20-Hydroxyeicosatetraenoic acid causes endothelial dysfunction via eNOS uncoupling

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Submitted 9 October 2007; accepted in final form 19 December 2007

Cheng J, Ou JS, Singh H, Falck JR, Narsimhaswamy D, Pritchard KA Jr, Schwartzman ML. 20-Hydroxyeicosatetraenoic acid causes endothelial dysfunction via eNOS uncoupling. Am J Physiol Heart Circ Physiol 294: H1018–H1026, 2008. First published December 21, 2007; doi:10.1152/ajpheart.01172.2007.—Nitric oxide (NO), generated from l-arginine by endothelial nitric oxide synthase (eNOS), is a key endothelial-derived factor whose bioavailability is essential to the normal function of the endothelium. Endothelium dysfunction is characterized by loss of NO bioavailability because of either reduced formation or accelerated degradation of NO. We have recently reported that overexpression of vascular cytochrome P-450 (CYP) 4A in rats caused hypertension and endothelial dysfunction driven by increased production of 20-hydroxyeicosatetraenoic acid (20-HETE), a major vasoconstrictor eicosanoid in the microcirculation. To further explore cellular mechanisms underlying CYP4A-20-HETE-driven endothelial dysfunction, the interactions between 20-HETE and the eNOS-NO system were examined in vitro. Addition of 20-HETE to endothelial cells at concentrations as low as 1 nM reduced calcium ionophore-stimulated NO release by 50%. This reduction was associated with a significant increase in superoxide production. The increase in superoxide in response to 20-HETE was prevented by Nω-nitro-l-arginine methyl ester, suggesting that uncoupled eNOS is a source of this superoxide. The response to 20-HETE was specific in that 19-HETE did not affect NO or superoxide production, and, in fact, the response to 20-HETE could be competitively antagonized by 19(R)-HETE. 20-HETE had no effect on phosphorylation of eNOS protein at serine-1179 or threonine-497 following addition of calcium ionophore; however, 20-HETE inhibited association of eNOS with 90-kDa heat shock protein (HSP90). In vivo, impaired acetylcholine-induced relaxation in arteries overexpressing CYP4A was associated with a marked reduction in the levels of phosphorylated vasodilator-stimulated phosphoprotein, an indicator of bioactive NO, that was reversed by inhibition of 20-HETE synthesis or action. Because association of HSP90 with eNOS is critical for eNOS activation and coupled enzyme activity, inhibition of this association by 20-HETE may underlie the mechanism, at least in part, by which increased CYP4A expression and activity cause endothelial dysfunction.

nitric oxide; vascular endothelium; vasodilator-stimulated phosphoprotein; cytochrome P-450; superoxide; endothelial nitric oxide synthase

THE INTEGRITY OF THE VASCULAR endothelium is critical for the maintenance of vascular homeostasis. The endothelium actively participates in the regulation of vascular tone, vessel diameter, and blood flow, as well as in presenting the first layer of defense against injurious stimuli. Nitric oxide (NO), generated from l-arginine by endothelial nitric oxide synthase (eNOS), is a key endothelial-derived factor whose bioavailability is essential to the normal function of the endothelium. Endothelial dysfunction is a major risk factor and a very early indicator of cardiovascular disease. It is characterized by a loss of NO bioavailability due to either reduced formation or accelerated degradation of NO. NO is scavenged by reactive oxygen species (ROS), including superoxide anion, and this is thought to be a major mechanism contributing to decreased NO availability and endothelial dysfunction (5).

20-Hydroxyeicosatetraenoic acid (HETE) is one of the primary eicosanoids produced in the microcirculation. It participates in the regulation of vascular tone by sensitizing the smooth muscle cells to constrictor stimuli (46) and contributes to myogenic, mitogenic, and angiogenic responses (3, 13, 16, 25). The synthesis of 20-HETE is catalyzed primarily by enzymes of the cytochrome P-450 (CYP) 4A family. CYP4A proteins are present in vascular tissues and show distinct distribution along the vascular tree (20). Suppression and overexpression of CYP4A proteins in small arteries and arterioles decreased and increased vascular reactivity and myogenic tone, respectively (17, 40, 47); these effects can be reversed, respectively, by the addition of 20-HETE or inhibition of its synthesis.

