2B), and COX-2 protein was not expressed in these vessels (Fig. 4), suggesting that, under normal conditions, COX-2-derived PGs do not contribute to the mediation of flow-induced dilation.

In adapting to the lack of endothelium-derived NO, flow-induced dilation of arterioles of male eNOS-KO mice is mediated solely by PGs that are derived from COX-1 and COX-2. This is evidenced by the fact that each inhibitor of the COX isoforms blocks flow-induced dilation by ~50% (Fig. 1, A and B) and a combination of both inhibitors eliminates the dilations (Fig. 1C). Moreover, as shown in Fig. 3, PGI₂ is the major mediator responsible for dilation in these vessels. The cascade of events, involving conversion of the physical stimulus of shear stress to chemical signals, followed by the synthesis/release of vasoactive autacoids, takes place in the vascular endothelium. Signaling cascades responsible for shear stress-stimulated PG release have been demonstrated to be linked to the activation of phospholipase C at the cell membrane, as well as increased intracellular Ca²⁺, followed by stimulation of downstream COX enzymes to initiate conversion of arachidonic acid to PGs (5, 7). Our results provide evidence of a predominant expression of COX-2 protein (Figs. 4 and 6) in endothelial cells of eNOS-KO, but not WT, arterioles, suggesting that a COX-2-dependent signaling cascade is responsible for the shear stress-initiated release of PGs. Interestingly, COX-2 was originally believed to be induced only in response to cytokines to act continuously to release increased amounts of PGs, as observed during the process of inflammation (11). In the present study, however, COX-2 seems to be functional only in response to shear stress, not in basal conditions, because although NS-389 inhibited flow-induced dilation by 50%, it had no effect on the basal tone of arterioles. An increased expression of endothelial COX-2 in response to shear stress has previously been reported (25, 33). On the other hand, we, as well as others, have noted that, in eNOS-KO and WT mice, treatment of arteries/arterioles with L-NAME or indomethacin or removal of the endothelium barely affected basal vascular diameters or smooth muscle resting membrane potential, indicating that basal release of endothelial NO and PGs may not contribute significantly to the maintenance of vascular tone (6, 28, 31). Together, these data suggest that, in response to NO deficiency, COX-2 is upregulated and accounts for the compensatory preservation of flow-induced dilation.

The literature regarding the cross talk between the NOS and COX pathways is divided with respect to whether NO activates or inhibits PG synthesis. Although our findings are likely to be consistent with the studies that support the hypothesis of a negative correlation between the two mediators (2, 3, 11, 26), still the question arises as to why this compensatory activity depends specifically on COX-2. This issue could perhaps be best explained by the evidence showing that NO exerts divergent effects on the COX isoforms, with potentiating COX-1

but inhibiting COX-2 (8). With the use of cultured fibroblasts of COX-1-KO and COX-2-KO mice, it was demonstrated that exposure of COX-2-KO cells to NO significantly increases the release of PGE₂ without changing COX-1 mRNA or protein expression, suggesting a nongenomic regulation of COX-1 enzymatic activity. In contrast, NO inhibited COX-2-derived PGE₂ production in COX-1-KO cells and LPS-stimulated macrophages, associated with a decreased expression of COX-2 mRNA and protein, as well as nitration of COX-2, leading to an impaired catalytic activity of the enzyme (10). In addition, a recent study reported a role for L-NAME in an initiation of damage to the intestine of rats via upregulation of COX-2 mRNA, further suggesting a negative relation between NOS and COX-2 (24). Thus these studies support our findings that an upregulation of endothelial COX-2 expression (gene and protein) in response to shear stress could be attributable to NO deficiency (25, 33). Consistent with the present findings, upregulation of COX-2 in the course of adaptation of an attenuated dilation to shear stress in mesenteric arterioles of L-NAME-treated rats has also been reported (12). Interestingly, we also found that the VS-sensitive portion of flow-induced dilation, as well as COX-1 protein expression, was comparable in arterioles of WT and eNOS-KO mice, indicating that the function of COX-1 in the mediation of flow-induced dilation of microvessels seems to be relatively stable and less affected by NO than by COX-2, a phenomenon that has also been observed in previous studies (2, 11, 12, 14–16, 29, 30, 32). It has also been suggested that COX-1 is more resistant than COX-2 to nitration by NO, because micromolar concentrations of NO elicit COX-2 nitration without effects on COX-1 (8). Results of the present study lead to the conclusion that COX-2-derived PGs, together with those derived from COX-1, compensate for the deficiency of eNOS to preserve shear stress-dependent mechanisms in the microvascular endothelium.

