20-Hydroxy-5,8,11,14-eicosatetraenoic Acid Mediates Endothelial Dysfunction via I κ B Kinase-Dependent Endothelial Nitric-Oxide Synthase Uncoupling

Jennifer Cheng, Cheng-Chia Wu, Katherine H. Gotlinger, Frank Zhang, John R. Falck, Dubasi Narsimhaswamy, and Michal Laniado Schwartzman

Department of Pharmacology, New York Medical College, Valhalla, New York (J.C., C.-C.W., K.H.G., F.Z., M.L.S.); and Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas (J.R.F., D.N.)

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ABSTRACT

Endothelial dysfunction and activation occur in the vasculature and are believed to contribute to the pathogenesis of cardiovascular diseases. We have shown that 20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE), a cytochrome P450 4A-derived eicosanoid that promotes vasoconstriction in the microcirculation, uncouples endothelial nitric-oxide synthase (eNOS) and reduces nitric oxide (NO) levels via the dissociation of the 90-kDa heat shock protein (HSP90) from eNOS. It also causes endothelial activation by stimulating nuclear factor- κ B (NF- κ B) and increasing levels of pro-inflammatory cytokines. In this study, we examined signaling mechanisms that may link 20-HETE-induced endothelial dysfunction and activation. Under conditions in which 20-HETE inhibited NO production, it also stimulated inhibitor of NF- κ B (I κ B) phosphorylation. Both effects were prevented by inhibition of tyrosine kinases and mitogen-activated protein

kinase (MAPK)/extracellular signal-regulated kinase (ERK). It is noteworthy that inhibitor of I κ B kinase (IKK) activity negated the 20-HETE-mediated inhibition of NO production. Immunoprecipitation experiments revealed that treatment of ionophore-stimulated cells with 20-HETE brings about a decrease in HSP90-eNOS association and an increase in HSP90-IKK β association, suggesting that the activation by 20-HETE of NF- κ B is linked to its action on eNOS. Furthermore, addition of inhibitors of tyrosine kinase MAPK and IKK restored the 20-HETE-mediated impairment of acetylcholine-induced relaxation in rat renal interlobar arteries. The results indicate that 20-HETE mediates eNOS uncoupling and endothelial dysfunction via the activation of tyrosine kinase, MAPK, and IKK, and these effects are linked to 20-HETE-mediated endothelial activation.

The integrity of the vascular endothelium is vital to the regulation of the cardiovascular system. The endothelium serves as a protective barrier between tissues and circulating blood and functions to maintain vascular homeostasis by releasing bioactive factors in response to hemody-

namic changes and blood-borne signals. An uncontrolled endothelial cell response is involved in many disease processes, including hypertension, atherosclerosis, and diabetes. These diseases are related to endothelial injury, dysfunction and activation. Nitric oxide (NO), generated from L-arginine by endothelial nitric-oxide synthase (eNOS), is a key endothelial-derived factor, the bioavailability of which is essential to the integrity and function of the endothelium. Endothelial dysfunction occurs as a result of a loss of NO bioavailability that is due to either reduced formation or accelerated degradation of NO, and it is often associated with endothelial activation. NO actively mediates many functions of the endothelium. In addition to its

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ABBREVIATIONS: NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; SMC, smooth muscle cell; 20-HETE, 20-hydroxyeicosatetraenoic acid; HSP90, 90-kDa heat shock protein; MAPK, mitogen-associated protein kinase; ERK, extracellular signal-regulated kinase; BAEC, bovine aortic endothelial cell; FBS, fetal bovine serum; L-NMMA, N^G-monomethyl-L-arginine; AG82, cyano-(3,4,5-trihydroxy)cinnamionitrile; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; 17-AAG, 17-(allylamino)-17-demethoxygeldanamycin; SC514, 4-amino-[2',3'-bi-thiophene]-5-carboxamide; NBD, NEMO-binding domain peptide; NEMO, NF- κ B essential modulating protein; A23187, calcium ionophore; tiron, 4,5-dihydroxy-1,3-benzene disulfonic acid; IKK, inhibitor of I κ B kinase; EET, epoxyeicosatrienoic acid; EGFR, epidermal growth factor receptor; IKK, I κ B kinase; I κ B, inhibitor of NF- κ B.

potent vasorelaxing effect, NO counteracts leukocyte adhesion to the endothelium, vascular smooth muscle cell (SMC) proliferation, and platelet aggregation that typically characterize endothelial activation.