It has long been known that NO and NO donors inhibit the catalytic activities of CYP enzymes (24). NO directly binds to the heme moiety of recombinant CYP4A (39) and inhibits the formation of 20-HETE and epoxyeicosatetraenoic acids (EETs) (1, 29, 34). In vivo, chronic inhibition of NO synthesis has been shown to increase expression of CYP4A protein and the synthesis of 20-HETE (14, 29). The inhibitory action of NO on the synthesis of 20-HETE has been shown to contribute to the cGMP-independent vasodilatory effect of NO in the renal and cerebral microcirculation (34, 36). Collectively, these studies implicate NO in the regulation of 20-HETE synthesis.

Recent reports, however, have indicated that the opposite also occurs; 20-HETE affects both the release and actions of NO. Frisbee et al. (9) showed that 20-HETE attenuated acetylcholine-induced relaxation of cremasteric arterioles of normotensive rats fed a low-salt diet and that inhibition of the synthesis of 20-HETE enhanced the vasodilator response to acetylcholine in hypertensive rats on a high-salt diet. On the other hand, 20-HETE-induced relaxation of bovine pulmonary...
arteries has been linked to its ability to increase Ca\textsuperscript{2+} in pulmonary artery endothelial cells and to activate eNOS by stimulating phosphorylation of serine (6, 43). Ward et al. (42) demonstrated a correlation between elevated urinary 20-HETE and endothelial dysfunction in humans, and a study in our laboratory provided evidence for a causative link between the CYP4A-20-HETE pathway and endothelial dysfunction in rats. We showed that intravenous injection of an adenosinergic to overexpress CYP4A2 caused hypertension and that renal arteries from these rats featured increased vascular CYP4A expression and 20-HETE production and displayed endothelial dysfunction exemplified by a reduced vasodilator response to acetylcholine, reduced levels of NO, and increased levels of superoxide anion (38). Similar results were obtained in a rat model of androgen-induced hypertension in which the vascular CYP4A expression is upregulated (33).

It has been observed that increased CYP4A protein levels (33) or transduction with CYP4A2 eDNA (38) impaired the acetylcholine-induced relaxation displayed by renal arteries. This was offset by ex vivo treatment of the vessels with a known inhibitor of 20-HETE synthesis and readily reinstated by application of exogenous 20-HETE. Hence it was concluded that the NO-dependent component of acetylcholine-induced relaxation is suppressed by increased 20-HETE levels. Consequently, the question of how 20-HETE promotes endothelial dysfunction was raised, leading to the current study that examines the effect of 20-HETE on the release of NO in endothelial cells in vitro and NO bioavailability in vivo.

**METHODS**

**Cell culture.** Bovine aortic endothelial cells (BAECs) were obtained from Cambrex (Walkersville, MD) and cultured in microvascular endothelial cell medium supplemented with 5% FBS and with bovine brain extract, human epidermal growth factor, hydrocortisone, and GA-1000 (amphotericin B; Gentamicin). Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics and maintained in endothelial cell medium 2 growth medium. Passages 3–5 were used in all experiments. Cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO\textsubscript{2}. BAECs were plated on 6-, 12- or 96-well plates. After reaching 70% confluence, cells were placed in FBS-free media for 24 h. On the day of the experiment, the cells were washed with Hanks' balanced salt solution (HBSS) and preincubated with or without 0.1–1 mM N\textsubscript{ω}-monomethyl-l-arginine (l-NMMA) or N\textsubscript{ω}-nitro-l-arginine methyl ester (l-NAME) and 1–1,000 nM 20-HETE (or its vehicle, ethanol). The medium was removed, and the cells, in the presence of l-arginine (25 μM), were incubated with or without l-NAME or l-NMMA (0.1–1 mM) and the calcium ionophore A-23187 (5 μM) for 30 min at 37°C. In some experiments, 5 nM of 19(S)-HETE or 19(R)-HETE were added to the cells 20 min before the addition of 20-HETE and calcium ionophore.

**Measurement of NO.** NO levels were evaluated by measuring total nitrite and nitrate content in culture medium using the NO quantitation kit and following the manufacturer's instructions (Active Motif). The levels were validated using the chemiluminescence method (44).

