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20-HETE Activates the Transcription of Angiotensin-Converting Enzyme via Nuclear Factor-κB Translocation and Promoter Binding

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ABSTRACT

Increased vascular 20-hydroxyeicosatetraenoic acid (20-HETE), a cytochrome P450 arachidonic acid metabolite, promotes vascular dysfunction, injury, and hypertension that is dependent, in part, on the renin angiotensin system (RAS). We have shown that, in human microvascular endothelial cells, 20-HETE increases angiotensin-converting enzyme (ACE) mRNA, protein, and ACE activity via an epidermal growth factor receptor (EGFR)/tyrosine kinase/mitogen-activated protein kinase (MAPK)/inhibitor of κB kinase (IKK)β–mediated signaling pathway. In this work, we show that, similar to epidermal growth factor (EGF), 20-HETE (10 nM) activates EGFR by stimulating tyrosine phosphorylation; however, unlike 20-HETE, EGF does not induce ACE expression, and pretreatment with a neutralizing antibody against EGF does not prevent the 20-HETE–mediated ACE induction. Inhibition of nuclear factor κB (NF-κB) activation prevented the 4.58-fold (±0.78; $P < 0.05$) 20-HETE–mediated induction of ACE. The 20-HETE increased NF-κB–binding activity in nuclear extracts and the activity of both the somatic and germinal ACE promoters by 4.37-fold (±0.18; $P < 0.05$) and 2.53-fold (± 0.24; $P < 0.05$), respectively. The 20-HETE–stimulated ACE promoter activity was abrogated by the 20-HETE antagonist 20-hydroxy-6,15-eicosadienoic acid and by inhibitors of EGFR, MAPK, IKKβ, and NF-κB activation. Sequence analysis demonstrated the presence of two and one putative NF-κB binding sites on the human somatic and germinal ACE promoters, respectively. Chromatin immunoprecipitation assay indicated that 20-HETE stimulates the translocation and subsequent binding of NF-κB to each of the putative binding sites (S1, 3.43 ± 0.3-fold enrichment versus vehicle; S2, 3.72 ± 0.68-fold enrichment versus vehicle; S3, 3.20 ± 0.18-fold enrichment versus vehicle; $P < 0.05$). This is the first study to identify NF-κB as a transcriptional factor for ACE and to implicate a distinct EGFR/MAPK/IKK/NF-κB signaling cascade underlying 20-HETE–mediated transcriptional activation of ACE mRNA and stimulation of ACE activity.

Introduction

Angiotensin-converting enzyme (ACE) is a critical catalytic enzyme in the renin angiotensin system (RAS), primarily involved in the conversion of the decapeptide angiotensin (Ang) I to the vasoactive octopeptide Ang II (Ng and Vane, 1967). In addition to Ang I, it also catalyzes the breakdown of peptides such as bradykinin and substance P (Skidgel and Erdos, 1987). Within the vasculature, ACE expression and activity are predominantly localized to the endothelium and undergo a systematic shedding process propagated by a yet-to-be-identified shedase (Ramchandran et al., 1994). Sequence analysis of the ACE gene located on chromosome 17 demonstrated the presence of two unique promoter regions required for the transcriptional activation of ACE (Shai et al., 1990). The first region, termed the somatic ACE promoter, is critical for the production of endothelial/vascular ACE, whereas the second, the germinal ACE promoter, is involved in the formation of the testis ACE protein that contains only a single catalytic N-terminal domain and is localized to the testis and not involved in the conversion of Ang I to Ang II (Bernstein et al., 2013). Vascular ACE expression requires the use of both the somatic and germinal ACE promoter regions, and disruption in these locations results in changes to protein structure (Fuchs et al., 2008) and localization (Bernstein et al., 2005; Shen et al., 2008). We have recently identified 20-hydroxyeicosatetraenoic acid (20-HETE) as a potent inducer of endothelial ACE (Cheng et al., 2012).

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ABBREVIATIONS: ACE, angiotensin-converting enzyme; Ang, angiotensin; ChIP, chromatin immunoprecipitation; CYP, cytochrome P450; EGF, epidermal growth factor; EGFR, EGF receptor; EMSA, electrophoretic mobility shift assay; HBSS, Hanks' buffered salt solution; HMVEC, human microvessel endothelial cell; IKK, inhibitor of κB kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; PCR, polymerase chain reaction; RAS, renin angiotensin system; TNF-α, tumor necrosis factor α; 20-HEDE, 20-hydroxy-6,15-eicosadienoic acid; 20-HETE, 20-hydroxy-5,8,11,14-eicosatetraenoic acid.

The 20-HETE is the ω-hydroxylation product of arachidonic acid metabolism by enzymes of the cytochrome P450 (CYP) 4 families. It elicits a variety of effects on the vasculature promoting the onset of hypertension. Synthesized by vascular smooth muscle cells along the vessel wall, 20-HETE stimulates vasoconstriction through inhibition of the smooth muscle large conductance Ca^{2+}-activated K^+ channels, leading to depolarization and elevation in cytosolic Ca^{2+} (Miyata and Roman, 2005). In addition, 20-HETE is a potent inducer of endothelial dysfunction and activation via uncoupling of endothelial nitric oxide synthase (Cheng et al., 2008, 2009), stimulation of NADPH oxidase activity (Zeng et al., 2010), and activation of the nuclear factor κB (NF-κB)–mediated inflammatory program (Ishizuka et al., 2008).

Changes in vascular 20-HETE have been demonstrated to contribute to increases in blood pressure in spontaneously hypertensive rats, androgen-treated rats and mice, and animal models in which specific 20-HETE–producing CYP enzymes are overexpressed (Wu et al., 2014). Studies in our laboratory uncovered a close relationship between 20-HETE and the RAS. These studies showed that blood pressure increase in models of 20-HETE–dependent is associated with marked induction of the vascular ACE and is prevented by administration of ACE inhibitors or Ang II receptor blockers (Sodhi et al., 2010). Administration of a 20-HETE synthesis inhibitor or a 20-HETE antagonist has been shown to prevent or reverse the endothelial dysfunction, oxidative stress, and RAS activation, all of which are associated with hypertension, thus positioning 20-HETE as a potent upstream effector (Wang et al., 2006; Singh and Schwartzman, 2008; Sodhi et al., 2010).