20-Hydroxyeicosatetraenoic acid (20-HETE), the ω -hydroxylation product of arachidonic acid, is a primary eicosanoid in the microcirculation. Its synthesis is catalyzed by enzymes of the cytochrome P450 (P450) 4 gene family, which encodes multiple structurally and functionally similar P450 proteins (CYP4A, -4B, and -4F) that are controlled by factors such as age and sex hormones (Hardwick, 2008). The importance of this metabolic pathway emerged with the seminal observation that reduction in its activity lowers blood pressure in spontaneously hypertensive rats (Sacerdoti et al., 1989). Consequently, the hypothesis that this pathway participates in the regulation of blood pressure was substantiated in experimental animal models and in humans (Miyata and Roman, 2005; Ward et al., 2008). Its role in promoting pro-hypertensive mechanisms and participating in the regulation of vascular tone and homeostasis (Miyata and Roman, 2005) stems from findings that 20-HETE is a potent vasoactive eicosanoid.

20-HETE is a key constrictor eicosanoid in microcirculatory districts, most notably the renal and cerebral microcirculations (Miyata and Roman, 2005); in the pulmonary circulation, it is vasodilatory (Zhu et al., 2002). Its synthesis within the vascular wall is primarily localized to the SMCs, increases with decreased vessel diameter, is stimulated by vasoactive hormones such as angiotensin II and endothelin-1, and is inhibited by nitric oxide (Miyata and Roman, 2005). It elicits vasoconstriction largely via inhibition of the SMC large conductance Ca^{2+} -activated K^{+} channel, leading to depolarization and elevation in cytosolic $[\text{Ca}^{2+}]$, and in some blood vessels via the Rho-kinase phosphorylation of MLC20 and the sensitization of the contractile apparatus to Ca^{2+} (Kroetz and Xu, 2005; Miyata and Roman, 2005). Besides its vasoactivity, 20-HETE has been shown to stimulate SMC migration and proliferation (Muthalif et al., 1998; Stec et al., 2007).

Although endothelial synthesis of 20-HETE is questionable in most vascular beds, except for the pulmonary circulation (Zhu et al., 2002), its actions on endothelial function have been recently documented. 20-HETE is a potent angiogenic factor in vitro and in vivo (Medhora et al., 2007) and a mitogen to endothelial cells (Guo et al., 2007) as well as a modulator of eNOS-NO activation and function (Chen et al., 2006; Wang et al., 2006; Singh et al., 2007; Cheng et al., 2008). It is noteworthy that changes in the production of 20-HETE have been observed in numerous pathological conditions, including ischemic cerebrovascular diseases, kidney diseases, hypertension, diabetes, and toxemia of pregnancy (Miyata and Roman, 2005). The vascular phenotype in many of these conditions is that of injury typified by endothelial dysfunction and activation.

We have provided substantial evidence that 20-HETE contributes to both endothelial dysfunction and endothelial activation. In two experimental models of increased vascular synthesis of 20-HETE, the resulting endothelial dysfunction (i.e., reduced acetylcholine-induced relaxation, oxidative stress, and hypertension) (Wang et al., 2006; Singh et al., 2007) has been linked to vascular 20-HETE; administration of a 20-HETE synthesis inhibitor in vivo reversed endothelial

dysfunction and normalized blood pressure. We further demonstrated that 20-HETE impairs NO production in vitro and its function in vivo by uncoupling the eNOS activation process via inhibition of HSP90 association (Cheng et al., 2008). In addition to its actions on eNOS, 20-HETE potently stimulates endothelial activation by activating the NF- κ B and MAPK-ERK1/2 pathways, leading to increased levels of pro-inflammatory cytokines such as interleukin-8 and adhesion molecules (Ishizuka et al., 2008). In the current study, we explore whether 20-HETE-induced endothelial dysfunction and activation are linked. The results suggest that 20-HETE-mediated eNOS uncoupling and impairment of acetylcholine-induced relaxations are dependent on tyrosine kinase, MAPK, and I κ B kinase (IKK); these steps link 20-HETE-induced endothelial dysfunction to activation.

Materials and Methods

Cell Culture. Bovine aortic endothelial cells (BAEC; passages 3–5), cultured in microvascular endothelial cell growth medium (Lonza, Walkersville, MD), were placed in FBS-free medium for 24 h after achieving 70% confluence. On the day of the experiment, they were preincubated with or without 20-HETE (5 nM) and N^G -monomethyl-L-arginine (L-NMMA; 100 μM) in the presence and absence of inhibitors for 30 min. The following inhibitors were used: genistein (30 μM), AG82 (10 μM), U0126 (10 μM), wedelolactone (50 μM), SC514 (10 μM), geldanamycin (10 μM ; Calbiochem, Gibbstown, NJ), radicicol (10 μM), 17-(allylamino)-17-demethoxygeldanamycin (17-AAG; 10 μM), NEMO-binding domain peptide (NBD; 100 μM), and NBD control peptide (100 μM ; BIOMOL Research Laboratories, Plymouth Meeting, PA). Cells were then incubated in the presence of L-arginine (25 μM) with and without calcium ionophore (A23187; 5 μM) for 10 to 30 min. Cells and media were processed for the measurements described below.

Measurement of NO. NO levels were evaluated by measuring total nitrite and nitrate (NO_x) content in culture medium using the NO quantitation kit and following the manufacturer's instructions (Active Motif Inc., Carlsbad, CA) as described previously (Cheng et al., 2008).

