Contribution of 20-HETE to Augmented Myogenic Constriction in Coronary Arteries of Endothelial NO Synthase Knockout Mice

An Huang, Dong Sun, Changdong Yan, John R. Falck, Gabor Kaley

Abstract—Previous studies suggested an important role for 20-HETE in the regulation of myogenic responses. Thus, pressure–diameter relationships were investigated in isolated, cannulated coronary arteries (≈100 μm) from male endothelial NO synthase knockout (eNOS-KO) and wild-type (WT) mice. All arteries constricted in response to step increases in perfusate pressure from 20 to 100 mm Hg. This constriction was significantly enhanced from 40 to 100 mm Hg in arteries of eNOS-KO compared with those of WT mice. For example, at 60 and 100 mm Hg, respectively, the normalized diameter (expressed as a percentage of the corresponding passive diameter) of arteries of eNOS-KO mice was 10% and 12% smaller than that of WT mice. Removal of the endothelium did not significantly affect the responses of vessels from either strain of mice. However, N-methylsulfonyl-12,12-dibromododec-11-enamide (5 × 10⁻⁶ M), an inhibitor of cytochrome P-450 (CYP)/ω-hydroxylase, significantly attenuated the greater myogenic constriction of arteries from eNOS-KO mice by ≈12% at each pressure step but did not significantly affect responses of those from WT mice, leading to a comparable myogenic response in the 2 strains. Western blot analysis demonstrated a comparable CYP4A protein content in coronary arteries of the 2 strains of mice. However, production of 20-HETE, measured by fluorescent high-performance liquid chromatography assay was ≈2.7-fold greater in eNOS-KO compared to WT mice. Thus, as a function of eNOS deficiency, the enhanced coronary artery constriction to pressure is attributable to an increased activity of ω-hydroxylase, which, consequently, increases the synthesis of 20-HETE in vascular smooth muscle. (Hypertension. 2005;46:607-613.)

Key Words: constriction • nitric oxide • coronary artery disease

The issue as to how the vascular system maintains basal tone and autoregulates blood flow and organ perfusion, as well as capillary hydrostatic pressure, has been investigated extensively. As demonstrated, one of the processes participating in these vital functions is the myogenic response, which is characterized by membrane depolarization and consequently constriction of vascular smooth muscle in response to increases in transmural pressure. The myogenic response has been demonstrated to be principally dependent of smooth muscle, although a role of the endothelium in the mediation of the response in certain pathophysiological conditions has also been reported. Since first described by Bayliss in 1902, significant advances in the understanding of mechanisms underlying the myogenic response have been made. More recently, the integrin family of cell adhesion molecules as possible "transducers" or "sensors" of changes in vascular smooth muscle tension has attracted attention, by which related downstream factors, such as peptides containing integrin-specific amino acids, calcium/L-type Ca²⁺ channels, tyrosine phosphorylation, mitogen-activated protein kinase (MAPK) activation, as well as 20-HETE, the CYP450/ω-hydroxylation product of arachidonic acid, may become involved. As one of the primary eicosanoids in certain microcirculatory beds, 20-HETE acts as an intracellular second messenger that plays an integral role in the signal transduction processes underlying the development of pressure-dependent myogenic tone, as indicated by the fact that inhibition of 20-HETE formation in vascular smooth muscle activates K⁺ channels, resulting in hyperpolarization and attenuation of myogenic tone. One of the modulators of 20-HETE formation is NO, which inhibits CYP4A/ω-hydroxylase expression and function. In the absence or in the presence of an impaired synthesis of NO, vasoconstriction in response to a variety of stimuli is enhanced, mediated by an increased production of 20-HETE.

In the coronary circulation, myogenic mechanisms contribute significantly to the control of coronary resistance, and NO plays a role in the modulation of coronary vascular tone. Whereas 20-HETE has been demonstrated to participate in the mediation of myogenic responses in cerebral, renal, skeletal muscle, and mesenteric arteries, there is scant evidence regarding the specific role of 20-HETE in the
modulation of the response in coronary arteries, especially when bioactivity of NO is impaired. Recently, the blockade of CYP450/ω-hydroxylase has been shown to stimulate an endogenous cardioprotective pathway, as indicated by a profound reduction in myocardial infarct size after inhibition of 20-HETE production during the process of myocardial ischemia-reperfusion.15

Given the negative modulatory effect of NO on CYP450/ω-hydroxylase activity as well as a role of 20-HETE in the regulation of myogenic tone, we tested the hypothesis that ischemia-reperfusion.15 profound reduction in myocardial infarct size after inhibition endogenous cardioprotective pathway, as indicated by a

Role of the Endothelium
Myogenic responses were assessed before and after removal of the endothelium by injection of air into the vessel lumen.2

Role of 20-HETE
Role of 20-HETE in the regulation of the myogenic response was assessed by incubation of vessels with N-methylsulfonyl-12,12-dibromomondec-11-enamide (DDMS), a specific inhibitor of 20-HETE synthesis.17 After control pressure–diameter curves were obtained, vessels were subjected to DDMS (5×10⁻⁷ mol/L) for 30 minutes before the pressure–diameter relationships were assessed once more.