**Measurement of superoxide.** BAECs were cultured on 96-well plates until they achieved ~70% confluence. After treatments with or without l-NAME (1 mM), 20-HETE (5 nM), calcium ionophore A-23187 (5 μM), 19(R)-HETE (5 nM), and 19(S)-HETE (5 nM) and in the presence of l-arginine (25 μM), the cells were incubated with 10 μM dihydroethidium (DHE) for 30 min at 37°C. Fluorescence intensity was measured using a Perkin-Elmer Luminescence Spectrometer at excitation/emission filters of 530/620 nm. In addition, HUVECs were cultured on four-well slide chambers, treated, and loaded with 5 μM DHE for 20 min at 37°C. After the incubation with DHE, cells were washed with Dulbecco’s PBS and imaged via fluorescence microscopy. The intensity of DHE fluorescence of cells was imaged under the same conditions for 30 ms at 30-s intervals using an automatic shutter on a Nikon epifluorescence inverted microscope (Diaphot).

**Immunoprecipitation of eNOS.** BAECs were cultured on four 60-mm dishes. When cells reached 100% confluence, they were preincubated with 20-HETE for 30 min. After being washed three times with HBSS, cells were stimulated with calcium ionophore A-23187 (5 μM) for 10 min in the presence of l-arginine (10 μM). Cells were lysed in modified RIPA buffer, scraped, and collected in Eppendorf tubes as previously described (27). Immunoprecipitation was performed with anti-eNOS antibody H32 (BioMol, Plymouth Meeting, PA), and immunoprecipitated fractions were used for Western blot analysis.

**Animal studies.** All experimental protocols were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats at 8–9 wk old were used. 5α-Dihydrotestosterone (DHT) (56 mg·kg·body wt·day\textsuperscript{-1}) or its vehicle (20% benzyl alcohol in corn oil) was administered for 14 days. Some of the DHT and control rats were chronically treated with the selective inhibitor of the synthesis of 20-HETE, N-hydroxy-N’-(4-butyl-2-methylphenyl)-formamidine (HET0016, 10 mg·kg·body wt·day\textsuperscript{-1}) or its vehicle (10% wt/vol lecithin in saline). At this dose, HET0016 inhibits 20-HETE production in renal interlobar arteries by 80–90% without significantly affecting the production of EETs (33). On day 14, the rats were anesthetized with phenobarbital (50 mg/kg body wt), and laparotomy was performed. The kidneys were removed, and renal interlobar arteries were microdissected for functional studies and Western blot analysis.

**Agonist-induced vasorelaxation.** Microdissected renal interlobar arteries were mounted on wires in myograph chambers (J.P. Trading) for measurement of isometric tension as previously described (33). The arteries were precontracted with phenylephrine (5 × 10\textsuperscript{-6} M for vehicle, vehicle + HET0016, and DHT + HET0016, 10–6 M for DHT-treated), and the vasodilator responses to acetylcholine (5 × 10\textsuperscript{-6} M or sodium nitroprusside (SNP, 5 × 10\textsuperscript{-6} M) were studied in the absence and presence of 20-HETE (50 nM), 19(R)-HETE (50–1,000 nM), or HET0016 (1 μM). Arteries were removed promptly after relaxation and stored in homogenizing buffer for Western blot analysis.

**Western blot analysis.** Western blot analysis of immunoprecipitated fractions, cell lysates, and renal interlobar arterial segments were performed as previously described (27, 33) using primary antibodies against eNOS (Zymed, San Francisco, CA), phosphorylated eNOS (p-eNOS) at serine-1179 (Cell Signaling, Beverly, MA), p-eNOS at threonine-497 (Upstate, Lake Placid, NY), HSP90 (Transduction, Lexington, KY), phosphorylated VASP (pVASP; Alexis Biochemicals, San Diego, CA), and β-actin (Sigma, St. Louis, MO).

**Statistical analysis.** Data are expressed as means ± SD. Significance of difference in mean values was determined using one-way ANOVA followed by the Newman-Keuls’s post hoc test. \(P < 0.05\) was considered to be significant.