Perspectives

PGs accompanied by NO participate in the mediation of shear stress-sensitive regulation of microvascular tone. Whereas many aspects of the action of shear stress on endothelial NO production remain open to question, even less is known about the equally potent ability of shear stress to stimulate endothelial PG synthesis, an issue that becomes especially important when NO synthesis is impaired or absent. The interactions between NO and PGs are complex and depend on the local environment in which these endothelial mediators are synthesized. Also, changes in the expression of endothelial mediators in response to NO deficiency are species, tissue, and cell specific, as well as sex and stimulus dependent (13, 17). For example, in skeletal muscle arterioles of eNOS-KO mice, dilation to ACh is counterbalanced by endothelium-derived hyperpolarizing factor-mediated responses (4, 16) and by neuronal NOS-derived NO in cerebral arteries (23) and PGs in mesenteric arteries (6), whereas flow-induced dilation in coronary arteries is counterbalanced by a neuronal NOS-dependent mechanism (15). Thus it is tempting to speculate that perhaps one or more redundant compensatory mechanisms become activated after selective gene deletion or enzyme inactivation. Moreover, it is important to point out that, in contrast to other NO deficiency models, such as hypertension or heart failure, in which NO deficiency is a consequence of the pathological process, in animals that underwent deletion of the eNOS gene or were treated with an NOS inhibitor, NO deficiency acts as an initial cause to activate the compensatory process. Therefore, it could be expected that in the former, the

compensation, if any, is usually not complete and is eventually followed by decompensation. In this context, we found previously that, in coronary arterioles of dogs, maintenance/compensation of flow-induced dilation was only observed in the early stage of heart failure (3 wk) and was followed quickly by permanent impairment of the responses (32). In adult spontaneously hypertensive rats, shear stress-induced dilation of skeletal muscle arterioles was attenuated by ~50%, attributable simply to the loss of the NO-mediated portion of the response (18). However, in rats of the same age treated chronically with L-NAME, high blood pressure was associated with a preserved shear stress-induced dilation that was mediated by PGs (34), a response similar to that observed in the present study. Although we do not have information about the time course of PG-dependent compensation of shear stress-induced responses in eNOS-KO mice and L-NAME-treated rats, our results suggest a relatively complete compensation in these NO deficiency models. Thus an understanding of different compensatory mechanisms in response to NO deficiency may be important in the investigation of adaptive mechanisms, as well as interactions among the various endothelial mediators, to maintain normal vascular function.

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Fig. 1.

Flow-induced dilation of gracilis muscle arterioles of endothelial nitric oxide synthase (eNOS)-knockout (KO) mice in control conditions and after inhibition of cyclooxygenase (COX)-1 with 3 mM valeryl salicylate (VS, $n = 6$; A), COX-2 with 10 μM NS-398 ($n = 8$; B), and NS-398 + VS (C). PD, passive diameter. $*P < 0.05$ (repeated-measures 2-way ANOVA).

Fig. 2.

Flow-induced dilation of gracilis muscle arterioles of wild-type (WT) mice in control conditions and after inhibition of COX-1 with VS ($n = 6$; A), COX-2 with NS-398 and COX-2 and COX-1 with NS-398 + VS ($n = 5$; B), and COX-1 and NOS with VS + N^G -nitro-L-arginine methyl ester (L-NAME, 100 μM , $n = 6$; C). * $P < 0.05$.

Fig. 3.

Effect of the PGI₂ receptor antagonist CAY-10441 (CAY, 10⁻⁶ M) on flow (10 µl/min)-induced dilation and dilation to the PGI₂ analog iloprost (10⁻⁷ M) in arterioles of WT and eNOS-KO mice (*n* = 5). **P* < 0.05 vs. control (Ctr). #*P* < 0.05 vs. WT.

Fig. 4.

A: representative Western blot of COX-1 and COX-2 in gracilis muscle arterioles of eNOS-KO and WT mice. *B* and *C*: normalized data of densitometry ratio of COX-1 to β -actin and COX-2 to β -actin from two blots. * $P < 0.05$ vs. WT.

Fig. 5.

COX-1 and COX-2 mRNA expression in gracilis muscle arterioles of WT ($n = 4$) and eNOS-KO ($n = 4$) mice as measured by real-time RT-PCR. Data are presented as fold change in COX-1 and COX-2 gene expression in KO mice normalized to GAPDH and relative to WT mice. * $P < 0.05$ vs. WT.

Fig. 6.

Cross sections of gracilis muscle arterioles labeled for COX-1 (*a* and *e*) and COX-2 (*c* and *g*) from WT (*a–d*) and eNOS-KO (*e–h*) mice. COX-1 and COX-2 were stained by 3,3'-diaminobenzidine (brown). Negative controls were obtained by treatment with blocking peptides of anti-COX-1 (*b* and *f*) and anti-COX-2 (*d* and *h*) antibodies. *i*: objective micrometer (10 μm per unit) showing final magnification. Staining of COX-1 in endothelial and smooth muscle layers of vessels is comparable in eNOS-KO and WT mice and is blocked by anti-COX-1 blocking peptide. COX-2 is prominently stained in endothelial, as well as smooth muscle, cells of arterioles from eNOS-KO mice and is abolished by COX-2 blocking peptide.