Induction of Angiotensin-Converting Enzyme and Activation of the Renin–Angiotensin System Contribute to 20-Hydroxyeicosatetraenoic Acid–Mediated Endothelial Dysfunction

Jennifer Cheng, Victor Garcia,* Yan Ding,* Cheng-Chia Wu, Krutanjali Thakar, John R. Falck, Errabelli Ramu, Michal Laniado Schwartzman

Objective—20-hydroxyeicosatetraenoic acid (20-HETE) promotes endothelial dysfunction by uncoupling endothelial NO synthase, stimulating O$_2^-$ production, and reducing NO bioavailability. Moreover, 20-HETE–dependent vascular dysfunction and hypertension are associated with upregulation of the renin–angiotensin system. This study was undertaken to examine the contribution of renin–angiotensin system to 20-HETE actions in the vascular endothelium.

Methods and Results—In endothelial cells, 20-HETE induced angiotensin-converting enzyme (ACE) mRNA levels and increased ACE protein and activity by 2- to 3-fold; these effects were negated with addition of the 20-HETE antagonist, 20-hydroxyeicosa-6(Z),15(Z)-dienoic acid (20 HEDE). 20-HETE induced ACE expression was protein kinase C independent and epidermal growth factor receptor tyrosine kinase and IκB kinase β dependent. ACE short interfering RNA abolished 20-HETE–mediated inhibition of NO production and stimulation of O$_2^-$ generation, whereas angiotensin II type 1 receptor short interfering RNA attenuated these effects by 40%. 20-HETE–stimulated O$_2^-$ production was negated by 20-HEDE and was attenuated by lisinopril and losartan. Importantly, 20-HETE–mediated impairment of acetylcholine-induced relaxation in rat renal interlobar arteries was also attenuated by lisinopril and losartan.

Conclusion—These results indicate that ACE and angiotensin II type 1 receptor activation contribute to 20-HETE–mediated endothelial cell and vascular dysfunction and further enforce the notion that excessive production of 20-HETE within the vasculature leads to hypertension via mechanisms that include the induction of endothelial ACE, thus, perpetuating an increase in vascular angiotensin which, together with 20-HETE, promotes vascular dysfunction.


Key Words: angiotensin II eicosanoids hypertension nitric oxide superoxide
wall are believed to constitute the mechanisms by which 20-HETE contributes to the development of hypertension.

In a recent study, we identified the angiotensin-converting enzyme (ACE) as one of the few genes that are markedly upregulated upon addition of 20-HETE to cultured endothelial cells. Additional studies indicated that increased vascular synthesis of 20-HETE in vivo is also associated with increased vascular ACE expression and circulating Ang II levels. Moreover, in an experimental model of 20-HETE–dependent hypertension, administration of ACE inhibitors or Ang II type 1 receptor (AT1R) blockers prevented and reversed vascular dysfunction and media was determined in the presence and absence of losinopril (100 nmol/L) using the BÜHLMANN ACE kinetic test from ALPCO (Salem, NH).

**Methods and Materials**

**Cell Culture**

Human microvascular endothelial cells (HMVECs) were grown in Medium 131 containing 5% microvascular growth supplement (Invitrogen, Carlsbad, CA) and 5% fetal bovine serum (USA Scientific, Ocala, FL). Passages 3–5 were used for all experiments. Cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO2:95% O2. For most experiments, cells were grown in 6-well plates with an equimolar concentration of 20-HEDE, a 20-HETE antagonist, 5 nmol/L, with or without actinomycin D (10 μmol/L) for 2 hours, after which they were preconstricted with phenylephrine (5 μmol/L), and the relaxing responses to increasing concentrations (5×10−5–5×10−4 mol/L) of acetylcholine were measured.

**Measurement of NO, cGMP, O2−, and H2O2**

For NO measurements, cells were preincubated with 20-HETE (5 nmol/L) or its vehicle (0.1% ethanol) for 2 hours and treated with or without calcium ionophore A23187 (5 μmol/L) in the presence of L-arginine (25 μmol/L) for 30 minutes. NO levels were measured using a NO quantitation kit (Active Motif, Carlsbad, CA), and cGMP levels were measured by immunoassay (R&D systems, Minneapolis, MN). For O2− measurements, cells were treated with or without Tiron (10 μmol/L), 20-HETE (5 nmol/L), 20-HEDE (5 nmol/L), angiotensin II (100 nmol/L), losartan (10 μmol/L), and lisinopril (10 μmol/L) for 2 hours. O2− levels were measured using dihydroethidium (DHE) (5 μmol/L) (Calbiochem, Gibbstown, NJ) as described. For measurements of H2O2 levels, polyethylene-glycolated superoxide dismutase (100 U/ml; Sigma Aldrich, St. Louis, MO) or polyethylene-glycolated catalase (500 U/ml; Sigma Aldrich) was added 30 minutes before staining with 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, 5 μmol/L; Sigma Aldrich) for 20 minutes. Fluorescence intensity was measured with excitation/emission filters of 485 nm/530 nm.

**ACE Protein and Activity Assays**

HMVECs were cultured on 6-well plates and placed in a serum-free media for 24 hours. Cells were then treated with 20-HETE (5 nmol/L) with or without 20-HEDE (5 nmol/L) for 6 to 24 hours. ACE protein was measured in cell lysates by immunoblotting with ACE N-terminus, epiope (N-20) goat polyclonal IgG (1:200, Santa Cruz Biotechnology, Santa Cruz, CA). ACE activity in cell lysates and media was determined in the presence and absence of losinopril (100 nmol/L) using the BÜHLMANN ACE kinetic test from ALPCO (Salem, NH).

**Statistical Analysis**

Data are expressed as means±SEM. Significance of difference in mean values was determined using t test and 1-way ANOVA, followed by the Newman–Keul post hoc test. P<0.05 was considered to be significant.