**RESULTS**

20-HETE decreases NO levels in BAEC. The effects of 20-HETE on agonist-stimulated NO release in BAEC are depicted in Fig. 1. At basal levels without any treatments, control cells produced 0.34 ± 0.07 μM of NO (Fig. 1A). Upon stimulation with the calcium ionophore A-23187, which dissociates eNOS from caveolin and activates eNOS (24), NO levels increased by twofold (\(P < 0.05\)) compared with control cells. Addition of 20-HETE (5 nM) to ionophore-stimulated
cells inhibited NO levels by 76% compared with the ionophore-stimulated cells. In the absence of ionophore, 20-HETE decreased basal NO levels by 40% (0.28 ± 0.06 μM) (Fig. 1A). The inhibitory effect of 20-HETE was observed over a wide range from 1 to 1,000 nM (Fig. 1B).

To determine whether the decrease in levels of NO was related to the inactivation of or interference with eNOS activity, the effect of 20-HETE was measured in the presence of the eNOS inhibitor L-NMMA. Treatment with L-NMMA alone did not change levels of NO compared with control, unstimulated cells. However, pretreatment with L-NMMA reduced ionophore-stimulated NO levels by 63% (P < 0.05), a level similar to that achieved with ionophore-stimulated 20-HETE-treated cells. Addition of 20-HETE after pretreatment with L-NMMA had no further effect on the levels of NO. Cells treated with 20-HETE and L-NMMA (0.27 ± 0.06 μM) and 20-HETE, L-NMMA, and ionophore (0.32 ± 0.07 μM) produced levels of NO that were similar to those achieved with 20-HETE-treated stimulated cells (Fig. 1). The inability of 20-HETE to exhibit further decreases in levels of NO in L-NMMA-treated cells suggests that 20-HETE decreases NO by interfering with eNOS activity.

**20-HETE disrupts the association of eNOS with 90-kDa heat shock protein.** The phosphorylation of eNOS at threonine-497 (T497) and serine-1179 (S1179) and its association with 90-kDa heat shock protein (HSP90) are both required for eNOS activation (8). To determine whether 20-HETE affects the activation of eNOS, we incubated BAECs with or without 20-HETE and ionophore, immunoprecipitated the cell lysates with eNOS antibody, and tested for the presence of eNOS, HSP90, and p-eNOS (S1179 and T497) in eNOS-immunoprecipitated fractions (Fig. 2A). As seen in Fig. 2, A–C, activation of NO production with ionophore was associated with a marked 2.3-fold increase in p-eNOS (S1179) (Fig. 2B) with no significant change in eNOS phosphorylation at T497 (Fig. 2C). 20-HETE had no effect on basal and ionophore-stimulated levels of eNOS phosphorylation at either S1179 or T497. On the other hand, ionophore activation of HSP90 association to eNOS, which amounted to 2.6-fold over control untreated cells, was completely negated by pretreatment with 20-HETE (Fig. 2D).

**20-HETE increases levels of superoxide.** Inactivation or uncoupling of eNOS is directly associated with increases in levels of ROS, most notably, superoxide anion (30). Therefore, we investigated the effects of 20-HETE on superoxide levels. Single cell intravital monitoring of superoxide (by monitoring DHE fluorescence) showed increased amounts of superoxide in endothelial cells treated with 10 nM of 20-HETE (Fig. 3, A and B). These increases were time dependent, as can be denoted by the increase in DHE fluorescence from Fig. 3C. Additional experiments with DHE fluorescence imaging showed greater intensities of DHE in cells treated with 20-HETE alone and 20-HETE with ionophore compared with control and stimulated cells in the absence of 20-HETE. The stimulatory effect of 20-HETE on superoxide was concentration dependent, as seen in Fig. 3D. We also measured the effect of 20-HETE in cells incubated with l-arginine and with or without ionophore and l-NAME. As seen in Fig. 3E, 20-HETE increased (P < 0.001) superoxide levels by 49% in control unstimulated cells. Stimulation of cells with ionophore did not significantly change levels of superoxide compared with that of control cells. However, addition of 20-HETE to ionophore-stimulated cells increased (P < 0.001) superoxide levels by 25% compared with that of ionophore-stimulated cells (Fig. 3A). Importantly, pretreatment with l-NAME abolished 20-HETE-induced increases in superoxide levels in both control unstimulated cells and cells stimulated with ionophore, suggesting that the increase in superoxide in response to 20-HETE is primarily driven by uncoupled eNOS as a consequence of 20-HETE-mediated inhibition of its association with HSP90.