Measurement of Superoxide. Cells were cultured on 96-well plates until they achieved ~70% confluence and were placed in FBS-free medium for 24 h. After treatments with or without 4,5-dihydroxy-1,3-benzene disulfonic acid (tiron; 10 mM), L-NMMA (0.1 mM), 20-HETE (5 nM), calcium ionophore A23187 (5 μM), and L-arginine (25 μM) for 30 min, the cells were incubated with 10 μM dihydroethidium (superoxide; Calbiochem) for 30 min at 37°C. Fluorescence intensity was measured using a luminescence spectrometer (PerkinElmer Life and Analytical Sciences, Waltham, MA) at excitation/emission filters of 530 nm/620 nm as described previously (Cheng et al., 2008). The fluorescence obtained in tiron-treated cells was used to represent nonspecific/background fluorescence and was subtracted from all values.

Measurement of cGMP. Cells were cultured on six-well plates and placed in FBS-free medium for 24 h after achieving 70% confluence. On the day of the experiment, cells were pretreated with 3-isobutyl-1-methylxanthine (30 μM ; Sigma, St. Louis, MO) for 30 min, washed with Hanks' balanced salt solution, and incubated in the presence and absence of 20-HETE (5 nM) (or its vehicle) and L-NMMA (0.1 mM) for 30 min. L-Arginine (25 μM) was then added with and without calcium ionophore A23187 (5 μM) for 30 min. The medium was removed and cells were lysed in 0.1 M hydrochloric acid. Cyclic GMP levels were determined using an enzyme immunoassay kit and following the manufacturer's instructions (Cayman, Ann Arbor, MI).

Immunoprecipitation. Cells were lysed and precleared with protein A Sepharose beads for 1 h. Lysates were incubated with

antibodies against IKK β , eNOS (Cell Signaling Technology, Danvers, MA) or HSP90 (Abcam, Cambridge, MA) overnight. Beads were added to the samples, incubated, washed with phosphate-buffered saline, and resuspended in loading buffer for Western blot analysis as described previously (Cheng et al., 2008).

Western Blot Analysis. Western blot analysis of immunoprecipitated fractions and cell lysates were performed as described previously (Cheng et al., 2008) using primary antibodies against eNOS, I κ B- α , phospho-I κ B- α , IKK β , and HSP90.

Animal Studies. All experimental protocols were approved by the Institutional Animal Care and Use Committee. Male 7- to 8-week-old Sprague-Dawley rats were used. Rats were anesthetized with ketamine (70 mg/kg) and xylazine (7 mg/kg), and laparotomy was performed. Renal interlobar arteries were microdissected for functional studies and eicosanoid measurements as described previously (Cheng et al., 2008).

Agonist-Induced Vasorelaxation. Renal interlobar arteries were mounted on wires in myograph chambers (J. P. Trading, Aarhus, Denmark) for measurement of isometric tension as described previously (Singh et al., 2007; Cheng et al., 2008). We have previously analyzed the relaxation response of these arteries to acetylcholine at different doses from 1 nM to 100 μ M; at a dose of 5 μ M, acetylcholine elicited 80% of maximal relaxation response in phenylephrine-precontracted arteries (Singh et al., 2007). Thus, after precontraction with phenylephrine (5 μ M), vasodilator responses to acetylcholine (5 μ M) were assessed in the presence and absence of 20-HETE (50 nM) and various kinase inhibitors.

Measurement of 20-HETE and Epoxyeicosatrienoic Acids. Cells were cultured on six-well plates and pretreated with L-NMMA (0.1 mM) for 30 min followed by addition of L-arginine (25 μ M) and calcium ionophore A23187 (5 μ M) for an additional 30 min, after which the cells were lysed. Renal interlobar arteries were isolated and incubated in oxygenated Krebs bicarbonate buffer, pH 7.4, with 1 mM NADPH for 1 h at 37°C with gentle shaking. After 1 h, the reaction was stopped by acidification with acetic acid, pH 4.0. Internal standards (d_6 -20-HETE and d_8 -11,12-EET, 500 pg each) were added to the lysates and arteries, followed by extraction with ethyl acetate. Identification and quantification of 20-HETE and EETs were performed with a Q-trap 3200 linear ion trap quadrupole liquid chromatography/tandem mass spectrometry equipped with a Turbo V ion source operated in negative electrospray mode (Applied Biosystems, Foster City, CA). Multiple reaction monitoring was used with a dwell time of 25 or 50 ms for each compound, with source parameters: ion spray voltage, -4500 V; curtain gas, 40 U; ion source gas flow rate 1, 70 U, and flow rate 2, 50 U; and temperature of 600°C. Synthetic standards were used to obtain standard curves (5–500 pg) for each eicosanoid.