**Results**

**20-HETE Increases ACE Expression and Angiotensin II Production in Endothelial Cells**

Addition of 20-HETE (5 nmol/L) to HMVECs resulted in a time-dependent induction of ACE mRNA expression with a maximal 2.5-fold increase at 2 hours (Figure 1A). The increase in ACE mRNA expression was abrogated by cotreatment with an equimolar concentration of 20-HEDE, a 20-HETE antagonist, suggesting that the increase in ACE expression is 20-HETE dependent. The effect of 20-HETE on ACE mRNA was observed over a wide range of concentrations with a significant 2.5- and 3-fold induction at 1 and 5 nmol/L, respectively, and a maximum induction of 4-fold at 10 nmol/L (Figure 1B). Additional experiments comparing the effect of 20-HETE and the known ACE inducer phorbol 12-myristate 13-acetate clearly indicated that, whereas phorbol 12-myristate 13-acetate significantly increased ACE mRNA 24 hours after its addition, the increase in ACE mRNA in response to 20-HETE was maximal at 2 hours (Figure II in the online-only Data Supplement). Moreover, the increase in ACE mRNA in response to 20-HETE appeared to be at the level of transcription because addition of actinomycin D abolished 20-HETE’s effect (Figure IIIA in the online-only Data Supplement).

The 20-HETE–stimulated increase in ACE mRNA was associated with an increase in ACE protein. 20-HETE–increased ACE immunoreactive protein (∼170 kDa) levels by 2.1±1.15 and 4.2±0.89 at 6 and 12 hours; ACE protein levels remained
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Moreover, the increase in ACE protein was negated by coadministration of the 20-HETE antagonist 20-HEDE (Figure 1D). Addition of 20-HETE to HMVECs stimulated cellular and extracellular ACE activity by 2-fold at 24 hours. Importantly, the 20-HETE–mediated increase in ACE activity was blocked by addition of 20-HEDE (5 nmol/L) and was inhibited by lisinopril (100 nmol/L), suggesting that the increase in ACE activity is 20-HETE dependent (Figure 1E).

The vascular wall, including the endothelium, expresses components of the renin–angiotensin system (RAS), including angiotensinogen, renin, ACE, ACE2, AT1R, and AT2R.17 Real-time polymerase chain reaction analysis revealed that 20-HETE at 5 nmol/L increased angiotensinogen mRNA by 2.2-fold at 2 hours. At the same time frame, 20-HETE did not affect levels of ACE2 mRNA but did reduce AT1R and AT2R expression by 30% to 40% (Figure V in the online-only Data Supplement).

The 20-HETE–Mediated Increase in ACE Expression Is Tyrosine Kinase and IKK β Dependent

Previous studies in our lab have demonstrated that 20-HETE–mediated endothelial cell dysfunction is tyrosine kinase (EGFR) and IKK β dependent18 but PKC independent (Figure VI in the online-only Data Supplement). Moreover, inhibition of tyrosine kinase EGFR activation abrogated 20-HETE–stimulated IKK β, as well as IκB phosphorylation (Figure VII in the online-only Data Supplement), placing EGFR activation as the upstream step in the 20-HETE signaling pathway.18 Therefore, we examined whether 20-HETE–mediated ACE induction is also tyrosine kinase and IKK β dependent. Pretreatment with either 2-[(3,4,5-trihydroxyphenyl)methylene]-propanedinitrile, a tyrosine kinase inhibitor specific for EGFR, or 4-amino-[2,3'-bithiophene]-5-carboxamide, a specific IKK β inhibitor, prevented 20-HETE from increasing ACE expression. The PKC inhibitor calphostin C had no effect on 20-HETE–induced ACE mRNA (Figure 2A) or ACE activity (Figure VIB in the online-only Data Supplement). 11,12-epoxyeicosatrienoic acid, an endothelial-derived cytochrome P450 eicosanoid which has been shown to activate EGFR in endothelial cells,19 had no effect on ACE mRNA (Figure IIIB in the online-only Data Supplement).

To ascertain that 20-HETE–mediated ACE induction is dependent on IKK β activation, a specific IKK β short interfering RNA (siRNA) was used. IKK β siRNA suppressed the expression of IKK β by 70±6% and ACE by 49±7% when compared with control siRNA (Figure 2B). Furthermore, incubation with IKK β siRNA prevented 20-HETE–mediated induction of ACE, whereas incubation with the control siRNA had no effect on 20-HETE–induced ACE expression; in the latter, ACE expression in response to 20-HETE increased by 2.5-fold (Figure 2C).

Figure 1. Angiotensin-converting enzyme (ACE) mRNA levels in human microvascular endothelial cells (HMVECs) treated with (A) 20-hydroxyeicosatetraenoic acid (20-HETE) (5 nmol/L) for 30 minutes to 24 hours (cells treated with 20-HETE and 20-HEDE (5 nmol/L) were incubated for 2 hours) or with (B) increasing concentrations of 20-HETE for 2 hours (n=5; * P<0.05 vs vehicle; † P<0.05 vs 2 hours). C, Effect of 20-HETE (5 μmol/L) on ACE protein levels in HMVECs (n=4; * P<0.05 vs vehicle). D, ACE protein levels in HMVECs treated with 20-HETE (5 nmol/L) in the presence and absence of 20-HEDE (5 nmol/L) for 12 hours (n=4; * P<0.05 vs vehicle). E, ACE activity in HMVECs treated with and without 20-HETE (5 nmol/L) and 20-HEDE (5 nmol/L) for 24 hours. Lisinopril (100 nmol/L) was added to the reaction mixture. The basal (vehicle) cellular and extracellular specific activities for ACE were 41.77±8.35 and 208.30±61.36 pmol hippuric acid/min per milligram protein cell lysate, respectively (n=4–5; * P<0.05 vs vehicle, † P<0.05 vs 20-HETE).