Passive Diameter
At the conclusion of each experiment, the suffusion solution was changed to a Ca²⁺-free solution containing 1 mmol/L EGTA. Vessels were incubated for 10 minutes followed by recording of diameter at each pressure step.

Western Blotting18
Isolated coronary arteries (~3 mm in length) were solubilized in modified Laemmli buffer and sonicated (2 minutes) to denature the proteins.

Samples were loaded on a SDS-PAGE gel. Membranes were probed with primary antibody (polyclonal antibody of CYP450-4A1) dissolved in PBS with 1% nonfat dry milk and 0.1% Tween 20 overnight at 4°C, and then were probed with secondary antibody after washing with PBS. The secondary antibody was conjugated to horseradish peroxidase. The exposed film was developed in a Kodak X-Omat developer. Specific bands were normalized to β-actin.

Fluorescence High-Performance Liquid Chromatography Assay for 20-HETE

Purification of 20-HETE
As described previously,19 coronary arteries (~2 to 6 µg protein per vessel) were isolated and incubated with indomethacin (3×10⁻⁵ mol/L), N⁶-nitro-l-arginine methyl ester (l-NNAME; 10⁻⁴ mol/L), 6-(2-proparglyoxyphenyl)hexanoic acid (5×10⁻³ mol/L), NaNPH (10⁻³ mol/L), and arachidonic acid (10⁻³ mol/L) for 1 hour. Identical amounts of 20(S,Z),14(Z)-hydroxyeoicosadienoic acid (WIT-002) were added to each sample as internal standards. After extraction, the samples were evaporated to dryness under argon.

Fluorescent Derivation Reaction
The method used is similar to that described previously.19 Samples were resuspended in 136 µL anhydrous acetonitrile (ACN) plus 4 µL N,N-diisopropylethylamine catalyst. Freshly prepared 10 µL fluorescent dye, 2-(2,3-naphthalimino)ethyl-trifluoromethanesuphanonate (NE-OTf; 2 mg/mL) dissolved in ACN was added and vortexed gently for ~2 seconds. Samples were kept at 4°C for 30 minutes followed by evaporation to dryness with argon.

Fluorescence-Detected High-Performance Liquid Chromatography
The method used was the same as that described previously.20 Samples were resuspended in 20 µL methanol following by injection of 18 µL into the Jasco high-performance liquid chromatography (HPLC) system. The sample was separated on a 4.6×250 mm Symmetry C18 reverse-phase HPLC column (Waters) isocratically, at a rate of 1.3 mL/min using methanol:water:acetic acid at 82:18:0.1 vol/vol/vol as a mobile phase. Fluorescence intensity was monitored continuously using a fluorescence detector (model L-7480; Hitachi) at a gain of 100 nm, with excitation wavelength 260 nm and emission wavelength 396 nm. Final fluorescence intensity of the 20-HETE in the sample was determined by comparing the area of 20-HETE peak to that of internal standards (WIT-002) and was further normalized to the protein content of each sample.

Chemicals
Solutions related to HPLC analysis were purchased from Fisher Scientific. N,N-diisopropylethylamine and NE-OTf were obtained from Aldrich and Molecular Probes Inc., respectively. Antibody to CYP450-4A1 was obtained from Affinity BioReagents. Standard 20-HETE was obtained from Cayman Chemical. DDMs and WIT-002 were synthesized by J.R. Falck. All other chemicals were purchased from Sigma.

Statistics
Changes in vessel diameter in response to increases in pressure were normalized, at each pressure step, to the passive diameter (PD). Data obtained by HPLC assay were analyzed by using Borwin 1.30.08 version software. Statistical significance was calculated by repeated-measures ANOVA followed by the Tukey–Kramer multiple-comparison test and Student t test. Values are means±SE. Significance level was taken at P<0.05. n indicates the number of mice.