We have recently identified the inhibitor of κB kinase (IKK)–NF-κB signaling pathway as a critical component of the cellular mechanisms underlying 20-HETE actions in the vascular endothelium, including endothelial nitric oxide synthase uncoupling and ACE induction (Cheng et al., 2009, 2012). The IKK complex and NF-κB have been implicated in a variety of vascular diseases and complications, including obesity (Kassan et al., 2013), renal disease, and hypertension (Cardinale et al., 2012). In animal models, hypertension-induced renal damage can be attenuated by endothelial-specific cell suppression of NF-κB (Henke et al., 2007). Additionally, in mice, inhibition of IKK complex has been demonstrated to ameliorate the hypertension and changes in vascular reactivity observed in the androgen-induced hypertension model, a model of 20-HETE–dependent hypertension (Wu et al., 2011). Targeting of the IKK complex has been shown to inhibit 20-HETE–dependent endothelial activation and dysfunction (Cheng et al., 2009, 2012), placing IKK and NF-κB as two major effectors downstream of 20-HETE. The link between NF-κB and ACE remains unclear, although ACE inhibitors have been demonstrated to decrease NF-κB gene signaling (Hernandez-Presa et al., 1997). To date, no studies have linked NF-κB as a regulator of ACE gene expression.

The present study is the first to identify NF-κB as a transcription factor regulating ACE expression and to implicate a distinct epidermal growth factor receptor (EGFR)/mitogen-activated protein kinase (MAPK)/IKK/NF-κB signaling cascade underlying 20-HETE–mediated transcriptional activation of ACE mRNA, protein, and stimulation of ACE activity.

Materials and Methods

Cell Culture. Human microvascular endothelial cells (HMVECs) were grown in Medium 131 containing microvascular growth supplement (Invitrogen, Waltham, MA). Passages 2–4 were used for all experiments. Cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO_2:95% O_2. For most experiments, cells were grown in six-well plates to 80–90% confluence and placed in serum-free Hanks' buffered salt solution (HBSS) media for 24 hours prior to addition of compounds. Compounds used in this study are the following: 20-hydroxyeicosatetraenoic acid (20-HETE; 5–10 nmol/L), 20-hydroxyeicosa-6(Z),15(Z)-dienoic acid (20-HEDE, a 20-HETE antagonist; 10 nmol/L), tumor necrosis factor α (TNF-α; 10 ng/ml), epidermal growth factor (EGF; 100 ng/ml), an anti-EGF neutralizing antibody (EGF_AB-236-NA; 5 μg/ml) (R&D Systems, Minneapolis, MN), AG1478 (an EGFR-tyrosine kinase inhibitor; 10 μmol/L), PD168393 (an irreversible EGFR-tyrosine kinase inhibitor; 10 μmol/L), U0126 (MAPK inhibitor; 10 μmol/L), SC-514 (an IKKβ inhibitor; 25 μmol/L), and JSH-23 (a NF-κB translocation inhibitor; 25 μmol/L).

Real-Time Polymerase Chain Reaction. Cells were cultured on six-well plates to 80–90% confluence and starved in serum-free media for 24 hours. Cells were incubated with and without 20-HETE (10 nmol/L), an anti-EGF neutralizing antibody (5 μg/ml), PD168393 (10 μmol/L), SC-514 (25 μmol/L), and JSH-23 (25 μmol/L). Cells were washed with 1× phosphate-buffered saline, followed by lysis buffer (Denville Scientific, Metuchen, NJ). Total RNA was isolated using the SpinSmart RNA Purification kit (Denville Scientific) and quantified using the Synergy HT Take3 Microplate Reader (BioTek, Winooski, VT). Reverse-transcriptase reaction of total RNA (500 ng) was performed using the qScript cDNA Synthesis kit (Quanta Biosciences, Gaithersburg, MD). Sequences for polymerase chain reaction (PCR) primers used are as follows: ACE sense, 5′-CGC TGA AAC CGC TGT ACG A-3′; ACE antisense, 5′-TGG GGG AGT TGT ACC AGG AG -3′; 18S rRNA sense, 5′-GAT GGG CGG CGG AAA ATA G -3′; 18S rRNA antisense, 5′-GCG TGG ATT CTG CAT AAT GGT-3′. Quantitative real-time PCR was performed using the PerfeCTa SYBR Green FastMix Low ROX kit (Quanta Biosciences) and the Mx3000p Real-Time PCR System (Stratagene, Santa Clara, CA), as previously described (Cheng et al., 2012).

Immunoprecipitation. HMVECs were cultured on six-well plates to 80–90% confluence and starved in serum-free media for 24 hours. Cells were pretreated with an anti-EGF neutralizing antibody EGF_AB-236-NA (5 μg/ml) (R&D Systems) for 40 minutes prior to incubation with 20-HETE (10 nmol/L) or its vehicle (phosphate-buffered saline) for 5 minutes. The neutralization dose for EGF_AB-236-NA measured by its ability to neutralize EGF-induced proliferation of fibroblasts is 0.04–0.08 μg/mL in the presence of 2 ng/mL recombinant human EGF (R&D Systems). EGF (100 ng/ml) treatment of 5 minutes was used as a positive control. Cells were lysed with 1× radioimmunoprecipitation assay buffer (Sigma-Aldrich, St. Louis, MO) containing protease and phosphatase inhibitor cocktails (Roche Applied Sciences, New York, NY). Protein concentrations were determined using the Bradford protein assay (Eppendorf BioPhotometer). Immunoprecipitation was conducted using the Dynabeads Protein G Immunoprecipitation kit (Life Technologies, Grand Island, NY). Dynabeads were incubated with the anti-human EGFR antibody (AHR5062; Invitrogen, Camarillo, CA) for 1 hour prior to washing and incubation with 10 μg cell lysate overnight. Samples were then washed and eluted per manufacturer's protocol and loaded onto a 4–20% Mini-PROTEAN TGX precast gel (Bio-Rad, Hercules, CA). Immunoblotting for phosphorylated tyrosine and EGFR was conducted using the phosphorylated tyrosine antibody (SC-7020; Santa Cruz Biotechnology, Dallas, TX) and EGFR antibody AHR5062, respectively. Membrane fluorescence-based immunodetection was conducted using the LI-COR Odyssey Infrared Imaging System (LI-COR, Lincoln, NE), and respective band density was quantified using the Odyssey Application Software Version 3.0.21.

Western Blot Analysis. Cells were cultured on six-well plates to 80–90% confluence and starved in serum-free HBSS media for 24 hours prior to incubation with and without 20-HETE (5-10 nmol/L) in the presence and absence of the NF-κB translocation inhibitor JSH-23 (25 μmol/L) for 12 hours. Cells were lysed with 1× radioimmunoprecipitation assay buffer (Sigma-Aldrich) containing protease and phosphatase inhibitor cocktails (Roche Applied Sciences). Protein concentrations were determined using the Bradford protein assay (Eppendorf BioPhotometer). Protein samples (20 μg) were loaded onto a 4–20% Mini-PROTEAN TGX precast gel (Bio-Rad) with respective loaded EZ-Run Prestained Rec Protein Ladder markers (Fisher BioReagents, Waltham, MA). SDS-polyacrylamide gels were transferred to Tran-Blot Turbo Mini polyvinylidene difluoride membranes (Bio-Rad), followed by blocking buffer (LI-COR) and subsequent incubation with primary and secondary antibodies. Antibodies included the following: ACE (N-20) (SC-12184; Santa Cruz Biotechnology), anti–β-actin mouse monoclonal IgG (Sigma-Aldrich), donkey anti-goat IRDye 800CW (LI-COR), and goat anti-mouse IRDye 800CW (LI-COR). Membrane fluorescence-based immunodetection was conducted using the LI-COR Odyssey Infrared Imaging System (LI-COR), and respective band density was quantified using Odyssey Application Software Version 3.0.21.