The vascular wall, including the endothelium, expresses components of the renin–angiotensin system (RAS), including angiotensinogen, renin, ACE, ACE2, AT1R, and AT2R.17
Effect of ACE and AT1R Knockdown on 20-HETE–Mediated Inhibition of NO and Stimulation of O2⁻ Production

We have shown that in short-term experiments, 20-HETE inhibits NO production (and cGMP production, Figure VIII in the online-only Data Supplement) and stimulates O2⁻ formation primarily by uncoupling endothelial NO synthase14 (Figure IXA in the online-only Data Supplement). In long-term conditions, it also stimulates NADPH oxidase activity and increases O2⁻, which is rapidly converted to H2O2 in some cells.8,11,20 The role of ACE induction in 20-HETE–mediated NO inhibition and O2⁻ production was assessed using ACE- and AT1R-specific siRNAs. In cells transfected with the ACE siRNA, ACE mRNA was reduced by 42%, whereas levels of AT1R mRNA were unaffected. Transfection of cells with AT1R siRNA reduced AT1R mRNA by 47% but had no significant effect on ACE mRNA (Figure 3A). As expected, in cells transfected with the ACE siRNA, 20-HETE failed to increase ACE mRNA but did decrease AT1R mRNA by 45% (P<0.05). However, in cells transfected with the AT1R siRNA, 20-HETE had no further effect on AT1R mRNA, whereas its inducing effect on ACE was unaffected; ACE mRNA increased by 2-fold in response to 20-HETE in AT1R siRNA-transfected cells (Figure 3A). The efficacy of the siRNAs was further confirmed by Western blot (Figure 3B).

The effect of downregulating ACE and AT1R expression on the 20-HETE–mediated NO production is shown in Figure 3C. Treatment of cells with 20-HETE inhibited ionophore-stimulated NO production by 11.7- and 6.3-fold in nontransfected and control siRNA-transfected cells, respectively. ACE siRNA transfection negated the ability of 20-HETE to inhibit NO production. However, 20-HETE–mediated inhibition of NO production was attenuated by 40% in AT1R siRNA-transfected cells (Figure 3C), suggesting that Ang II contributes to 20-HETE–mediated inhibition of NO production via AT1R–dependent mechanisms.

The effect of downregulation of ACE and AT1R expression on the 20-HETE–mediated increase in O2⁻ formation was further evaluated using lisinopril and losartan. As shown in Figure 4A, incubation of cells with Ang II increased O2⁻ levels in HMVECs (Figure IXB in the online-only Data Supplement).
of 20-HEDE inhibited 20-HETE–mediated \( \mathrm{O}_2^- \) generation, it did not affect Ang II–mediated \( \mathrm{O}_2^- \) generation. However, losartan negated Ang II–stimulated \( \mathrm{O}_2^- \) generation and reduced 20-HETE–stimulated \( \mathrm{O}_2^- \) levels by 30%. Lisinopril also reduced 20-HETE–stimulated \( \mathrm{O}_2^- \) levels by 40% but had no effect on \( \mathrm{O}_2^- \) production in response to Ang II. Lisinopril and losartan had no significant effect on \( \mathrm{O}_2^- \) production in the absence of 20-HETE or Ang II (Figure 4A). These results further support the notion that Ang II contributes to the 20-HETE–mediated stimulation of \( \mathrm{O}_2^- \) production.

20-HETE–Mediated Impairment in Acetylcholine-Induced Vasorelaxation Is Partially Restored With Inhibitors of ACE and AT\(_1\)R

The results in endothelial cells suggested that ACE and AT\(_1\)R may play a role in the 20-HETE–mediated inhibition of acetylcholine-induced relaxation. As shown in Figure 4B, incubation of rat renal interlobar arteries with 20-HETE markedly impaired the relaxing response to acetylcholine, with maximal relaxation reduced from 76.8±3.8 to 19.6±2.6%. Treatment of arteries with lisinopril or losartan significantly improved 20-HETE–impaired relaxation responses to 46.4±3.5 and 58.5±5.2%, respectively. Addition of lisinopril to the bath in the absence of 20-HETE did not alter acetylcholine-induced relaxation response (74.3±10.0%). A similar response was achieved in arteries treated with losartan alone (75.7±0.1%) (Figure 4B).

Discussion

The relationship between RAS and 20-HETE has been suggested in several studies but has not been fully elucidated. Ang II has been shown to stimulate the synthesis of 20-HETE in isolated human neutrophils and platelets\(^ {21}\) and in rat kidney and preglomerular vessels.\(^ {22,23}\) In hypertensive humans, increased plasma levels of 20-HETE are correlated with increased plasma renin activity,\(^ {24}\) whereas in high-salt diet–fed rats in which the RAS is suppressed, CYP4A expression is reduced.\(^ {25}\) Other studies demonstrated that increased 20-HETE in the peripheral vasculature contributes to the acute vasoconstrictor response to Ang II,\(^ {26}\) whereas acute and chronic inhibition of 20-HETE synthesis, attenuates the renal pressor response to Ang II,\(^ {22}\) and the development of Ang II–dependent hypertension.\(^ {27}\)