**20-HETE's effects on NO and superoxide are reversed with 19(R)-HETE.** Previous studies have indicated that 19(R)-HETE and other structurally similar analogs act as competitive inhibitors of the vasoconstrictor response to 20-HETE in various vascular beds (2, 45). We tested whether preincubating the cells with 19(R)-HETE would attenuate the 20-HETE-mediated decrease in NO levels and increase in superoxide levels. As seen in Fig. 4A, 20-HETE decreased levels of ionophore-stimulated NO by 79%. 19(R)-HETE, but not 19(S)-HETE, completely reversed the inhibitory action of 20-HETE on ionophore-stimulated NO production (Fig. 4A). A similar reversal was seen in superoxide levels. 19(S)-HETE and 19(R)-HETE alone had no effect on DHE fluorescence. However, 19(R)-HETE but not 19(S)-HETE, significantly decreased the
20-HETE-stimulated increase in DHE fluorescence by 24% compared with cells treated with 20-HETE alone (Fig. 4B).

Vasodilator-stimulated phosphoprotein phosphorylation is regulated by the levels of expression and activity of the CYP4A-20-HETE pathway. The phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at Ser 239 has been shown to be an indicator of bioactive NO and the activity of the NO-cGMP-protein kinase G signaling pathway (26). To further examine the role for the CYP4A-20-HETE system as an endogenous regulator of the eNOS-NO pathway, we evaluated the levels of pVASP in renal interlobar arteries isolated from rats chronically treated with DHT. This is a model of hypertension and endothelial dysfunction that is dependent on up-regulation of vascular CYP4A expression and synthesis of 20-HETE (33). As seen in Fig. 5, renal interlobar arteries obtained from rats treated with DHT expressed relatively lower levels of pVASP, detected at both 46 and 50 kDa (23, 26), compared with arteries from vehicle-treated rats. That the lower level of pVASP expression in arteries from DHT-treated rats is linked to the CYP4A-20-HETE pathway is evident from the observation that the expression levels of pVASP returned to their control levels following chronic treatment of the rats with HET0016, a selective inhibitor of the synthesis of 20-HETE (22, 33) (Fig. 5). Interestingly, arteries from rats treated with HET0016 alone in which endogenous levels of 20-HETE are significantly reduced (33) displayed slightly higher levels of pVASP (Fig. 5).

VASP phosphorylation as a function of CYP4A-20-HETE activity was further compared with the vasodilator response to acetylcholine in arteries from rats treated with vehicle, DHT, DHT + HET0016, or HET0016. As seen in Fig. 6, arteries from vehicle-treated rats relaxed to 5 × 10⁻⁶ M acetylcholine with a maximal response of 55.6 ± 1.3% (n = 5); addition of 20-HETE to the bath greatly diminished the acetylcholine-induced relaxation by fivefold to 9.8 ± 5.1% (n = 3; P < 0.05) (Fig. 6A). These arteries were taken from the myograph bath at the peak of the relaxation and subjected to Western blot analysis for pVASP. The inset in Fig. 6A clearly shows that pVASP levels were reduced upon the addition of 20-HETE, suggesting a functional correlation between the relaxing response and the diminished levels of NO brought about by the addition of 20-HETE. Arteries from rats chronically treated with DHT showed a diminished relaxing response (Fig. 6B) that somewhat increased by addition of HET0016 to the bath (from 6.4 ± 2.7 to 28.8 ± 8.9%, respectively, n = 3, P = 0.15). The levels of pVASP in these arteries seemed to reflect the relaxing responses. The slight effect of HET0016 suggests that the short time frame of this type of experiment is not sufficient for significant reduction in 20-HETE levels. However, arteries from rats treated with DHT and HET0016 in vivo