Statistical Analysis. All experiments were run in duplicates and repeated at least three times. Data are expressed as means $\pm$ S.E.M. Significance of difference in mean values was determined using one-way analysis of variance followed by the Newman-Keul's post hoc test. $P < 0.05$ was considered to be significant.

Results

20-HETE Production in BAECs and Renal Interlobar Arteries. Levels of 20-HETE and EETs were measured in BAECs in the presence and absence of ionophore and L-NMMA by liquid chromatography/tandem mass spectrometry. 20-HETE was undetectable in all experimental conditions, whereas measurable levels of EETs (primarily 14,15-EET and, to a lesser extent, 11,12-EET) were detected. EET levels amounted to 30.1 ± 12.0 pg/mg protein ($n = 5$). There were no differences in EET levels among the different experimental conditions. The fact that we were not able to detect 20-HETE despite a relatively sensitive assay may be attributed to the source of cells, the aorta, which has been shown to

lack the capacity to synthesize 20-HETE (Marji et al., 2002). Hence, the BAECs, which are commonly used as a rich source of eNOS, were used in this study as a bioassay preparation.

Except for the pulmonary circulation (Zhu et al., 2002), the ability of the endothelium to produce 20-HETE is questionable. We examined the cellular source of 20-HETE in freshly isolated renal interlobar arteries, which were used for functional studies. 20-HETE levels in these arteries were unaffected by the removal of the endothelium (45.5 ± 4.6 and 43.8 ± 9.0 ng/mg 20-HETE in endothelium-intact and endothelium-denuded, respectively) whereas, as expected, EET levels were reduced from 123.9 ± 27.4 to 71.2 ± 7.0 ng/mg. These results indicate that, in the renal arteries, 20-HETE synthesis is endothelium-independent, as suggested previously (Miyata and Roman, 2005).

20-HETE Causes eNOS Uncoupling in Endothelial Cells. We have recently demonstrated that 20-HETE inhibits ionophore-stimulated NO production in endothelial cells (Fig. 1A). The 20-HETE-mediated inhibition of NO was associated with increased L-NMMA-sensitive superoxide production (Fig. 1B) and HSP90-eNOS dissociation, indicating

Fig. 1. Effect of 20-HETE on NO production (A), superoxide generation (B), and cGMP production (C) in endothelial cells. Cells were preincubated with and without L-NMMA (0.1 mM) and 20-HETE (5 nM) for 30 min, followed by incubation with the calcium ionophore A23187 (5 μ M) in the presence of L-arginine (25 μ M) for 30 min. Results are mean $\pm$ S.E.M., $n = 3$ –12; *, $p = 0.05$ versus untreated; †, $p < 0.05$ versus A23187; ‡, $p < 0.05$ versus 20-HETE + A23187.

that 20-HETE uncouples eNOS (Cheng et al., 2008). The inhibitory action of 20-HETE on eNOS was also evident by a concomitant reduction in cGMP levels. 20-HETE abolished ionophore-stimulated cGMP levels and had no further effect in cells treated with L-NMMA, indicating that 20-HETE decreased cGMP levels through the inhibition of eNOS (Fig. 1C). This effect of 20-HETE was associated with inhibition of HSP90-eNOS association and no changes in eNOS phosphorylation at serine 1179 or threonine 497 (Cheng et al., 2008).

20-HETE-Mediated Inhibition of NO Production Is Tyrosine Kinase- and ERK1/2-Sensitive. Addition of genistein, a nonselective tyrosine kinase inhibitor, or AG82, an EGFR kinase inhibitor, to endothelial cells prevented the inhibitory action of 20-HETE on ionophore-stimulated NO production (Fig. 2, A and B). We have previously shown that the 20-HETE-mediated activation of NF- κ B and production of inflammatory cytokines is mediated, in part, by the MAPK-ERK1/2 pathway (Ishizuka et al., 2008). In this study, addition of the MAPK inhibitor U0126 prevented the inhibition of ionophore-stimulated NO production by 20-HETE (Fig. 2C). These results suggest that both tyrosine kinase (EGFR activation) and MAPK-ERK1/2 signaling pathways

are involved in the 20-HETE-mediated inhibition of NO production in endothelial cells.

20-HETE-Mediated Inhibition of NO Production Is IKK-Sensitive. We showed recently that 20-HETE uncouples eNOS by inhibiting HSP90-eNOS association (Cheng et al., 2008). HSP90 is a heat shock protein that functions as a molecular chaperone to numerous signaling proteins. Among them is the cytoplasmic IKK complex, which consists of the scaffold protein, NF- κ B essential modulator (NEMO/IKK γ), and the IKK α and IKK β kinases (Broemer et al., 2004; Hayden and Ghosh, 2008). It is possible that the uncoupling of eNOS by 20-HETE is the consequence of 20-HETE-induced NF- κ B activation, for which HSP90 is required. Consequently, we examined whether NF- κ B is activated under conditions in which 20-HETE inhibits NO production. As seen in Fig. 3A, phosphorylated I κ B levels were barely detected in untreated cells and were slightly, but not significantly, elevated in cells treated with ionophore. However, as shown previously (Ishizuka et al., 2008), addition of 20-HETE to untreated cells increased I κ B phosphorylation by $214 \pm 22\%$. Likewise, in ionophore-stimulated cells, 20-HETE increased I κ B phosphorylation by $214 \pm 25\%$ compared with ionophore alone. Addition of L-NMMA did not significantly affect 20-HETE-stimulated I κ B phosphorylation (data not shown).