20-HETE is known to mediate Ang II–induced mitogenic effects in cultured aortic vascular smooth muscle cells and contribute to the vascular injury, hypertrophy, and hypertension caused by Ang II in rats.\(^ {28–30}\) Interestingly, experimental models of hypertension that show increased vascular 20-HETE production, such as the spontaneously hypertensive rats\(^ {31,32}\) and the androgen-induced hypertensive rats,\(^ {11,33–35}\) are also RAS mediated. The nature of these interactions is yet to be fully identified. In a recent study, we showed that endothelial–specific CYP4A2 expression produced 20-HETE–dependent hypertension,\(^ {36}\) which was abrogated by either
The actions of 20-HETE, where it interferes with NO synthesis and stimulates O$_2^-$ production. We have recently demonstrated that, in endothelial cells, 20-HETE uncouples endothelial NO synthase via an EGFR-mitogen–activated protein kinase-IKKβ signaling pathway leading to decreased NO levels and increased O$_2^-$ production. Furthermore, in the long-term, 20-HETE also increases O$_2^-$ production by activating NADPH oxidase. Here, we demonstrated that 20-HETE induces ACE expression and increases its activity, an effect abrogated by cotreatment with the 20-HETE antagonist 20-HEDE. The ability of 20-HETE to increase ACE mRNA within 2 hours and the demonstration that inhibition of EGFR or IKKβ activation, both of which mediate 20-HETE actions in endothelial cells, abolished the ability of 20-HETE to increase ACE mRNA levels further points to the specificity of 20-HETE effect and suggests a novel mechanism not shared with known ACE inducers, such as phorbol 12-myristate 13-acetate and vascular endothelial growth factor. Phorbol 12-myristate 13-acetate-activated PKC has been shown to increase ACE mRNA level and ACE gene transcription, as well as ACE secretion in human umbilical vein endothelial cells. The demonstration that a PKC inhibitor had no effect on 20-HETE–mediated increases in ACE mRNA and activity further substantiate the notion of a distinct mechanism for 20-HETE actions on ACE expression and activity. Moreover, the fact that 11,12-epoxyeicosatrienoic acid, which has been shown to activate EGFR in endothelial cells, does not induce ACE mRNA suggests that activation of IKK downstream of EGFR is a key feature of 20-HETE action. A recent study demonstrated that inhibition of IKK activation in vivo abrogates 20-HETE–dependent vascular dysfunction and hypertension. The mechanism that links activation of EGFR-IKK and possibly nuclear factor-kappa B (NF-kB), the downstream effector molecule of IKKβ activation, to ACE expression is unclear. There are no reports regarding the presence of NF-kB response elements on the ACE promoter, and studies to examine the effect of NF-kB inhibition on intrarenal RAS expression in a model of proteinuric renal injury suggested that ACE expression is not subjected to regulation by NF-kB. However, IKKβ and NF-kB may activate a distinct ACE-responsive transcriptional pathway, such as activator protein 1. A connection between NF-kB and activator protein 1 has been documented, and activator protein 1 has been shown to activate the transcription of endothelial ACE. Clearly, further studies are needed to fully understand the mechanism by which 20-HETE induces endothelial ACE.

This study demonstrated that induction of ACE expression and activity is an important mechanism by which 20-HETE affects endothelial and vascular function. In cells transfected with ACE siRNA, 20-HETE–mediated inhibition of NO and stimulation of O$_2^-$ production were completely prevented. However, suppression of AT$_1$R expression by siRNA attenuated but did not prevent the 20-HETE–mediated effects on NO and O$_2^-$ production, suggesting that Ang II actions through the AT$_1$R (eg, stimulation of NADPH oxidase) are required but not necessary for achieving the maximum effect of 20-HETE. As for ACE, it is possible that its induction by 20-HETE elicits actions independent of Ang II and its actions through the AT$_1$R such as degradation of bradykinin, a key mediator of NO production in the endothelium and a substrate for ACE. However, we cannot exclude the possibility that ACE
contribution could be independent of its catalytic activity.\textsuperscript{47} Certainly, additional studies are needed to fully determine the mechanism by which ACE affects 20-HETE. The use of lisinopril and losartan to inhibit ACE activity and block the AT\(_R\), respectively, provided additional evidence that ACE and AT\(_R\) contribute to the stimulatory effect of 20-HETE on O\(_2^−\) levels. In addition, treatment of renal interlobar arteries with either lisinopril or losartan improved the 20-HETE–induced impairment in relaxation response to acetylcholine, an effect that has been attributed to 20-HETE–induced reduction in NO production through endothelial NO synthase uncoupling.\textsuperscript{10,14}

We also found that 20-HETE induces angiotensinogen expression in endothelial cells. Angiotensinogen is transcriptionally activated by NF-κB, and the IKK–NF-κB pathway may be the mechanism by which 20-HETE induces its expression. All together, the ability of 20-HETE to induce angiotensinogen in the same time frame as ACE suggests that 20-HETE contributes to activation of the RAS at multiple points. It also raises the possibility that 20-HETE perpetuates at least 2 positive feedback loops. As shown in Figure 5, there are several ways by which the initial response to 20-HETE can be amplified. We argue that ACE induction is an important component of 20-HETE–induced endothelial and vascular dysfunction.

We further postulate that induction of endothelial ACE sets in motion a functional amplification circuit that combines the actions of 20-HETE, ACE (generation of Ang II and degradation of bradykinin), and Ang II (reactive oxygen species–derived endothelial dysfunction, vasoconstriction), and further increases vascular dysfunction. The ability of 20-HETE to increase angiotensinogen expression provides an additional amplification of the aforementioned processes. The relevance of this study to human health is supported by numerous reports indicating a correlation between urinary 20-HETE, oxidative stress, and endothelial dysfunction in hypertensive subjects,\textsuperscript{46,49} as well as associations among CYP4F2 (a major 20-HETE–synthesizing enzyme in humans) polymorphisms and hypertension\textsuperscript{40,51} and ischemic stroke.\textsuperscript{72}

**Sources of Funding**

This study was supported by National Institutes of Health grants HL34300 (M.L. Schwartzman), DK38226 (J.R. Falck), and F30 HL097402 (C.C. Wu) and National Heart, Lung, and Blood Institute Diversity Supplement HL34300 (V. Garcia), the Robert A. Welch Foundation (GL625910) (J.R. Falck), and American Heart Association predoctoral fellowship (0715781T) to J. Cheng.

**Disclosures**

None.