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Fig. 2. Effect of 20-HETE on endothelial nitric oxide synthase (eNOS) phosphorylation and association with 90-kDa heat shock protein (HSP90) in BAEC. A: representative immunoblots of eNOS-immunoprecipitated cells with antibodies against eNOS, HSP90, threonine-497 (T497), and serine-1179 (S1179). Densitometry analysis of the relative expression levels of phosphorylated (p)-eNOS¹¹⁷⁹ (B), p-eNOS⁴⁹⁵ (C), and HSP90 (D) in eNOS-immunoprecipitated cells. Results are means ± SE; n = 5. P < 0.05 from control (*) and from ionophore-stimulated cells (†).
displayed a relaxing response to acetylcholine that was similar to that seen in arteries obtained from control, vehicle-treated rats (Fig. 6C). Addition of 20-HETE to the bath attenuated the relaxing response from 55.1 ± 27.9 to 11.1 ± 2.5% (n = 3, P < 0.05) and reduced pVASP levels (Fig. 6C). Likewise, addition of 20-HETE to arteries from rats treated with HET0016 alone reduced the response to acetylcholine from 52.2 ± 18.1 to 8.1 ± 5.3% (n = 3, P < 0.05) and the levels of pVASP (Fig. 6D). That 20-HETE contributed to the impaired relaxing response to acetylcholine in arteries from DHT-treated rats is further substantiated by the demonstration that addition of 19(R)-HETE, the functional antagonist of 20-HETE, to the bath completely reversed this impairment (Fig. 6E). The effect of 19(R)-HETE was dose dependent, e.g., 27.1 ± 4.6 and 57.9 ± 8.9% relaxation at 50 and 1,000 nM, respectively (n = 3). At the higher dose of 19(R)-HETE, maximal relaxation was similar to that seen in arteries from vehicle-treated rats (Fig. 6A). Notably, the relaxing response to the NO donor SNP was not significantly different between arteries from DHT-treated rats and those from vehicle-treated rats (49.3 ± 5.8 and 50.7 ± 3.0% maximal response in arteries from DHT- and vehicle-treated rats, respectively, n = 3) (Fig. 6F), suggesting that NO bioavailability rather than the response to NO is affected by the increased 20-HETE synthesis. The relaxing response to SNP was accompanied by a marked increase in VASP phosphorylation (Fig. 6F), further indicating the validity of using pVASP as an index of NO bioavailability.

**DISCUSSION**

The current study provides strong evidence for a mechanistic link between the vascular CYP4A-20-HETE pathway and...
endothelial dysfunction; it clearly demonstrates that 20-HETE impairs NO production in vitro and its function in vivo by uncoupling the eNOS activation process. These findings are novel in that they implicate 20-HETE, a major eicosanoid of the microcirculation, as a key regulator of eNOS and, thereby, endothelial function.

20-HETE has been recognized for its presence in the vasculature, its role in the regulation of vascular tone, and its contributions to mitogenic and angiogenic responses. In the renal and cerebral microcirculation, 20-HETE elicits vasoconstriction via protein kinase C (PKC)- or tyrosine kinase-sensitive inhibition of the Ca^{2+}/H^+ -activated K channel, vascular smooth muscle cell depolarization, and elevation in cytosolic Ca^{2+} concentration (21). In small porcine coronary arteries, 20-HETE induces contraction by a mechanism involving the activation of the Rho-kinase phosphorylation of myosin light chain 20 and the sensitization of the contractile apparatus to Ca^{2+} (31). The current study is the first to demonstrate that 20-HETE inhibits NO production in aortic endothelial cells. The inhibitory action of 20-HETE was obtained at concentrations as low as 1 nM, indicating potency and relevancy since such concentrations are easily detected in vascular beds, including the renal (20) and cerebral (12). The effect of 20-HETE on ionophore-stimulated NO production was specific in that it was not reproduced by the structurally similar 19-HETE enantiomers.

This specificity and potency, along with its established presence within the vascular wall and blood (18, 21), position 20-HETE as an ideal small molecule regulator of NO production and function. Moreover, the fact that 20-HETE’s inhibitory effect on NO production was negated by 19(R)-HETE, a functional antagonist of 20-HETE (45), further substantiates the specificity of 20-HETE action.