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assay (EMSA) experiments were conducted using the LI-COR

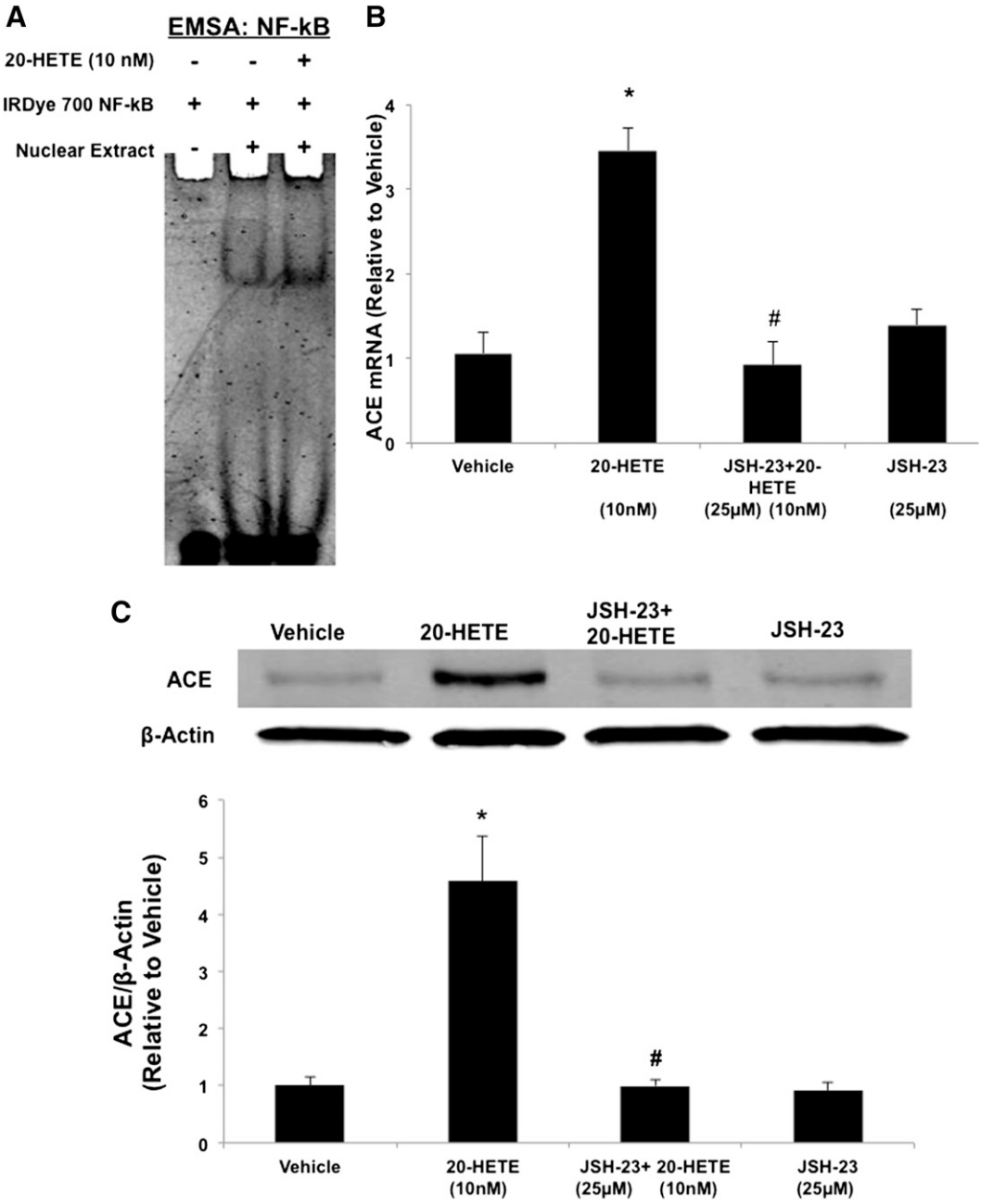

Fig. 1. NF-κB is required for the 20-HETE–mediated transcriptional activation of ACE in HMVECs. (A) EMSA analysis of nuclear extracts from cells treated with 20-HETE (10 nmol/L) for 40 minutes. (B) ACE mRNA in cells pretreated with the NF-κB translocation inhibitor, JSH-23 (25 μmol/L), for 40 minutes prior to addition of 20-HETE (10 nmol/L) for 2 hours ($n = 3$; $^{*}P < 0.05$ versus vehicle, $^{\#}P < 0.05$ versus 20-HETE). (C) A representative Western blot and densitometry analysis of ACE protein levels in cells treated with 20-HETE (10 nmol/L) for 12 hours in the presence and absence of JSH-23 (25 μmol/L) ($n = 3$; $^{*}P < 0.05$ versus vehicle, $^{\#}P < 0.05$ versus 20-HETE).

EMSA kit (LI-COR), following the manufacturer's instructions, to assess nuclear DNA-protein interactions. This kit uses specific transcription factor-binding sequence oligonucleotides labeled with an infrared dye. The total nuclear transcription factor-binding complex was visualized using the Odyssey Infrared Imaging System (LI-COR). The following NF-κB oligonucleotides were used: sense, 5′-AGT TGA **GGG GAC TTT CCC**AGGC-3′, and antisense, 3′-TCA ACT **CCC CTG AAA GGG** TCC G-5′ (bold sequence: NF-κB binding site). NF-κB IRDye 700 (LI-COR) EMSA oligonucleotides were incubated with nuclear extracts from HMVEC treated with 20-HETE (10 nM) for 40 minutes. The binding reaction mixture included the following: 10× binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5), 1 μg/μL poly(dI•dC) in 10 mM Tris, 1 mM EDTA, pH 7.5, 25 mM DTT/2.5% Tween 20, Water, IRDye 700 NF-κB, and 10 μg/μL nuclear extract. This mixture was then incubated for 30 minutes at room temperature under dark conditions as the contents are light-sensitive. Following this incubation, samples underwent electrophoresis on a 4–12% Tris/borate/EDTA polyacrylamide gel (Bio-Rad) and imaged on the Odyssey Infrared Imaging System (LI-COR).