**References**


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Arterioscler Thromb Vasc Biol. 2012;32:1917-1924; originally published online June 21, 2012; doi: 10.1161/ATVBAHA.112.248344

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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SUPPLEMENT MATERIAL

METHODS AND MATERIALS

Cell culture. Human microvascular endothelial cells (HMVECs) were grown in Medium 131 containing 5% microvascular growth supplement (Invitrogen) and 5% fetal bovine serum (FBS, USA Scientific). Passages 3-5 were used for all experiments. Ea.hy926 cells were grown in Dulbecco’s Modified Eagle Medium with 5% FBS, 1% penicillin-streptomycin and HAT supplement (sodium hypoxanthine, aminopterin and thymidine). All cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂/95% O₂.

Transfection with siRNA. Cells were grown on 12-well plates to 70% confluence and placed in serum-free media for 24 h. Cells were washed with siRNA transfection medium (Santa Cruz) and incubated with a mixture of ACE, AT₁-R, IKKβ or control siRNAs (100 nmol/L, Santa Cruz) and siRNA transfection reagent (Santa Cruz) for 6 h. The transfection solutions were replaced with fresh growth medium for an additional 36 h before assaying for NO or O₂⁻ levels. Cells were also assayed for ACE and AT₁-R mRNA expression by real-time PCR and for IKKβ protein levels by Western blot analysis as previously described 1.

Real-Time PCR. Cells were cultured on 6-well plates to 80-90% confluence and starved in serum-free media for 24 h. Cells were incubated with and without 20-HETE (5 nmol/L), 20-hydroxyeicosa-6(Z),15(Z)-dienoic acid (20-HEDE, a 20-HETE antagonist, 5 nmol/L), 19(R)-HETE (a 20-HETE antagonist, 5 nmol/L), actinomycin D (a DNA transcription inhibitor, 10 µmol/L), calphostin C (a PKC inhibitor, 100 nmol/L) or AG82 (an EGFR-tyrosine kinase inhibitor, 10 µmol/L) for 0.5-4 h in serum-free media. Cells were washed with 1X PBS, trypsinized, centrifuged and lysed in 350 µl Buffer RLT (Qiagen). Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and quantified with NanoDrop (ThermoScientific). RT reaction of total RNA (500 ng) was performed using the qScriptDNA Synthesis Kit (Quanta Biosciences). Sequences for PCR primers used are as follows:

angiotensin converting enzyme (ACE) sense: 5'- CGCTGAAACCCTGTACG A -3'; ACE antisense: 5'- TGGGGGAGTTGTACCAGG AG -3'; ACE2 sense: 5'- GAG CTA ATG CAT GCC ATT CTC A -3'; angiotensinogen antisense: 5'- GCCTGCTTACATCTT CAG C -3'; AT₁-R sense: 5'- GTCACTGCAATCATC ACC TGGTTG -3'; AT₂-R antisense: 5'- TCATAAGCTTCTTCTAGGGGCTTC -3'; AT₂-R sense: 5'- TTC ACC ACCTGAA ATA TGC C -3'; AT₂-R antisense: 5'- GGTCACGGGTTATCCGTCTTCT T -3'; renin antisense: 5'- TCGGAGGAGTGTGTACCAGG AG -3'; renin antisense: 5'- TCTCGGAAT CTC TGTGT AG -3'; CYP4A11 sense: 5'- CYP4A11 antisense: 5'- ATT GTT TCC CGA TGC AGT TC -3'; CYP4A22 sense: 5'- ATG GAT TGG GTA CAG CTT GC -3'; CYP4A22 antisense: 5'- CAT CAA GGA GAC GTG CTG AA -3'; CYP4F2 sense: 5'- GAG TGC TGG TGA CAA GTG GA -3'; CYP4F2 antisense: 5'- TGA GCC TGA TGT GCT GAA AC -3'; CYP4F3 sense: 5'- AGG AGG TTG TGT GGG ACA AG -3'; CYP4F3 antisense: 5'- CYP4F3 antisense: 5'- TGA CAG CAG TGG TCA TAG AA -3'; CYP4F8 sense: 5'- CAT CTT CAG TTT TGA CAG CAA -3'; CYP4F8 antisense: 5'- TGA GCT CCA TGA TCG CAG TA -3'; 18S rRNA sense: 5'- CYP4F8 sense: 5'- CAT CTT CAG TTT TGA CAG CAA -3'; CYP4F8 antisense: TGA GCT CCA TGA TCG CAG TA -3'; 18S rRNA antisense: 5'- GCCTGG ATT CTT CAT AATGGT - 3'. Quantitative Real-Time PCR was performed using the PerfeCTaSYBR Green FastMix Low ROX Kit (Quanta Biosciences) and the Mx3000p Real-Time PCR System (Stratagene) as previously described 2.

Western blot analysis: Cells were cultured on 6-well plates to 80-90% confluence and starved in serum-free media for 24 h prior to incubation with and without 20-HETE (5 nmol/L) in the
presence and absence of 20-HEDE (5 nmol/L) for 6-24h. Cells were lysed with 1X RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma) containing protease and phosphatase inhibitor cocktails (Roche Applied Sciences.) Protein concentrations were determined using the Bradford protein assay (Eppendorf BioPhotometer). Proteins (20 µg) were loaded onto a SDS-polyacrylamide gel: 4% Stacking/8%Resolving gel (Bio-Rad Laboratories) with respective loaded Precision Plus Protein Dual Color Standard (Bio-Rad Laboratories) markers. SDS-polyacrylamide gels were transferred to Immobilon-FL transfer membranes (Millipore) followed by blocking buffer (Odyssey Infrared Imaging System Li-Cor) and subsequent incubation with primary and secondary antibodies. Antibodies included: ACE (N-20) goat polyclonal IgG raised against a peptide mapping near the end terminus of the human ACE (1:200, Santa Cruz Biotechnology), anti-β-Actin mouse monoclonal IgG (Sigma), Alexa Fluor 680 donkey anti-goat IgG (H+L) (1:5000, Invitrogen Molecular Probes), and goat anti-mouse IRDye 800CW (1:10000, Odyssey Infrared Imaging System Li-Cor). Membrane fluorescence-based immunodetection was conducted using the Li-Cor Odyssey Infrared Imaging System and respective band density was quantified using the Odyssey Application Software Version 3.0.21.