Our recent studies (33, 38) clearly indicated a deficiency in agonist (acetylcholine)-induced NO bioavailability in arteries that expressed more CYP4A and produced higher levels of 20-HETE; inhibition of CYP4A activity by DDMS reversed this effect, and addition of 20-HETE to N-methylsulfonyl-12, 12-dibromododec-11-enamide treated arteries restored the apparent endothelial dysfunction. These results suggested that the deficiency in NO bioavailability is transient and reversible. Mechanisms that may account for such an effect include an effect on the phosphorylation state of eNOS (19) and/or uncoupling of eNOS activity via interference with HSP90 association (10) and/or rapid activation of ROS generation that, in turn, scavenge NO (37). Other mechanisms that may alter eNOS activity include cofactor (tetrahydrobiopterin) or substrate accessibility (37) and increased expression of caveolin-1, a negative regulator of eNOS (8). However, the latter are more likely to be less transient. Therefore, we concentrated on the state of eNOS phosphorylation and HSP90 association.

There are two major phosphorylation sites on eNOS that have been extensively studied with regard to its activity. These are T495 (Thr^{497} in bovine) and S1177 (Ser^{1179} in bovine). Recent studies suggested that dual phosphorylation of Thr^{495} and Ser^{1177} determines the activity of eNOS in agonist-stimulated endothelial cells. Phosphorylation at Ser^{1177} increases eNOS specific activity at any given Ca^{2+} concentration, whereas the association of calmodulin with eNOS in agonist-stimulated endothelial cells and initiation of NO production are regulated by Thr^{495} dephosphorylation (8). Signaling through...
PKC has been shown to inhibit eNOS activity by phosphorylation of Thr<sup>495</sup> and Ser<sup>1177</sup> dephosphorylation (8). 20-HETE is a known activator of PKC (35). Taken together, disruption in agonist-induced phosphorylation/dephosphorylation of eNOS could account for 20-HETE’s effect on endothelial function. The results of the current study indicate that the mechanism by which 20-HETE causes a reduction in NO levels does not include interference with phosphorylation/dephosphorylation of eNOS at Thr<sup>497</sup> and Ser<sup>1179</sup>. 20-HETE had no effect on the levels of p-eNOS at either residues in control or ionophore-stimulated cells. These findings are in contrast to those reported in the pulmonary endothelial cells in which addition of 20-HETE, albeit at a 200-fold higher concentration than that used in the current study (5 nM), resulted in a twofold increase in basal p-eNOS at Ser<sup>1179</sup>. 20-HETE had no effect on the levels of p-eNOS at either residues in control or ionophore-stimulated cells. These findings are in contrast to those reported in the pulmonary endothelial cells in which addition of 20-HETE, albeit at a 200-fold higher concentration than that used in the current study (5 nM), resulted in a twofold increase in basal p-eNOS at Ser<sup>1179</sup>. 20-HETE had no effect on the levels of p-eNOS at either residues. These findings are in contrast to those reported in the pulmonary endothelial cells in which addition of 20-HETE, albeit at a 200-fold higher concentration than that used in the current study (5 nM), resulted in a twofold increase in basal p-eNOS at Ser<sup>1179</sup>. 20-HETE had no effect on the levels of p-eNOS at either residues. Hence, it is possible that disruption in the association of HSP90 with eNOS constitutes, at least in part, the mechanisms by which 20-HETE interferes with NO production and endothelial function. 20-HETE has been shown to decrease HSP90 association with eNOS. Addition of 20-HETE at 5 nM did not alter basal levels of eNOS-associated HSP90; however, it completely blocked ionophore-stimulated HSP90 association with eNOS. Hence, it is possible that disruption in the association of HSP90 with eNOS constitutes, at least in part, the mechanisms by which 20-HETE interferes with NO production and endothelial function. 20-HETE has been shown to decrease HSP90 association with eNOS. Addition of 20-HETE at 5 nM did not alter basal levels of eNOS-associated HSP90; however, it completely blocked ionophore-stimulated HSP90 association with eNOS. Hence, it is possible that disruption in the association of HSP90 with eNOS constitutes, at least in part, the mechanisms by which 20-HETE interferes with NO production and endothelial function. 20-HETE has been shown to decrease HSP90 association with eNOS. Addition of 20-HETE at 5 nM did not alter basal levels of eNOS-associated HSP90; however, it completely blocked ionophore-stimulated HSP90 association with eNOS.
Rapid activation of superoxide generation may also constitute a mechanism by which 20-HETE interferes with eNOS/NO function. Our recent studies indicate that arterial preparations overexpressing CYP4A produce more superoxide than control preparations (33, 38). The fact that 20-HETE reversed acetylcholine-induced relaxations in DDMS-treated CYP4A-overexpressed arteries raised the possibility of a direct effect of 20-HETE on either eNOS-dependent or NAD(P)H oxidase-derived superoxide generation or both. Indeed, cells treated with 20-HETE showed increased production of superoxide, and fluorescence imaging suggested that this effect is relatively rapid. However, inhibition of eNOS by l-NAME prevented the 20-HETE-induced increase in superoxide levels. Consequently, the increased superoxide levels brought about by 20-HETE may be because of eNOS uncoupling via disruption of HSP90-eNOS association (30). Alternatively, the increase in superoxide may simply be a function of reduced scavenging of superoxide secondary to a reduction in NO production. Similarly, we cannot exclude the possibility that 20-HETE exhibits a direct stimulatory action on a superoxide-producing system as suggested by Guo et al. (11).