Gene Promoter/Reporter Activation. Promoter reporter constructs for the ACE GoClone somatic (S721661) and germinal (S721660) promoter regions and empty promoter controls were obtained from Switchgear Genomics (Carlsbad, CA) and used per manufacturer's instructions. Cells (passage 2–3) were cultured in 96-well format plates to 60–80% confluency. GoClone promoter construct plasmid DNA (30 ng/μL), FuGENE HD transfection reagent (Switchgear Genomics, Carlsbad, CA), and Opti-MEM serum-free media (Invitrogen, Camarillo, CA) were mixed and incubated at room temperature for 30 minutes. The reaction mixture was added to cells and incubated for 36 hours. Transfected cells were then preincubated with the following inhibitors: AG1478 (an EGFR-tyrosine kinase inhibitor; 10 μmol/L), U0126 (MAPK inhibitor; 10 μmol/L), SC-514 (an IKKβ inhibitor; 25 μmol/L), and JSH-23 (a NF-κB translocation inhibitor; 25 μmol/L) for 40 minutes prior to 20-HETE (10 nmol/L) treatment of 1 hour and 20 minutes. Cells were also treated with 20-HETE (10 nmol/L) and the 20-HETE antagonist, 20-HEDE (10 nmol/L), for 1 hour and 20 minutes. Additional experiments were conducted with TNF-α (10 ng/ml) and EGF (100 ng/ml). LightSwitch Assay Reagents (LS010; Switchgear Genomics, Carlsbad, CA) were reconstituted, added to each sample, and incubated for 30 minutes protected from light at room temperature. Luciferase activity was measured using the LightSwitch Luciferase Assay System (Switchgear Genomics), which utilizes the RenSP luciferase. Each plate was read using the

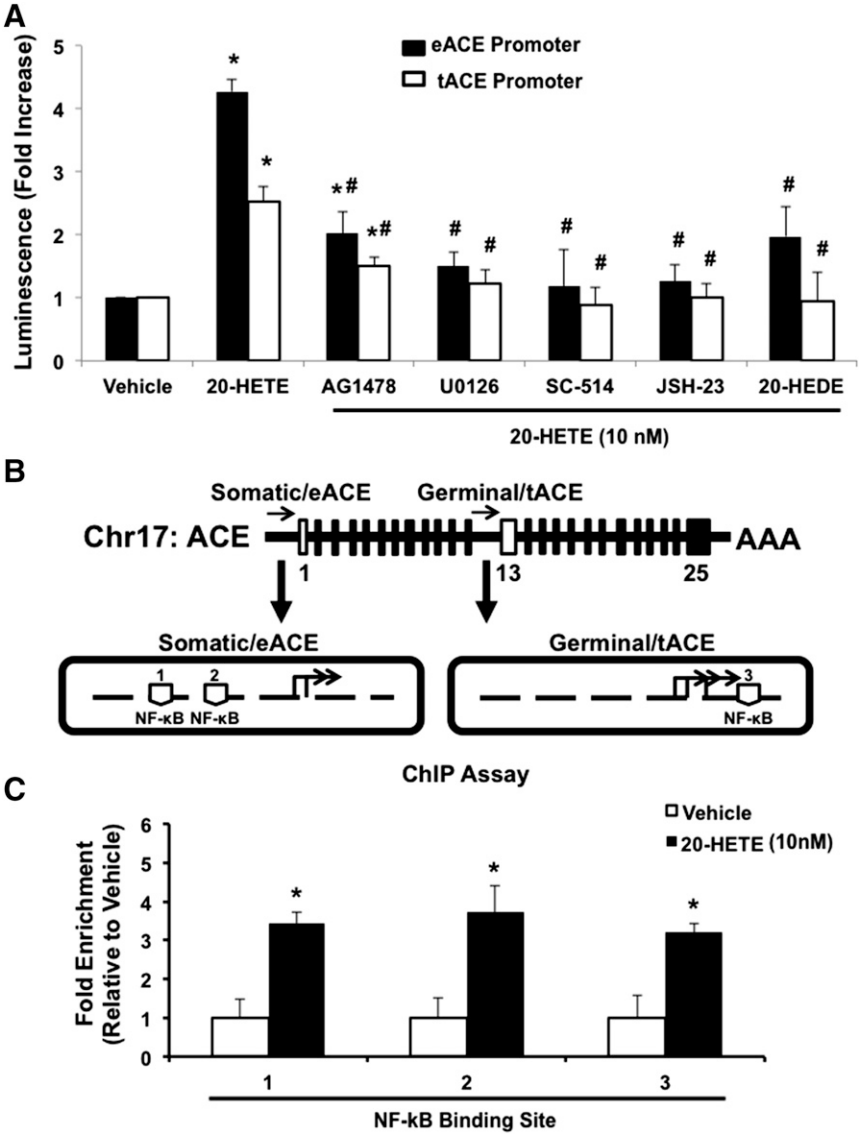

Fig. 2. The 20-HETE stimulates ACE promoter activation via an EGFR-/MAPK-/IKK-/NF-κB– dependent pathway. (A) Effect of 20-HETE (10 nmol/L) on endothelial (eACE) and testis (tACE) promoter-reporter luciferase activation. Cells transfected with either the eACE or tACE promoter regions were pretreated with specific inhibitors of EGFR (AG1478; 10 μmol/L), MAPK (U0126; 10 μmol/L), IKKβ (SC514; 25 μmol/L), or NF-κB (JSH-23; 25 μmol/L) translocation for 40 minutes before the addition of 20-HETE (10 nmol/L) or its vehicle, phosphate-buffered saline, for 1 hour and 20 minutes. Cells treated with 20-HETE and the 20-HETE antagonist, 20-HEDE, were also treated for 1 hour and 20 minutes under equimolar (10 nmol/L) concentrations (n = 4; $^{*}P < 0.05$ versus vehicle, $^{\#}P < 0.05$ versus 20-HETE). (B) Schematic illustration of the human ACE gene and respective somatic/endothelial and germinal/testis ACE promoter regions (eACE and tACE, respectively). The somatic/eACE contains two putative NF-κB binding sites, whereas the germinal/tACE contains one. (C) ChIP assay of NF-κB binding to putative binding sites on the ACE gene promoter regions. Cells were treated with 20-HETE (10 nmol/L) for 50 minutes prior to cross-linking of protein-DNA complexes, chromatin immunoprecipitation with anti–NF-κB p65 antibodies, and DNA purification. PCRs were performed using NF-κB primers created for each putative binding site along the ACE somatic and germinal promoter regions. The 20-HETE stimulates the translocation and subsequent binding of NF-κB to each of the three corresponding binding sites located along the ACE promoter regions (n = 3; $^{*}P < 0.05$ versus vehicle).

Synergy HT Microplate Reader (BioTek, Winooski, VT) (480 nM for 2 seconds), and fold luminescence was calculated.