Phosphorylation of I κ B is catalyzed by the cytoplasmic IKK complex (Hayden and Ghosh, 2008). Addition of wedelolactone, a nonspecific inhibitor of IKK α and IKK β , or SC514, a potent and highly selective inhibitor of IKK β , completely reversed the 20-HETE-induced inhibition of NO production but had no direct effect on ionophore-stimulated NO production (Figs. 3, B and C). The prevention of 20-HETE-induced inhibition of NO production was also observed with the cell-permeable NBD peptide, which blocks the interaction of NEMO with the IKK kinase complex and thereby prevents NF- κ B activation (May et al., 2000). Treatment of cells with NBD did not affect ionophore-stimulated NO production but negated the 20-HETE-induced inhibition of NO production (Fig. 4D). The analog of NBD, which is defective in binding to NEMO (May et al., 2000) and was used as a negative control, had no effect on NO production in the presence or absence of 20-HETE (Fig. 3D).

20-HETE Increases HSP90-IKK Association. First, we examined whether HSP90 is required for 20-HETE-induced NF- κ B activation. Pretreatment of cells with the HSP90 inhibitor geldanamycin (GA) inhibited 20-HETE-induced I κ B phosphorylation in cells stimulated with ionophore by $49 \pm 10\%$ but had no significant effect on 20-HETE-stimulated I κ B phosphorylation in the absence of ionophore (Fig. 4A). As expected, GA treatment prevented the increase in ionophore-stimulated NO production (from 0.61 ± 0.08 to 0.1 ± 0.08 μ M, $n = 3$, $p < 0.05$), thus supporting previous studies showing that the association of HSP90 with eNOS is essential for eNOS activation and NO production (Pritchard et al., 2001). Addition of 20-HETE to stimulated cells treated with GA had no further effects (0.07 ± 0.05 μ M, $n = 3$). Similar results were obtained in cells treated with two structurally different HSP90 inhibitors, radicicol and 17-AAG. Both radicicol and 17-AAG decreased ionophore-stimulated NO production from 0.52 ± 0.15 to 0.07 ± 0.06 and 0.08 ± 0.04 μ M, respectively ($n = 4$, $p < 0.05$) and prevented 20-HETE-induced I κ B phosphorylation in ionophore-stimulated cells

Fig. 2. Effect of tyrosine kinase and MAPK inhibitors on 20-HETE-mediated inhibition of ionophore (A23187)-stimulated NO production. Cells were pretreated with and without 20-HETE (20H; 5 nM) for 30 min in the presence and absence of the tyrosine kinase inhibitor Genistein (30 μ M) (A), the EGFR-specific kinase inhibitor AG82 (10 μ M) (B), and the MAPK inhibitor U0126 (10 μ M) (C), followed by incubation with A23187 (5 μ M) in the presence of L-arginine (25 μ M) for 30 min. Results are mean $\pm$ S.E.M., $n = 6$; *, $p < 0.05$ versus untreated; #, $p < 0.05$ versus A23187; †, $p < 0.05$ versus 20H+A23187.

Fig. 3. A, effect of 20-HETE on IκB phosphorylation. Cells were preincubated with and without 20-HETE (20H; 5 nM) for 30 min followed by incubation with and without A23187 (5 μM) in the presence of L-arginine (25 μM) for 10 min. Western blot and densitometry analysis of phosphorylated and total IκB proteins in cell lysates ($n = 4$, $*$, $p < 0.05$ versus untreated). Effect of the IKKα/IKKβ inhibitor wedelolactone (WED; 50 μM) (B), the IKKβ-specific inhibitor SC514 (10 μM) (C), and NBD peptide 100 nM and its control peptide (CP; 100 nM) (D) on 20-HETE-mediated inhibition of ionophore-stimulated NO production. Cells were preincubated with and without the above inhibitors and 20-HETE (5 nM) for 30 min, followed by incubation with A23187 (5 μM) in the presence of L-arginine (25 μM) for 30 min. Results are mean $\pm$ S.E.M., $n = 6$; $*$, $p < 0.05$ versus untreated; $\#$, $p < 0.05$ versus A23187; $\dagger$, $p < 0.05$ versus 20H+A23187; $\ddagger$, $p < 0.05$ versus A23187+CP.