**Measurement of NO and cGMP.** Cells were preincubated with or without 20-HETE (5 nmol/L) (or its vehicle, 0.1% ethanol) for 2 h and were treated with or without calcium ionophore A23187 (5 µmol/L) in the presence of L-arginine (25 µmol/L) for 30 min. In some experiments, L-NAME (1 mmol/L) was added to the preincubation period and NO levels were evaluated by measuring total nitrite and nitrate (NOx) content in the reaction medium using the NO quantitation kit and following the manufacturer’s instructions (Active Motif, Carlsbad, CA) as previously described 3. Levels of cGMP were assessed by immunoassay using a commercial kit (R&D Biosystems, Minneapolis, MN) as previously described 4.

**Measurement of superoxide (O2^-) and hydrogen peroxide (H2O2).** Cells were cultured on 96-well plates to ~70% confluence and placed in serum-free media for 24 h. Cells were treated with or without Tiron (10 mmol/L), 20-HETE (5 nmol/L) (or its vehicle, 0.1% ethanol), 20-HEDE (5 nmol/L), angiotensin II (100 nmol/L), losartan (10 µmol/L) and lisinopril (10 µmol/L) for 2 h. For measurements of O2^- levels, dihydroethidium (DHE, 5 µmol/L, Calbiochem, Gibbstown, NJ) was added for 20 min at 37°C and fluorescence intensity was measured with excitation/emission filters of 530 nm/620 nm as described 3. The fluorescence obtained in Tiron-treated cells was used to represent non-specific/background fluorescence and was subtracted from all values. For measurements of H2O2 levels, polyethylene-glycolated superoxide dismutase (PEG-SOD, 100U/ml, Sigma Aldrich) or polyethylene-glycolated catalase (PEG-catalase, 500U/ml, Sigma Aldrich) were added 30 min prior to staining with 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, 5 µmol/L, Sigma Aldrich) for 20 min. Fluorescence intensity was measured with excitation/emission filters of 485 nm/530 nm.

**ACE activity assay.** HMVECs were cultured on 6-well plates to ~90% confluence and placed in serum-free media for 24 h. Cells were then treated with 20-HETE (5 nmol/L) with or without 20-HEDE (5 nmol/L) for 24 h. Cell medium and cell lysate were used to determine ACE activity using the BÜHLMANN ACE kinetic test from ALPCO (Salem, NH) following the manufacturer’s instruction. Lisinopril (100 nmol/L) was added to the reaction mixture to ascertain ACE activity. The assay uses Furylacryloyl-phenylalanyl-glycyl-glycine (FAPGG) as the substrate. Hydrolysis of FAPGG to furylacryloylphenylalanine (FAP) and glycyglycine results in a decrease in absorbance at 340 nm. The ACE activity in the sample is determined by comparing the sample reaction rate to that obtained with the ACE Calibrator and expressed as pmol hippurric acid/min/mg protein of cell lysate.
**Agonist-induced vasorelaxation**—All experimental protocols were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (n=3-5) at 7-8 wks old were used. Rats were anesthetized with ketamine (70 mg/kg) and xylazine (7 mg/kg) and laparotomy was performed. Renal interlobar arteries were microdissected and mounted on wires in myograph chambers (J.P. Trading, Denmark) for measurement of isometric tension as described. Arteries were incubated in the presence and absence of 20-HETE (1 µmol/L), lisinopril (10 µmol/L) and losartan (10 µmol/L) for 2 h, after which they were preconstricted with phenylephrine (5 µmol/L) and the relaxing responses to increasing concentrations (5x10^{-8}-5x10^{-4} mol/L) of acetylcholine were measured.

**Statistical analysis.** Data are means±SEM. Significance of difference in mean values was P<0.05 was considered to be significant.

**RESULTS**

**HMVECs express CYP4 isoforms but do not have detectable levels of 20-HETE**

mRNA expression of CYP4F2, 4F3,4F8 and CYP4A22 were present in HUVECs as determined via real-time PCR analysis. CYP4A11 mRNA was undetectable. CYP4F2 expression was the highest with a Ct value of 25, followed by CYP4F8, 4F3 and 4A22 with Ct values of 31, 32 and 39, respectively (Figure I). LC-MS/MS analysis of eicosanoids in HMVECs, either with or without arachidonic acid supplementation, revealed no detectable levels of 20-HETE (sensitivity of the assay is 20 pg). These results support our previous report showing that the vascular endothelium is not a significant source of 20-HETE but rather a target for 20-HETE in the vasculature.

![Figure I: CYP4 isoform expression in HMVECs. Cells (passages 3 or 4) were grown to 80% confluency, harvested and processed for real-time PCR analysis as described in Methods. Levels of mRNA were normalized to 18S. Results are mean±SEM, n=3.](image)

**20-HETE-mediated increase in ACE mRNA is distinct from that of PMA**

Maximal increase of ACE expression in response to 20-HETE occurs at 2 h, a time frame that is distinct from that of known ACE inducers such as phorbol esters (PMA). We examined the time-dependent effects of PMA along with TNFα (a reported inhibitor of ACE expression) on ACE mRNA levels in HMVECs. As seen in Figure II, a significant 2-fold induction of ACE mRNA in response to PMA was only observed at 24 h. In contrast, 20-HETE increased ACE mRNA by 2-fold at 2 h and had no significant effect thereafter. TNFα had no significant effect on ACE mRNA
levels. These results suggest that the mechanism by which 20-HETE increases levels of ACE mRNA is different than that of PMA.