Chromatin Immunoprecipitation Assay. The SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling, Danvers, MA) was used to detect endogenous NF-κB protein-DNA interactions. Chromatin immunoprecipitation (ChIP) assay experiments were conducted following the manufacturer's instructions. In brief, cells were treated with 20-HETE (10 nM) for 50 minutes, followed by in vivo cross-linking, nuclei sample preparation, and microsomal nuclease digestion of chromatin. The cross-linked chromatin preparation was then analyzed to determine proper size and concentration and ensure the absence of overdigestion. ChIP was conducted using manufacturer's ChIP buffers and protocol. The NF-κB immunoprecipitating antibody Rb NF-κB p65 Ab7970 (Abcam, Cambridge, MA) was used. The reaction mixture was incubated overnight with rotation at 4°C. Following the incubation, samples were washed under low- and high-salt conditions. Elution of chromatin from antibody/protein G magnetic beads and reversal of cross-links was completed, and DNA purification was conducted using spin columns. Quantification of DNA was completed using the real-time quantitative PCR method. PCRs also included the positive control histone H3, a tube with no DNA to control for contamination, and a serial dilution of the 2% input chromatin DNA (undiluted, 1:5, 1:25, 1:125) to create a standard curve and determine the efficiency of amplification, as instructed. NF-κB primers (Gene Link, Hawthorne, NY) were created for each putative binding site along the ACE somatic and germinal promoter regions. The NF-κB binding site primers used are as follows: site 1 (somatic ACE promoter) forward, 5′-AGG CGG GAG GCT CCG GGG-3′, and reverse, 5′-CCC CGG AGC CTC CCG CCT-3′; site 2 (somatic ACE promoter) forward, 5′-GGC TCG GGT GTT CCG GCA A-3′, and reverse, 5′-TTG CCG GAA CAC CCG AGC C-3′; and site 3 (germinal ACE promoter) forward, 5′-CTG CAG GAC TTC CCA GCC T-3′, and reverse, 5′-AGG CTG GGA AGT CCT GCA G-3′. Quantitative real-time PCR was performed using the PerfeCTa SYBR Green FastMix Low ROX kit (Quanta Biosciences) and the Mx3000p Real-Time PCR System (Stratagene). The PCR reaction program included initial denaturation (95°C, 3 minutes), denaturation (95°C, 15 seconds), followed by annealing and extension (60°C, 60 seconds) steps for 40 cycles. Analysis of the quantified PCR results is expressed as fold enrichment.

ACE Activity Assay. HMVECs were cultured on six-well plates to ~90% confluency and placed in serum-free HBSS media for 24 hours. Cells were then preincubated with the following inhibitors: AG1478 (an EGFR-tyrosine kinase inhibitor; 10 μmol/L), U0126 (MAPK inhibitor; 10 μmol/L), SC-514 (an IKKβ inhibitor; 25 μmol/L), and JSH-23 (a NF-κB translocation inhibitor; 25 μmol/L) for 40 minutes prior to 20-HETE (5 nmol/L) for 24 hours. ACE activity in cell lysates (cellular) and media (extracellular) was determined using the BÜHLMANN ACE kinetic test from ALPCO (Salem, NH), following the manufacturer's instruction, as previously described (Cheng et al., 2012).

Statistical Analysis. Data are expressed as mean ± S.E.M. Significance of difference in mean values was determined using *t* test and one-way analysis of variance, followed by the Newman-Keul post hoc test. The *P* value <0.05 was considered to be significant.

Results

20-HETE–Mediated Increase in ACE Induction Requires Nuclear Translocation of NF-κB. The ability of 20-HETE to activate the IKK–NF-κB signaling pathway suggested the involvement of NF-κB in the transcriptional activation of ACE (Cheng et al., 2009; Wu et al., 2011). EMSA was used to assess the effect of 20-HETE on NF-κB nuclear translocation. EMSA blots displayed increased NF-κB translocation stimulated by 20-HETE (10 nmol/L) (Fig. 1A). Moreover, pretreatment with the specific NF-κB nuclear translocation inhibitor JSH-23 prevented the 20-HETE–stimulated 3.5-fold (±0.26; $P < 0.05$) increase in ACE mRNA (Fig. 1B) and the 4.58-fold (±0.78; $P < 0.05$) increase in endothelial ACE protein expression (Fig. 1C).

20-HETE Stimulates ACE Promoter Activation via an EGFR-/MAPK-/IKK-/NF-κB–Dependent Pathway. In cells transfected with either the endothelial or the testis ACE-luciferase promoter-reporter constructs 20-HETE (10 nmol/L) increased promoter activity by 4.37-fold (±0.18; $P < 0.05$) and 2.53-fold (±0.24; $P < 0.05$), respectively (Fig. 2A). The 20-HETE–stimulated ACE promoter activity was abrogated by equimolar concentrations of the 20-HETE antagonist 20-HEDE. Additionally, inhibitors of EGFR [AG1478 (10 μmol/L)], MAPK [U0126 (10 μmol/L)], IKKβ [SC-514 (25 μmol/L)], and NF-κB [JSH-23 (25 μmol/L)] abrogated the 20-HETE–mediated promoter activation (Fig. 2A).

20-HETE Stimulates NF-κB Binding to Human Endothelial ACE Promoter Regions. Sequence analysis of the human ACE gene demonstrated the presence of two (chromosome 17: site 1, 61554061-61554075; site 2, 61554157-61554171) and one (chromosome 17: site 3, 61562250-61562264) putative NF-κB binding site on the human somatic/endothelial and germinal/testis ACE promoters, respectively (Fig. 2B). To assess the effect of 20-HETE on NF-κB binding to specific sites on the ACE gene, we used the ChIP. Results of ChIP assay of HMVEC treated with 20-HETE (10 nmol/L) indicated that

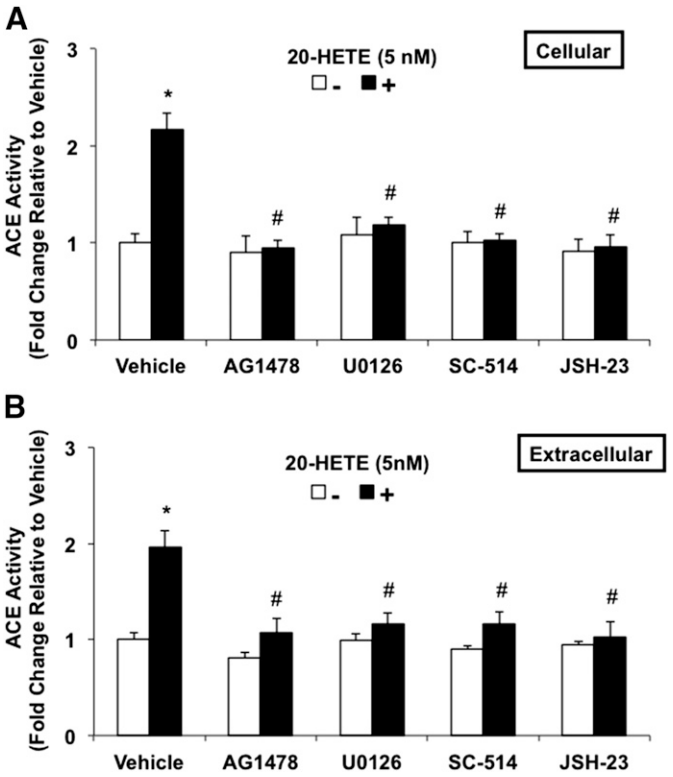

Fig. 3. The 20-HETE stimulates ACE activity via an EGFR-/MAPK-/IKK-/NF-κB–dependent pathway. Cellular (A) and extracellular (condition media) (B) ACE activity in human microvascular endothelial cells treated with and without 20-HETE (5 nmol/L) in the presence of specific inhibitors for EGFR (AG1478; 10 μmol/L), MAPK (U0126; 10 μmol/L), IKKβ (SC514; 25 μmol/L), and NF-κB (JSH-23; 25 μmol/L) translocation. Cells were pretreated for 40 minutes with each specific inhibitor prior to the addition of 20-HETE (5 nmol/L) for 24 hours ($n = 4–5$; $^{*}P < 0.05$ versus vehicle, $^{\#}P < 0.05$ versus 20-HETE).