The functional implication of 20-HETE’s effect on the eNOS/NO system in isolated endothelial cells is evident from the last set of experiments in which VASP phosphorylation, a surrogate marker for NO bioavailability and function (26), was examined in isolated renal arteries. The renal interlobar arteries are a rich source of 20-HETE where it amplifies constrictor responsiveness (17, 20). We found an inverse relationship between CYP4A-20-HETE expression and the levels of pVASP. Hence, pVASP was markedly reduced in arteries from androgen-treated rats, which displayed higher CYP4A expression and produced more 20-HETE (33). More importantly, pVASP levels returned to control levels in arteries from androgen-treated rats receiving the CYP4A inhibitor HETO016, suggesting a functional link between the CYP4A-20-HETE and NO bioavailability. That the deficiency in NO bioavailability underlies, at least in part, the 20-HETE-induced impairment in relaxations to acetylcholine was further substantiated by the concurrent measurements of the relaxing response to acetylcholine and the levels of arterial pVASP in the presence and absence of a 20-HETE inhibitor or antagonist. Hence, addition of 20-HETE ex vivo caused impaired relaxation and reduced pVASP, whereas addition of 19(R)-HETE reversed the impaired acetylcholine relaxations and increased pVASP in arteries from DHT-treated rats. These results, together with data derived from cultured cells, suggest that 20-HETE-mediated endothelial dysfunction is primarily driven by eNOS uncoupling and reduction in bioavailable NO. In addition, the findings in this study, including the observation that treatment with HETO016 by itself tended to increase pVASP levels, suggest a close functional interplay between these two systems. On one hand, NO has been shown to inhibit CYP4A and 20-HETE production (1, 29), and this action has been implicated as the cGMP-independent relaxing effect of NO (36). On the other hand, 20-HETE, which is released in response to numerous vasoactive hormones (7, 28), has the ability to control NO bioavailability. Hence, the levels of these two mediators, representing endothelium-derived relaxing and contracting factors, may determine vascular tone and homeostasis. However, further studies are required to substantiate this speculation and discern between the effect of 20-HETE on endothelial function and its smooth muscle cell contractile property.

In summary, the CYP4A proteins and their arachidonic acid metabolite 20-HETE have been implicated in the regulation of vascular function in health and disease. Of importance are the numerous studies linking CYP4A to the development of hypertension and to the severity of ischemic cerebral and cardiac disease in experimental models (21) and reports of correlation between urinary 20-HETE, oxidative stress, and endothelial dysfunction in human subjects (4, 41, 42). The findings in this study provide yet another mechanistic explanation for 20-HETE’s actions in the vasculature. Hence, 20-HETE may contribute to the development of hypertension and cardiovascular disease by increasing vascular reactivity, enhancing constrictor responsiveness, and bringing about endothelial dysfunction by uncoupling eNOS.

ACKNOWLEDGMENTS

We thank Dr. Jeffrey Williams and Dr. Sergey Brodsky for help in measuring NO and superoxide levels in cultured cells and Dr. Fan Zhang for assistance in setting up the myograph for relaxation measurements.

REFERENCES