![Graph of time-dependent effect of 20-HETE, PMA, and TNFα on ACE mRNA levels in HMVECs.](image)

**Figure II:** Time-dependent effect of 20-HETE, PMA, and TNFα on ACE mRNA levels in HMVECs. Cells were grown to 80% confluence and placed in serum-free media for 24 h prior to addition of 20-HETE (5 nmol/L) with and without 20-HEDE (5 nmol/L), PMA (1 µmol/L), and TNFα (10 ng/ml). Cells were incubated for 2, 4, 6, and 24 h. ACE mRNA levels were normalized to 18S RNA. Results are given as fold increase from corresponding controls. The inset depicts the time difference of ACE induction by 20-HETE and PMA. Results are mean±SEM, n=4, *p<0.05 vs corresponding controls.

**20-HETE-mediated increase ACE mRNA is inhibited by actinomycin D and is not shared by 11,12-EET**

As seen in Figure III, actinomycin D (10 µmol/L) negated the stimulatory effect of 20-HETE on ACE mRNA suggesting that 20-HETE-mediated increased in ACE mRNA is at the level of transcription. Actinomycin D alone decreased basal ACE mRNA by 30%.
Figure III: A) Effect of actinomycin D on 20-HETE-mediated increase in ACE mRNA. HMVECs were grown to 80% confluence and placed in serum-free media for 24 h prior to addition of 20-HETE (5 nmol/L) with and without actinomycin D (10 µmol/L). Cells were incubated for 2 h and ACE mRNA was measured as described in Methods (mean±SEM, n=5, *p<0.05 vs vehicle). B) Effect of 11,12-EET (5 nmol/L) on ACE mRNA (mean±SEM, n=5, *p<0.05 vs vehicle).

20-HETE-stimulated increase in ACE protein is negated by 20-HEDE

Figure IV: Effect of 20-HEDE on 20-HETE stimulated increase in ACE protein. HMVECs were grown to 80% confluence and placed in serum-free media for 24 h prior to addition of 20-HETE (5 nmol/L) with and without 20-HEDE (5 nmol/L). Cells were incubated for 12 h and ACE protein was measured by Western blot and densitometry analysis (mean±SEM, n=3, *p<0.05 vs vehicle).

Effect of 20-HETE on components of the renin-angiotensin system

The vascular wall, including the endothelium, expresses components of the RAS, including angiotensinogen, renin, ACE, ACE-2, AT₁R and AT₂R. While the initial gene microarray showed little or no effect of 20-HETE on the expression of RAS components other than ACE, a further time-dependent real-time PCR analysis indicated otherwise. Addition of 20-HETE to endothelial cells increased angiotensinogen expression as early as 1 h and peaked at 2 h. This increase in angiotensinogen levels at 2 h after addition of 20-HETE was prevented upon co-treatment with 20-HEDE (Figure VA). 20-HETE had no effect on ACE2 expression (Figure VB). Interestingly, AT₁R expression decreased by 40 and 30% at 1 and 2 h, respectively, and this decrease was prevented by co-treatment with 20-HEDE (Figure VC). Similarly, expression of AT₂R in response to 20-HETE was reduced by about 40%; this decrease was also prevented by co-treatment with 20-HEDE (FigureVD). The expression levels of renin were low (Ct values ranged between 32-33) when compared to Ct values (27-29) for all other RAS components and there was no apparent effect of 20-HETE on these levels (data not shown).
Figure V: Effect of 20-HETE on expression of RAS components. Ea.hy926 cells were treated with 20-HETE (5 nmol/L) for 30 min to 4 h in the presence and absence of 20-HEDE (5 nmol/L). Cells were probed for levels of (A) angiotensinogen, (B) ACE2, (C) angiotensin II type 1 receptor (AT1R) and (D) angiotensin II type 2 receptor (AT2R) mRNAs. Results are mean±SEM; n=3-6; *p<0.05 vs vehicle, †p<0.05 vs 2h.

20-HETE-mediated inhibition of NO production and stimulation of ACE activity is PKC-independent

PKC has been shown to participate in eNOS deactivation 9. Moreover, 20-HETE actions in the vasculature have been shown to be PKC-dependent 10, 11. Therefore, we assessed whether 20-HETE inhibits NO production via PKC-dependent mechanisms. As seen in Figure VI, addition of calphostin C, a PKC inhibitor, had no effect on 20-HETE-mediated inhibition of NO production in HMVECs. These data also corroborate with our previous finding that 20-HETE does not affect phosphorylation of eNOS at Thr-495 3. PMA-activated PKC has been shown to increase ACE mRNA level and ACE gene transcription in HUVEC, an effect associated with an increased ACE secretion 12. Our results indicate that the PKC inhibitor calphostin C did not affect 20-HETE-mediated induction of ACE mRNA (Figure 2 in the manuscript). Moreover, as seen in Figure VIB, addition of calphostin C to HMVECs incubated with 20-HETE had no effect on ACE activity, suggesting that the mechanism by which 20-HETE induces ACE mRNA and increases ACE activity does not include PKC activation.
Figure VI: (A) Effect of PKC on 20-HETE-mediated inhibition of NO production. HMVECs were incubated with 20-HETE (5 nmol/L) with and without calphostin C (CC, 100 nmol/L) and the calcium ionophore A23187 (5 µmol/L). NO levels were measured using the NO quantitation kit. Results are mean±SEM, n=6. p<0.05 from untreated control; †p<0.05 from A23187-treated cells. (B) Effect of PKC on 20-HETE-mediated increase in ACE activity. Cells were incubated with and without 20-HETE (5nmol/L) in the presence and absence of calphostin C (CC, 100 nmol/L) for 24 h. ACE activity was measured in the culture media. Results are mean±SEM relative to control, n=4, *p<0.05.