20-HETE stimulates the translocation and subsequent binding of NF-κB to each of the putative NF-κB binding sites: site 1, 3.43-fold (±0.3; $P < 0.05$); site 2, 3.72-fold (±0.68; $P < 0.05$); and site 3, 3.20-fold (±0.18; $P < 0.05$) enrichment versus vehicle (Fig. 2C). Together, these data support the notion that NF-κB is a critical transcription factor for the 20-HETE–mediated ACE induction.

20-HETE–Mediated Increase in ACE Activity is EGFR-/MAPK-/IKK-/NF-κB– Dependent. In vitro, ACE activity can be measured at the cellular compartment (ACE, which continues to be bound to the plasma membrane) and extracellular ACE found in the condition media that has undergone shedding through time. Addition of 5 nmol/L 20-HETE increased ACE activity in cellular and extracellular ACE activity by 2.16-fold (±0.17; $P < 0.05$) and 1.96-fold (±0.16; $P < 0.05$), respectively (Fig. 3), as previously reported (Cheng et al., 2012). Inhibitors of EGFR [AG1478 (10 μmol/L)], MAPK [U0126 (10 μmol/L)], IKKβ [SC-514 (25 μmol/L)], and NF-κB [JSH-23 (25 μmol/L)] also inhibited the 20-HETE–mediated increases in ACE activity in both cellular (Fig. 3A) and extracellular (Fig. 3B) compartments.

20-HETE–Mediated Increase in ACE Induction Does Not Require EGFR Ligand Binding. We further assess whether EGFR activation by 20-HETE requires ligand binding. As seen in Fig. 4A, treatment of HMVEC with 20-HETE at 10 nmol/L increased EGFR tyrosine phosphorylation by 2.4-fold (±0.13; $P < 0.05$) at 5 minutes compared with vehicle-treated cells. Moreover, pretreatment of cells with the EGF neutralizing antibody did not prevent the 20-HETE–mediated increase in EGFR tyrosine phosphorylation (Fig. 4A). The 20-HETE (10 nmol/L) induced ACE mRNA expression by 3.2-fold (±0.60; $P < 0.05$), and pretreatment with an irreversible EGFR tyrosine kinase inhibitor (PD168393; 10 μmol/L) prevented the 20-HETE–mediated ACE mRNA induction. In contrast, although EGF increased EGFR tyrosine phosphorylation, it did not stimulate ACE expression when added alone or in combination with 20-HETE. Additionally, pretreatment with a neutralizing antibody against EGF did not prevent the 20-HETE–mediated ACE induction, indicating that the induction of ACE by 20-HETE is EGF-independent (Fig. 4B). Interestingly, pretreatment with the neutralizing antibody against EGF, followed by treatment with EGF, stimulated a