(Fig. 4B). On the other hand, inhibition of IKKβ with SC514 had no effect on IκB phosphorylation by 20-HETE (Fig. 4C), indicating that 20-HETE can also phosphorylate IκB in an IKK-independent manner.

Immunoprecipitation of IKKβ in ionophore-stimulated cells treated with 20-HETE revealed a $222 \pm 41\%$ increase in HSP90-IKKβ association compared with untreated cells (Fig. 5A). Likewise, immunoprecipitation of HSP90 in cells treated with 20-HETE and stimulated with ionophore showed a $307 \pm 37\%$ increase in IKKβ-HSP90 association compared with untreated cells (Fig. 5B). HSP90-IKKβ association was unchanged in both ionophore-stimulated cells and 20-HETE-treated cells compared with that of untreated cells. That 20-HETE interferes with eNOS-HSP90 association is further documented in Fig. 6. Immunoprecipitation with either eNOS (Fig. 6A) or HSP90 (Fig. 6B) clearly demonstrated that ionophore stimulated eNOS-HSP90 association, which was completely inhibited by the addition of 20-HETE.

20-HETE-Induced Impairment of Acetylcholine-Induced Relaxations Is Sensitive to Tyrosine Kinase, ERK1/2, and IKK. We have previously shown that increased endogenous 20-HETE production or addition of exogenous 20-HETE impairs acetylcholine-induced relaxation in renal interlobar arteries and that this impairment is associated with reduced NO bioavailability (Cheng et al., 2008). As seen in

Fig. 7, addition of 20-HETE to renal interlobar arteries pre-constricted with phenylephrine reduced the relaxing response to 5 μM acetylcholine from 57.0 ± 4.8 to $16.1 \pm 3.5\%$. Pretreatment of arteries with AG82, U0126, or SC514 fully restored the impaired relaxing response to acetylcholine in the presence of 20-HETE, yielding responses to 5 μM acetylcholine of 51.6 ± 3.9 , 57.0 ± 9.1 , and $52.3 \pm 5.0\%$, respectively, which were not different from control (Figs. 7, A–C). However, addition of geldanamycin to 20-HETE-pretreated arteries resulted in a relaxation response of $20.6 \pm 8.5\%$, which was not different from the relaxation response seen in 20-HETE-pretreated arteries (Fig. 7D). This is in agreement with previous studies showing that inhibition of HSP90 attenuates the vasodilatory response to acetylcholine (García-Cardena et al., 1998).

Discussion

Endothelial dysfunction and activation are hallmarks of hypertension and cardiovascular diseases. Endothelial dysfunction refers to the diminished production or bioavailability of NO and/or an imbalance in the relative contribution of endothelium-derived relaxing and contracting factors. This decreased bioavailability of NO can be a consequence of eNOS uncoupling, which is brought about by many factors,

Fig. 4. Effect of HSP90 inhibitors on HSP90-induced I κ B phosphorylation. A, geldanamycin (GA; 10 μ M); B, radicicol (Rad; 10 μ M) and 17-AAG (10 μ M). Cells were preincubated with and without 20-HETE (20H; 5 nM) and the HSP90 inhibitors for 30 min, followed by incubation with and without A23187 (5 μ M) in the presence of L-arginine (25 μ M) for 10 min. C, effect of SC514 on 20-HETE-stimulated I κ B phosphorylation. Results are mean $\pm$ S.E.M., $n = 3-4$, $*$, $p < 0.05$ versus untreated; $\dagger$, $p = 0.05$ versus 20-HETE+A23187.

Fig. 5. Effect of 20-HETE on IKK β -HSP90 association. Cells were preincubated with and without 20-HETE (20H; 5 nM) for 30 min, followed by incubation with and without A23187 (5 μ M) in the presence of L-arginine (25 μ M) for 10 min. Cells were immunoprecipitated with either HSP90 or IKK β antibodies. Western blot and densitometry analysis of IKK β and HSP90 in IKK β - (A) and HSP90- (B) immunoprecipitated fractions ($n = 4$, $*$, $p < 0.05$ versus A23187).

Fig. 6. Effect of 20-HETE on eNOS-HSP90 association. Cells were preincubated with and without 20-HETE (20H; 5 nM) for 30 min followed by incubation with and without A23187 (5 μ M) in the presence of L-arginine (25 μ M) for 10 min. Cells were immunoprecipitated with either HSP90 or eNOS antibodies. Western blot and densitometry analysis of eNOS and HSP90 in eNOS- (A) and HSP90- (B) immunoprecipitated fractions ($n = 4$, $*$, $p < 0.05$ versus untreated; $\dagger$, $p = 0.05$ versus A23187).

including a reduction in HSP90-eNOS association, eNOS phosphorylation, and cofactor accessibility, as well as an increase in caveolin-1-eNOS binding (Fleming and Busse, 1999, 2003). Endothelial activation refers to a phenotype of inflammatory tissue that promotes leukocyte adhesion and increases vascular permeability via up-regulation of surface cell adhesion molecules and the release of inflammatory cytokines. These two processes are closely linked where endo-