20-HETE-induced NF-kB activation is tyrosine kinase-dependent.

To further support our working hypothesis indicating tyrosine kinase activation as the upstream signaling step of in endothelial cells (Figure 5), cells were pretreated with inhibitor of tyrosine kinase, prior to addition of 20-HETE (5 15 min. Western blot analysis indicated that inhibition of kinase activation abrogated the 20-HETE-induced activation of the NF-κB pathway, as measured by IκB phosphorylation (Figure VII).

Figure VII: 20-HETE-induced NF-κB activation is tyrosine kinase-dependent. A representative blot and densitometry analysis of phosphorylated IκB:total IκB are shown. HMVECs were grown to 80% confluence and placed in serum-free media for 24 h prior to the addition of 20-HETE (5 nmol/L) with and without the tyrosine kinase inhibitor, genistein (30 µmol/L). Results are mean±SEM, n=4, *p<0.05 vs vehicle; #p<0.05 vs 20-HETE.

20-HETE inhibition of NO production is associated with inhibition of cGMP in HMVECs

Measurement of cGMP are frequently used to assess eNOS-mediated NO production. As seen in Figure VIII, similar to L-NAME, 20-HETE inhibited ionophore-stimulated cGMP levels as it inhibited ionophore-stimulated NO levels.
Figure VIII: Effect of 20-HETE on ionophore-stimulated NO and cGMP production in HMVECs. Cells were incubated with 20-HETE (5 nmol/L) with and without L-NAME (1 mmol/L) and the calcium ionophore A23187 (5 µmol/L). NO levels were measured using the NO quantitation kit. cGMP levels were measured by immunoassay using a commercial kit. Results are mean±SEM, n=6. *p<0.05 from untreated control;

Effect of 20-HETE on H2O2 levels in HMVECs

We have previously shown that 20-HETE-stimulated superoxide (O2-) production is initially the result of eNOS uncoupling by 20-HETE3, 5. 20-HETE uncouples eNOS by interfering with its association with HSP90 via a EGFR-MAPK-IKKβ dependent pathway.5 Figure IXA further demonstrates that the stimulated production of O2- following addition of 20-HETE for 30 min is not inhibited by apocynin, an inhibitor of NADPH oxidase; it is, however, inhibited by L-NAME (Figure IXA). Long term, 20-HETE has been shown to activate NADPH oxidase and increase O2-, which rapidly dismutases to H2O2 in some cells1, 13, 14. We further examined whether 20-HETE affects H2O2 levels within the 2 h time frame of our experimental protocol. As seen in Figure IXB, 20-HETE had no effect on basal, SOD- or SOD+catalase-stimulated H2O2 production.

Figure IX: A) Effect of L-NAME and apocynin on 20-HETE-mediated stimulation of superoxide (O2-) production. Treatment with L-NAME inhibits while pretreatment with apocynin has no effect on the 20-HETE-mediated increase in O2- levels. HMVECs were preincubated with 20-HETE (5 nmol/L) with and without apocynin (NADPH oxidase inhibitor; 100 µmol/L) or L-NAME (1 mmol/L) for 30 min. O2- levels were measured using DHE fluorescence at excitation and
emission wavelengths of 530 nm and 620 nm. Results are mean±SEM, corrected for Tiron, n=5, \(^p<0.05\) from untreated. B) Effect of 20-HETE on \(\text{H}_2\text{O}_2\) levels in HMVECs. Cells were cultured on 96-well plates to ~70% confluence and placed in serum-free media for 24 h. Cells were treated with and without 20-HETE (5 nmol/L) (or its vehicle, 0.1% ethanol) for 2 h, after which PEG-SOD (100U/ml) and PEG-catalase (500U/ml) were added 30 min prior to staining with 2',7'-dichlorodihydrofluorescein diacetate (H\(_2\)DCF-DA, 5 \(\mu\)mol/L) for 20 min. Fluorescence intensity was measured with excitation/emission filters of 485 nm/530 nm. Results are mean±SEM; n=4.

Effect of ROS on 20-HETE-mediated impairment of acetylcholine-induced relaxation

We have previously shown that 20-HETE impairs the NO-dependent acetylcholine-induced relaxation and its action is associated with decreased NO production \(^3\). Using renal microvessels from the androgen-treated rat, a model of increased vascular 20-HETE production\(^1\), we further showed that scavenging ROS only partially attenuates the 20-HETE-mediated inhibition of acetylcholine-induced relaxation. Addition of Tiron (10 mmol/L) to arteries from control rats did not have any effect on the relaxation response to acetylcholine (67.4±5.7% without Tiron, 62.3±0.4% with Tiron). However, addition of Tiron to arteries from 5α-dihydrotestosterone (DHT)-treated rats partially improved the maximal relaxation response to acetylcholine from 14.1±2.3 to 34.2±3.1%, but not to the maximal response that is achieved with the 20-HETE antagonist, 19(R)-HETE (54.0±6.4%). Further addition of 20-HETE to the chamber bath impaired the relaxation response to its original state (21.7±6.7%) (Figure X).

![Figure X: Scavenging superoxide with Tiron partially restores acetylcholine-induced relaxation in arteries from DHT-treated rats. (A) Acetylcholine-induced relaxation in renal interlobar arteries from control and DHT-treated rats was monitored in the presence and absence of Tiron (10 mmol/L), 20-HETE (20H, 50 nmol/L) and the 20-HETE antagonist 19(R)-HETE (1 \(\mu\)mol/L). (B) Results are represented as maximal relaxation response after 30 sec (% of phenylephrine constriction) to acetylcholine, n=4. \(^*p<0.05\) vs. DHT; \(#p<0.05\) vs. DHT+Tiron; \(^†p<0.05\) vs. DHT+Tiron+20-HETE.](image-url)
REFERENCES