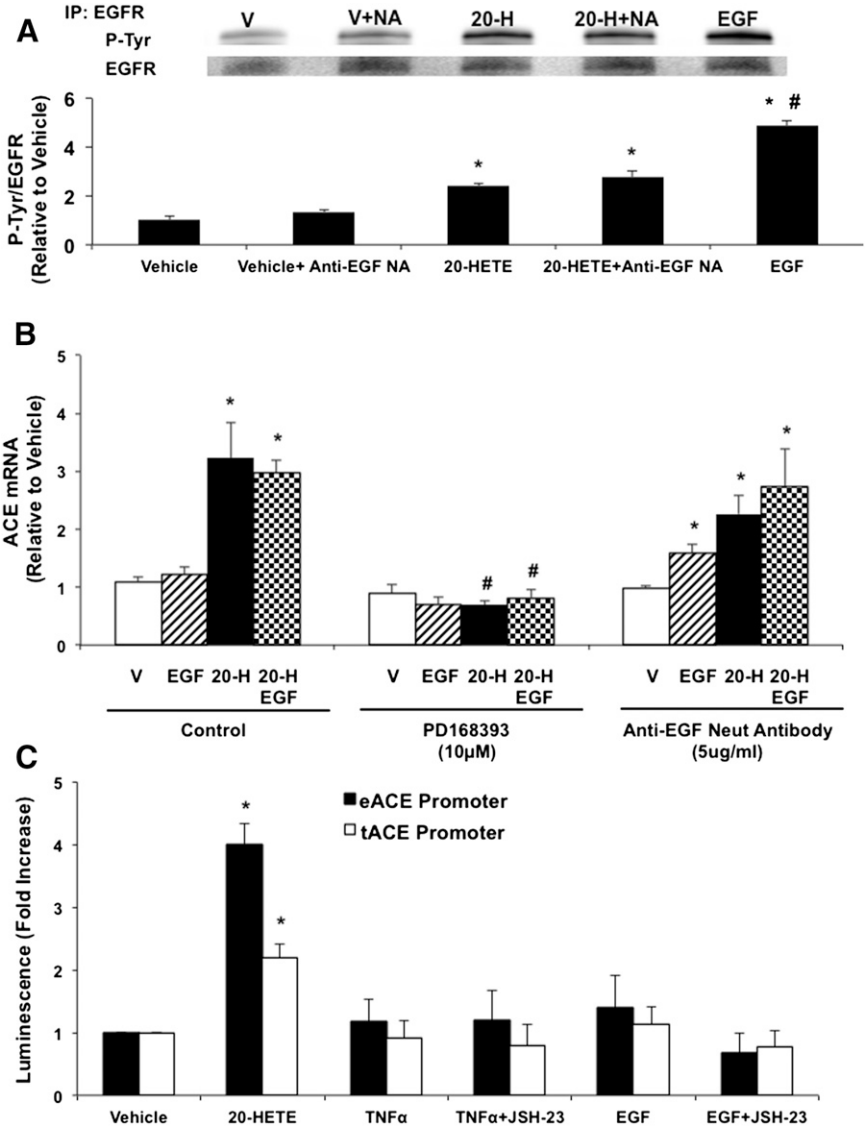

Fig. 4. The 20-HETE stimulates EGFR tyrosine (Tyr) phosphorylation and ACE mRNA in the absence of EGF. (A) Immunoprecipitation and Western blot analysis of EGFR Tyr phosphorylation in cells treated with vehicle (V; phosphate-buffered saline) or 20-HETE (10 nmol/L) in the presence and absence of anti-EGF neutralizing antibody (NA; 5 μg/ml) for 5 minutes ($n = 3$; $^*P < 0.05$ versus vehicle). EGF (100 ng/ml) was used as a positive control. (B) ACE mRNA in cells pretreated with the irreversible EGFR tyrosine kinase inhibitor, PD168393 (10 μmol/L), or an anti-EGF neutralizing antibody (5 μg/ml) for 40 minutes prior to addition of 20-HETE (10 nmol/L), EGF (100 ng/ml), or 20-HETE (10 nmol/L) + EGF (100 ng/ml) for 2 hours ($n = 4$; $^*P < 0.05$ versus vehicle, $^{\#}P < 0.05$ versus 20-HETE). (C) Effect of TNF-α and EGF on endothelial (eACE) or testis (tACE) promoter-reporter luciferase activation. Cells transfected with either the eACE or tACE ACE promoter regions were treated with 20-HETE (10 nmol/L), TNF-α (10 ng/ml), or EGF (100 ng/ml), for 1 hour and 20 minutes ($n = 3$; $^*P < 0.05$ versus vehicle).

small, but significant 1.6-fold (±0.20; $P < 0.05$) increase in ACE mRNA potentially, suggesting a stimulatory role of exogenous EGF when endogenous EGF is neutralized for an extended period of time. Additional endothelial or testis ACE promoter-reporter activation experiments once again illustrated that 20-HETE (10 nmol/L) increased promoter activity by 4.00-fold (±0.32; $P < 0.05$) and 2.20-fold (± 0.22; $P < 0.05$), respectively (Fig. 4C). A known inducer of NF-κB activation and translocation, TNF-α (10 ng/ml), did not increase promoter activation. In addition, EGF (100 ng/ml) also did not activate the endothelial or testis ACE promoters.

Discussion

Previous studies from our laboratory have identified 20-HETE as a potent inducer of ACE in endothelial cells (Cheng et al., 2012). The present study is the first to reveal that NF-κB is a transcription factor regulating endothelial ACE expression and to implicate a distinct signaling pathway for 20-HETE–mediated induction of the ACE gene, which requires the phosphorylation of the EGFR with subsequent activation of MAPK, IKKβ, and NF-κB nuclear translocation.

Two original findings are presented in the current study. The first is the demonstration that NF-κB is a transcriptional activator of the ACE gene. The second is the fact that 20-HETE activates the ACE promoter, both the somatic and germinal, through stimulation of NF-κB binding to ACE promoter regions. Interactions between 20-HETE and NF-κB have been documented in previous reports including demonstrations by Western blot and immunofluorescence analyses of 20-HETE–stimulated inhibitory NF-κB phosphorylation and NF-κB nuclear translocation in cultured endothelial cells (Ishizuka et al., 2008), as well as identification of IKK–NF-κB activation as a critical step in 20-HETE actions in vitro and in vivo (Ishizuka et al., 2008; Cheng et al., 2009, 2012; Wu et al., 2011). The current study corroborates previous findings and provides further evidence that NF-κB is a key component in the bioactions of 20-HETE in endothelial cells. In this work, we clearly demonstrated, using promoter-luciferase constructs, EMSA, and ChIP assays, that 20-HETE stimulates NF-κB translocation to the nucleus and its binding to three loci on the ACE promoter (two on the somatic and one on the germinal promoter) through a distinct EGFR-MAPK-IKK signaling pathway (Fig. 5). Importantly, the induction of ACE expression as well as activation of its promoter by 20-HETE was prevented under concurrent treatment with the 20-HETE antagonist, 20-HEDE, further illustrating the specificity of this interaction. Accordingly, these results implicate 20-HETE as a potent endogenous modulator of endothelial ACE expression. Studies in animal models showing that increased vascular 20-HETE production is associated with upregulation of vascular ACE (Sodhi et al., 2010) further support the notion that 20-HETE is an endogenous modulator of the ACE. Gene expression of endothelial ACE has been shown to be increased by phorbol 12-myristate 13-acetate (Villard et al., 1998; Eyries et al., 2002) and isoproterenol (Xavier-Neto et al., 1999). Interestingly, there are no known endogenous inducers of ACE expression, although activation of protein kinase C and the transcription factors EGR-1, AP-1, and ETS-1 has been demonstrated to mediate ACE upregulation (Villard et al., 1998; Mungunsukh et al., 2008). Conditions such as sheer stress or elevation in estrogen levels have been

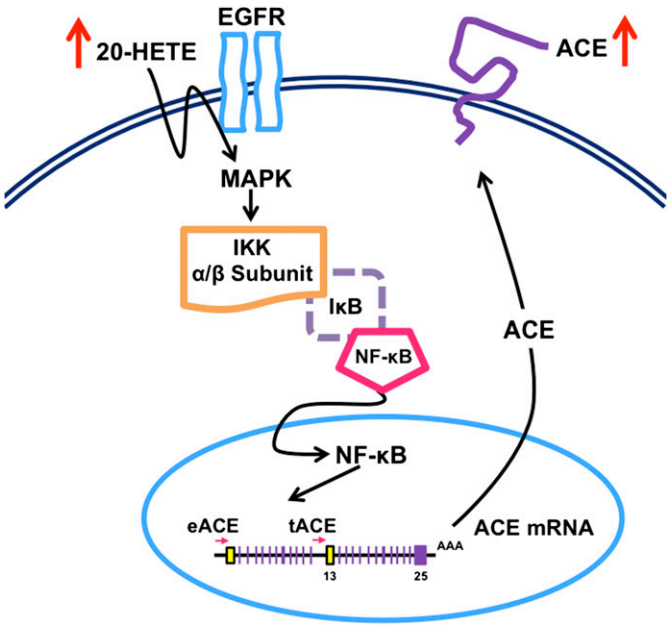

Fig. 5. Schematic diagram illustrating the cascade of signaling events contributing to the induction of ACE that is modulated by 20-HETE. The 20-HETE promotes the phosphorylation of EGFR, independent of EGF, followed by MAPK and subsequent IKKβ activation. The activation of the IKK complex promotes the release and nuclear translocation of the transcription factor NF-κB. Binding of NF-κB to transcription factor binding sites on the ACE promoter regions initiates transcriptional activation of the ACE gene, which results in increases in ACE mRNA, protein, and activity. The induction of endothelial ACE can lead to increased vascular production of angiotensin II, setting forth a feed-forward mechanism promoting the release of 20-HETE from vascular smooth muscle cells, which in turn further amplifies 20-HETE's biologic actions promoting endothelial and vascular dysfunction.

demonstrated to decrease ACE expression (Rieder et al., 1997; Gallagher et al., 1999). Recently, we have shown that known inducers of ACE or NF-κB such as phorbol 12-myristate 13-acetate or TNF-α fall short of inducing ACE gene expression within 2 hours, as observed with 20-HETE. Furthermore, these agents require 24 hours to elicit significant changes in ACE mRNA (Cheng et al., 2012). In this study, we demonstrate that TNF-α, although a known inducer of NF-κB (Duh et al., 1989), does not activate the promoter regions of ACE. This observation suggests that 20-HETE–induced NF-κB activation elicits a downstream signaling cascade that is different from other known activators of NF-κB. We have also demonstrated that activation of PKC is not involved in 20-HETE–mediated induction of ACE (Cheng et al., 2012). Hence, 20-HETE differs from the known ACE inducers not only in its potency, but also in its mechanism of action.