Results
Pressure-Induced Myogenic Constriction
The top of Figure 1 shows that active diameter of vessels of WT mice, at 20 and 40 mm Hg was comparable to their PD. The myogenic constriction of vessels in response to increases in perfusate pressure started at 60 mm Hg and was maintained as pressure was increased to 80 and 100 mm Hg. Thus, the
The active tone of vessels at these pressure steps was 74%, 71%, and 72%, respectively, of their PD. However, in vessels of eNOS-KO mice (bottom), unlike in WT arteries, basal diameter of vessels at 20 and 40 mm Hg was significantly smaller than their PD and was followed by a greater myogenic constriction when pressure reached 60, 80, and 100 mm Hg. This leads to a significantly enhanced myogenic tone at each pressure step of 64%, 61%, and 60% of PD, respectively, compared with that of WT mice. When active diameter at each pressure step was expressed as a percentage of PD and further compared between the 2 strains of mice (Figure 2a), there was a parallel but greater downward shift of the myogenic response curve in arteries of eNOS-KO mice. Also, myogenic constriction of eNOS-KO arteries started earlier (at 40 mm Hg) compared with that of WT arteries, as indicated by a smaller normalized active diameter at 40 mm Hg than at 20 mm Hg.

The role of endothelium in the myogenic constriction was assessed by comparison of the responses before and after removal of the endothelium. Data depicted in Figure 2 show that the significant difference between the 2 pressure-diameter curves observed in intact vessels (Figure 2a) was maintained after denudation of the endothelium (Figure 2b), indicating that the enhanced myogenic constriction in arteries of eNOS-KO mice is independent of the endothelium. Indeed, the comparable myogenic indexes in endothelium-intact (Figure 2c) and endothelium-denuded vessels (Figure 2d) of WT and eNOS-KO mice demonstrate a similar pattern of myogenic reactivity.

To determine whether 20-HETE produced from vascular smooth muscle accounts for the enhanced myogenic constriction of eNOS-KO arteries, the effect of inhibition of CYP450/ω-hydroxylase by DDMS was assessed. Figure 3 shows that the pressure-diameter curve of WT mice was not significantly affected by DDMS (top) but that it shifted significantly upward in vessels of eNOS-KO mice (bottom), leading to a comparable curve in the 2 strains of mice. In a separate experiment, the effects of 20-HETE on the potentiation of vascular myogenic constriction was further confirmed by the results that exogenous administration of 20-HETE reversed the attenuated myogenic constriction of eNOS-KO arteries after treatment with DDMS (data not shown).

Molecular Analyses
Protein content of CYP450-4A1 measured by Western blotting is shown in Figure 4, indicating that there is no significant difference between coronary arteries, as well as aorta (data not shown) of WT and eNOS-KO mice.

Fluorescence HPLC Analysis
Retention time of 20-HETE and WIT-002 was 32 minutes and 65 minutes, respectively, detected in the standard containing 40 ng of 20-HETE and 200 ng of WIT-002. Figure 5 shows the fluorescence intensity of 20-HETE, indicating that the production of 20-HETE in coronary arteries of eNOS-KO mice was significantly increased compared with that of WT mice, and that DDMS prevented its synthesis in vessels of both strains.
Discussion

A previous study provided indirect evidence showing that exogenous administration of 20-HETE potentiates porcine coronary arterial tone via endothelium-dependent and -independent mechanisms. In the present study, we provided direct evidence by using a pharmacological inhibitor, as well as measures of endogenous release of 20-HETE from isolated arteries, to indicate that as a result of eNOS deficiency, myogenic constriction of coronary arteries was enhanced via an endothelium-independent mechanism involving increased activity of CYP450/ω-hydroxylase.

We demonstrated previously in coronary arteries of eNOS-KO mice, that flow-induced dilation was maintained because of a compensatory increased expression of neuronal NOS (nNOS) as well as an augmented release of vasodilator prostaglandins (PGs) from endothelial cells. Interestingly, the maintained flow-induced dilation of coronary arteries of eNOS-KO mice was observed in the presence of an enhanced basal vascular tone that was not affected by removal of the endothelium or inhibition of the endothelial mediators by L-NAME and indomethacin. These findings led us to hypothesize the existence of an endothelium-independent mechanism by which coronary arteries of eNOS-KO mice exhibit greater contractility in response to pressure. To this end, experiments were conducted on isolated coronary arteries from male eNOS-KO and WT mice to compare the arterial responses to increases in intravascular pressure and furthermore to elucidate the mechanism responsible for the responses.