Fig. 7. Effects of inhibitors of tyrosine kinase (AG82; 10 μ M) (A), MAPK (U0126; 10 μ M) (B), IKK (SC514; 10 μ M) (C), and HSP90 (geldanamycin; 10 μ M) (D) on 20-HETE-impaired acetylcholine-induced relaxations. The responses to a single dose of acetylcholine (5 μ M) over a 30-s period are depicted. $n = 3-4$, *, $p < 0.05$ versus control + 20-HETE; †, $p < 0.05$ versus control.

thelial activation is thought to be the cause or consequence of endothelial dysfunction. This closed interdependence is best exemplified by the nature of NO bioactions in promoting vasodilation and anti-inflammation. In contrast, 20-HETE promotes vasoconstriction, endothelial dysfunction, and inflammation. 20-HETE is a smooth muscle-derived eicosanoid in most circulatory districts (Miyata and Roman, 2005); its synthesis in the vasculature is increased in experimental models of hypertension including the SHR (Zhang et al., 2001), CYP4A-transgenic rats (Wang et al., 2006), and androgen-induced hypertension (Singh et al., 2007). In these models, endothelial dysfunction and activation can be mitigated by inhibition of 20-HETE synthesis. In vitro, 20-HETE activates endothelial cells (Guo et al., 2007; Ishizuka et al., 2008) and interferes with eNOS activity (Cheng et al., 2008). These characteristics add 20-HETE to the list of factors implicated in the genesis of vascular complications that are initiated at the level of the endothelium. The prevalence and coexistence of dysfunction and activation and cellular mechanisms that link the two with regard to 20-HETE are the main subjects of this study.

Findings in this study clearly demonstrate that 20-HETE inhibits NO production and stimulates I κ B phosphorylation, a key step in the activation of the NF- κ B pathway, by a mechanism that involves the activation of the tyrosine kinase/EGFR and MAPK pathways. A general tyrosine kinase inhibitor, as well as an inhibitor specific for EGFR activation, completely prevented the 20-HETE-induced inhibition of NO synthesis. Likewise, inhibition of MAPK-ERK1/2 phosphorylation prevented the inhibitory action of 20-HETE on NO production. We have previously shown that 20-HETE stim-

ulates I κ B phosphorylation and nuclear translocation of NF- κ B in a MAPK/ERK-dependent manner (Ishizuka et al., 2008). This suggests that tyrosine kinase and MAPK activation are upstream signaling pathways for 20-HETE effects on NO production and NF- κ B activation. Numerous studies have documented the involvement of tyrosine kinase-EGFR transactivation and that of the MAPK-ERK1/2 in mediating 20-HETE effects, including vasoconstriction (Sun et al., 1999), proliferation, angiogenesis (Medhora et al., 2007), and superoxide generation (Guo et al., 2007). The ability of 20-HETE to concurrently uncouple eNOS and activate NF- κ B via a common cellular mechanism raises the question of the nature of interdependence between these two events. Recent studies have demonstrated that NO exerts an inhibitory action on NF- κ B activation through several mechanisms including I κ B stabilization and p50 nitrosylation (Peng et al., 1995; Grumbach et al., 2005). Alternatively, studies by Harrison and colleagues demonstrated that NF- κ B activation stimulates eNOS transcription under shear stress (Davis et al., 2004; Grumbach et al., 2005). It may be concluded that the activation of NF- κ B by 20-HETE is the consequence of removing the inhibitory action by NO; however, the 20-HETE-stimulated I κ B phosphorylation in the presence of ionophore or L-NMMA was not significantly different from that elicited by 20-HETE alone, suggesting that alterations in NO levels do not influence 20-HETE action on I κ B phosphorylation.

NF- κ B is activated upon phosphorylation of I κ B by IKK (Hayden and Ghosh, 2008). IKK is a complex that comprises α , β , and γ /NEMO subunits; IKK α and primarily IKK β function to phosphorylate I κ B, whereas IKK γ /NEMO is a

regulator of IKK complex assembly, which is required for IKK β activity (Hayden and Ghosh, 2008). It is noteworthy that pretreatment of cells with IKK β inhibitors completely prevented the effect of 20-HETE on NO production. Moreover, pretreatment of cells with an NBD, an inhibitor of IKK complex assembly, but not its inactive control peptide, blocked the effect of 20-HETE on decreasing NO levels. This suggests that 20-HETE-mediated eNOS uncoupling is IKK-dependent.