Our previous studies showed that 20-HETE–mediated endothelial cell dysfunction is mediated by a mechanism that requires the activation of EGFR, MAPK–extracellular signal-regulated kinase-1/2, and IKKβ (Cheng et al., 2012). In this study, we assess whether the same signaling pathway also mediates 20-HETE–induced transcriptional activation of the ACE gene and, consequently, ACE activity in endothelial cells. Results clearly showed that pharmacological inhibition of each of the signaling steps, including EGFR tyrosine phosphorylation, MAPK activation, IKKβ, and NF-κB, prevented 20-HETE–mediated promoter ACE activation and subsequently the increases in ACE activity. Importantly, we showed

in this work that 20-HETE potently and rapidly stimulates tyrosine phosphorylation of EGFR, an effect shared by its ubiquitous ligand EGF. However, unlike 20-HETE, EGF failed to induce ACE expression, suggesting that phosphorylation of EGFR in response to 20-HETE ignites a 20-HETE–specific signaling pathway. Previously, we demonstrated that 11,12-epoxy-5,8,14-eicosatrienoic acid, an endothelial-derived CYP-eicosanoid, which has been shown to activate EGFR in endothelial cells (Michaelis et al., 2003; Yan et al., 2008), had no effect on ACE mRNA, further emphasizing the specificity of this response to 20-HETE and not other known activators of EGFR (Cheng et al., 2012). Moreover, experiments using a neutralizing antibody against EGF clearly suggested that stimulation of EGFR tyrosine phosphorylation by 20-HETE does not require EGF receptor-ligand interactions. Interestingly, the neutralization of endogenous EGF, followed by addition of recombinant EGF, resulted in a 50% increase in ACE mRNA. We postulate that the neutralizing antibody reset a regulatory signaling driving ACE expression. Further experiments are necessary to explore the intricacies and nature of the relationship between EGFR and ACE.

The mechanism by which 20-HETE stimulates EGFR tyrosine phosphorylation is yet to be elucidated. As a small lipid molecule, 20-HETE can cross the cell membrane and stimulate tyrosine kinases within the intracellular milieu. Alternatively, 20-HETE may interact with a G-protein coupled receptor, the activation of which could lead to transactivation of the EGFR, as was documented for many autacoids, including Ang II (Mehta and Griendling, 2007; Okada, 2012). Such hypothesis has been postulated for the mitogenic action of 20-HETE in renal epithelial cells. Akbulut et al. (2009) showed that 20-HETE activates the Raf/MAPK kinase/extracellular signal-regulated kinase pathway in renal epithelial cells through an EGFR- and c-Src–dependent mechanism and proposed the presence of a 20-HETE–specific G-protein coupled receptor-EGFR transactivation through c-Src. To date, a receptor/binding site for 20-HETE has been implicated based on the use of specific agonists and antagonists, but it still needs to be identified.

The activation of NF-κB is primarily associated with the inflammatory program, and the finding that NF-κB is a transcriptional activator of the endothelial ACE gene may provide yet another link between inflammation and the development of hypertension and vascular complications. A growing number of reports supports the role of inflammation in the development of hypertension, placing ACE/Ang II at the intersection of this relationship. Ang II has been shown to induce the presence of adhesion molecules, vascular macrophage infiltration, T lymphocyte production of TNF-α, and inflammatory cytokine production (Savoia and Schiffrin, 2006; Harrison et al., 2011). Most notably, Ang II-induced inflammatory damage is ameliorated by the inhibition of NF-κB (Muller et al., 2000). The 20-HETE has been demonstrated to play an important role in the development of hypertension (Wu et al., 2014) and has been shown to be closely linked to vascular injury and inflammation through the stimulation of endothelial adhesion molecules, oxidative stress, and cytokine production, including interleukin-8, a potent neutrophil chemoattractant (Wang et al., 2006; Guo et al., 2007; Singh et al., 2007; Ishizuka et al., 2008; Cheng et al., 2012). Notably, administration of an IKK inhibitor prevents 20-HETE–mediated hypertension (Wu et al., 2014). These findings set the stage for synergy between the 20-HETE and RAS axis in the development and progression of vascular inflammation, hypertension, and cardiovascular disease.

Numerous reports have indicated correlations between increased 20-HETE–producing enzymes and levels in human diseases and conditions such as cancer (Panigrahy et al., 2010), endothelial dysfunction (Ward et al., 2004), oxidative stress (Ward et al., 2005), autosomal dominant polycystic kidney disease (Klawitter et al., 2013), ischemic stroke (Fava et al., 2008), and hypertension (Liu et al., 2008; Ward et al., 2008). The ability of 20-HETE to induce ACE and amplify the conversion of Ang I to Ang II highlights the need for new novel pharmacological therapuetics targeting the 20-HETE system in hypertension. In addition, the observed promiscuity of the ACE enzyme and newfound protective roles, including a role in the prevention of brain atrophy and cerebral small vessel disease, increased immune response, and a role in reducing the cognitive decline observed in Alzheimer's disease (Bernstein et al., 2013), raise further questions as to the interaction between 20-HETE and ACE and the implication of such a relationship in new and dynamic physiologic locations and conditions.

In summary, the finding that 20-HETE induces ACE transcription via stimulation of NF-κB binding to its promoters provides a mechanistic link for the synergy between 20-HETE and the RAS in the development and progression of vascular inflammation, hypertension, and cardiovascular disease. The 20-HETE may represent an upstream effector molecule for activation of the RAS and a target for therapeutic interventions in the treatment of vascular inflammation, injury, and hypertension. Moreover, given the growing evidence of multiple functions for ACE, the identification of a novel endogenous autacoid that specifically and potently induces its expression provides a powerful tool for further research and clinical applications.

Authorship Contributions

Participated in research design: Garcia, Schwartzman.
Conducted experiments: Garcia, Shkolnik, Milhau.
Performed data analysis: Garcia, Schwartzman.
Wrote or contributed to the writing of the manuscript: Garcia, Falck, Schwartzman.

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