Enhanced Myogenic Constriction of Coronary Arteries in eNOS-KO Mice

We found that in response to increases in perfusate pressure, coronary arteries of both strains of mice exhibited significant constrictions that started at 60 mm Hg in WT mice but started earlier (at 40 mm Hg) in eNOS-KO mice (Figures 1 and 2). The myogenic response curve of vessels of eNOS-KO mice shifted significantly downward, in parallel with that of WT mice (Figure 2a), indicating that an increased basal, but not pressure-induced, release of a vasoconstrictor is responsible for the enhanced myogenic tone. This conclusion was further confirmed by the identical myogenic index curves illustrated in Figure 2c, suggesting a similar myogenic reactivity to changes in pressure in vessels of both groups. This finding differs from previous studies showing that elevations in transmural pressure from 20 to 140 mm Hg produced dose-dependent increases in the release of 20-HETE in cerebral arteriolar smooth muscle of rats. Moreover, removal of the endothelium did not significantly affect the constrictions or the myogenic indexes (Figure 2b and 2d), indicating that the greater basal release of the constrictor mediator originates from vascular smooth muscle.
The above results are in keeping with previous findings 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. This depolarization is unlikely to be attributable to the absence of endothelial NO, per se, because in eNOS-KO and WT mice, treatment of the arteries with either L-NAME or indomethacin, or endothelial removal, did not affect the resting membrane potential, indicating that basal release of endothelial mediators does not modulate membrane potential or myogenic tone of vascular smooth muscle.

Role of 20-HETE in Enhanced Myogenic Constriction of Arteries of eNOS-KO Mice

Data shown in Figure 3 support the hypothesis that NO deficiency may elicit activation of CYP450/ω-hydroxylase that would otherwise be suppressed by NO, resulting in a 20-HETE–dependent augmented myogenic constriction in coronary arteries of eNOS-KO mice. 20-HETE has been demonstrated even in physiological conditions to be an essential component of arteriolar responses to elevations in transmural pressure in the cerebral, renal, and skeletal muscle microcirculation. However, in the present study, contribution of 20-HETE to the mediation of myogenic constriction was only observed in NO deficiency, as indicated by the fact that DDMS did not significantly affect the myogenic contractions of normal vessels but attenuated the response via inhibiting the enhanced portion of the response of vessels of eNOS-KO mice, normalizing, as a result, the myogenic response curve to that observed in WT vessels (Figure 3). Also, additional administration of 20-HETE to eNOS-KO vessels prevented the DDMS-induced reduction in vascular tone (data not shown), a response that is consistent with previous findings suggesting an increased myogenic tone caused by exogenous 20-HETE. Regarding the failure of nNOS-derived NO to buffer the enhanced myogenic tone in coronary arteries of eNOS-KO mice, we noted that unlike the arterioles isolated from rats in which endothelial mediators modulate myogenic tone in a variety of vascular beds, in mouse preparations, basal vascular tone was not significantly affected by the endothelium, a phenomenon that has also been observed by others. Previous studies also suggested an interaction between 20-HETE and PGs, both being metabolites of arachidonic acid via the CYP450 and cyclooxygenase pathways. For instance, in porcine coronary arteries, 20-HETE elicited vasoconstriction that was significantly attenuated by a cyclooxygenase inhibitor, suggesting the involvement of an endothelium-derived vasoconstrictor prostanoid. In bovine coronary arteries, 20-HETE paradoxically relaxed coronary rings, a response that was inhibited by indomethacin, revealing a role for a vasodilator PG. However, in our studies, as a function of eNOS deficiency, upregulation of vasodilator PGs did not buffer the greater myogenic tone, suggesting that the response is caused by 20-HETE. Although it is believed that the source of 20-HETE is primarily vascular smooth muscle, the release of 20-HETE and its metabolites from bovine and porcine coronary endothelial cells have also been reported, but the physiological relevance of this remains to be elucidated.

The expression of CYP4A protein in the vasculature is increased with decreasing vessel size, consistent with an important role for 20-HETE in the maintenance of vascular tone. Moreover, CYP4A has the highest catalytic activity toward arachidonic acid ω-hydroxylation and is well expressed in the vasculature. The expression of CYP4A (Figure 4) as well as the release of 20-HETE (Figure 5) was, to the best of our knowledge, first detected in mouse coronary arteries and arterioles. Because of a limitation in sample content, we could not test the vascular release of 20-HETE in response to different pressures, but our functional data excluded the involvement of pressure, per se, in the altered responses. We found that there was no significant difference in the expression of CYP4A protein in coronary arteries between the 2 strains of mice, but the activity of ω-hydroxylase was significantly increased in eNOS-KO mice, as evidenced by the >2-fold increase in 20-HETE fluorescence intensity. The inhibitory effect of NO on the activity of ω-hydroxylase, followed by a significantly attenuated release of 20-HETE, has been suggested to be attributable to a covalent attachment of the prosthetic heme group to CYP4A enzymes. This can also serve as an explanation for the observation that even in the presence of continuous expression of CYP4A in WT arteries, 20-HETE was not responsible for the generation of the myogenic response because the enzyme is inhibited by NO via the heme-binding mechanism. Deficiency of NO or impaired NO bioactivity disinhibits ω-hydroxylation, allowing it to be activated to synthesize 20-HETE. In the present study, we have as yet no specific explanations for the puzzle as to the failure of nNOS-derived NO to compensate for eNOS deficiency to efficiently inhibit the synthesis of 20-HETE, an issue that needs to be further clarified.

Possible Mechanisms Responsible for Regulation of Myogenic Tone by 20-HETE

The major biological role of 20-HETE, especially in the development of myogenic mechanisms, has been studied in detail. The question as to whether 20-HETE is the primary mediator responsible for the generation of myogenic response, or whether it simply serves as a modulator of the response is still open for discussion. 20-HETE does play a significant role in the myogenic response of renal, cerebral, mesenteric, and skeletal muscle arterioles of rats. However, our data (Figure 3) support the hypothesis that in certain pathophysiological conditions, 20-HETE functions to potentiate the responses. This view is consistent with the concept that the myogenic response is triggered by the influx of Ca²⁺ through stretch-sensitive channels when vessel tension changes. The rise in intracellular Ca²⁺ may initiate multiple downstream signaling cascades, including the activation of a receptor coupled to phospholipase A₂ to release arachidonic acid and 20-HETE, followed by activation of protein kinase C (PKC)/Rho or PKC/MAPK pathways. The signal-transduction pathway that predominates is also species-, tissue-, and cell-type–dependent and likely reflects diversity in the expression of the signaling transduction proteins and
types of ion channel expression within the tissue. Moreover, CYP450/6-hydroxylase is regulated by multiple factors, and so is the myogenic mechanism. The end point of the signaling pathway most likely involves inhibition of K\textsubscript{Ca} channels to cause depolarization of vascular smooth muscle. Indeed, previous studies showed an antagonistic relationship between myogenic constriction and endothelium-derived hyperpolarizing factor–mediated dilation in coronary arteries of rats.39

Together, this is the first study to provide direct evidence for a role of CYP450/6-hydroxylase in the potentiation of pressure-induced myogenic constriction in eNOS-deficient coronary arteries.

Perspectives
20-HETE serves as a second messenger that plays a critical role in the myogenic response, vascular hypertrophy, and vascular responses to constrictor and dilator agents by regulating K\textsuperscript{+} channel activity. 20-HETE is currently characterized as a prohypertensive eicosanoid on the basis of its capacity of potentiating vasoconstriction and the ensuing increases in peripheral resistance. Several studies have demonstrated that the expression of CYP4A and production of 20-HETE are altered in different models of hypertension,3,9,30 a condition characterized by an enhanced myogenic tone and endothelial dysfunction.2 In the present study, the 20-HETE–induced enhanced myogenic tone may be linked to the physical effect of elevated blood pressure24 on the vessel wall, but the causal relationship between hypertension and upregulation of 20-HETE synthesis, as a function of eNOS deficiency, is still unknown. Because a positive feedback between hypertension and 20-HETE production is established, and because enhanced coronary myogenic constriction results from an increased 20-HETE release, our studies suggest a possible beneficial intervention by blockade of 20-HETE synthesis to improve coronary perfusion in patients with ischemic coronary heart disease.

Acknowledgments
This study was supported by NIH grants HL 070653, HL 68813, and HL 43023.

References
10. Hercule HC, Oyekan AO. Cytochrome P450 omega/omega-1 hydroxylase-derived eicosanoids contribute to endothelin(A) and endothelin(B) receptor-mediated vasoconstriction to endothelin-1 in the rat preganglomular arteriole. J Pharmacol Exp Ther. 2000;292:1153–1160.


Contribution of 20-HETE to Augmented Myogenic Constriction in Coronary Arteries of Endothelial NO Synthase Knockout Mice
An Huang, Dong Sun, Changdong Yan, John R. Falck and Gabor Kaley

*Hypertension.* 2005;46:607-613; originally published online July 25, 2005;
doi: 10.1161/01.HYP.0000176745.04393.4d

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/46/3/607

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Hypertension* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Hypertension* is online at:
http://hyper.ahajournals.org//subscriptions/