HSP90, a molecular chaperone, is critical for the regulation of IKK homeostasis and is required for both IKK biosynthesis and constitutive and inducible IKK/NF- κ B activation (Broemer et al., 2004). Short-term inhibition of HSP90 with geldanamycin impairs IKK activation by stimuli such as tumor necrosis factor α ; long-term inhibition of HSP90 downmodulates all complexes of IKK (Chen et al., 2002; Broemer et al., 2004). The activity of eNOS is also dependent on HSP90, which acts as an intermediate in the signaling cascades leading to the activation of eNOS (Fleming and Busse, 1999). HSP90 coimmunoprecipitates with eNOS (Fleming and Busse, 1999); inhibition of HSP90 signaling attenuates the vasodilatory response to acetylcholine (García-Cardena et al., 1998) and inhibits eNOS-dependent ionophore-stimulated nitrite production in endothelial cell cultures (Pritchard et al., 2001). These studies provide substantial evidence for a central role of HSP90 in modulating key processes of endothelial dysfunction (eNOS-NO) and activation (NF- κ B). It has been demonstrated that high glucose impairs NO production by enhancing the association of IKK β -HSP90 in a IKK β -dependent manner (Mohan et al., 2009). Our previous study showed that 20-HETE uncouples eNOS via the dissociation of HSP90-eNOS (Cheng et al., 2008). In the current study, we showed that inhibition of HSP90 attenuated 20-HETE-stimulated I κ B phosphorylation, suggesting that HSP90 is a common component of 20-HETE-mediated eNOS-NO inhibition and NF- κ B activation. In addition, 20-HETE significantly increased HSP90-IKK β association under ionophore-stimulated conditions. It is noteworthy that 20-HETE did not affect HSP90-IKK β association in the absence of ionophore, suggesting that the increase in HSP90-IKK β association occurs only when eNOS is activated. Calcium ionophore by itself has been shown to be involved in the nuclear export of NEMO; inhibitors of calcium attenuate NF- κ B activation (Berchtold et al., 2007). However, treatment of cells with ionophore alone did not increase I κ B phosphorylation or HSP90-IKK β association. Moreover, HSP90 or IKK inhibitors did not affect 20-HETE-stimulated I κ B phosphorylation in the absence of ionophore, suggesting that 20-HETE activates NF- κ B through I κ B phosphorylation via IKK-dependent and -independent mechanisms.

It has long been shown that NO and NO donors inhibit the catalytic activities of CYP enzymes by forming stable nitrosyl complexes with the ferrous iron, nitration of tyrosine residues, or oxidation of thiols (Morgan et al., 2001). NO has been shown to directly bind to the heme moiety of recombinant CYP4A and microsomal P450 proteins and to inhibit the formation of 20-HETE (Wang et al., 2003). The inhibitory action of NO on 20-HETE synthesis has been implicated as the cGMP-independent component of NO vasodilatory effect (Alonso-Galicia et al., 1997). Mice lacking the eNOS gene displayed increased vascular 20-HETE synthesis (Huang et al., 2005). Inhibition of 20-HETE synthesis attenuated the

rise in blood pressure in *N*^ω-nitro-L-arginine methyl ester-treated rats (Hercule et al., 2003) and induced the NO/endothelium-dependent relaxation to acetylcholine (Kerkhof et al., 1999), whereas administration of a 20-HETE agonist prevented the fall in blood pressure after endotoxin shock (Tuncan et al., 2008). In all, these studies implicate NO in the regulation of 20-HETE synthesis. However, other studies including ours, suggest that 20-HETE significantly affects NO levels. For example, 20-HETE-induced relaxation in bovine pulmonary arteries has been linked to its ability to activate eNOS and increase NO production (Chen et al., 2006), whereas in the renal microcirculation, 20-HETE uncouples eNOS, inhibits NO production, and reduces NO-dependent relaxation to acetylcholine (Cheng et al., 2008). In human (Ward et al., 2004) and experimental animal models of hypertension, a correlation between endothelial dysfunction and 20-HETE levels has been documented. In these animal models, inhibition of 20-HETE synthesis or action improves endothelial function and reduces blood pressure (Frisbee et al., 2000; Singh et al., 2007; Dunn et al., 2008). Our findings add another feature to 20-HETE actions: its ability to induce the inflammatory program while removing the cytoprotection bestowed by NO. The relevance of NO-20-HETE-NF- κ B interactions to cardiovascular disease is still unclear. However, the studies cited above clearly implicate 20-HETE as an important regulator of vascular function and could portend a novel therapeutic strategy for cardiovascular disease that targets 20-HETE synthesis and/or action.

The bioactions of 20-HETE in endothelial cells are evident at low nanomolar concentrations and have been shown to be highly specific in that they were not reproduced by structurally similar eicosanoids (Cheng et al., 2008). This specificity

Fig. 8. Postulated mechanism linking 20-HETE-induced endothelial dysfunction and activation.

as well as potency, along with its established presence within the vascular wall and lumen and the synthetic capacity of the vascular smooth muscle (Kroetz and Xu, 2005; Miyata and Roman, 2005), position 20-HETE as an ideal small-molecule regulator of endothelial function. Here, we show that 20-HETE uncouples eNOS and impairs acetylcholine-induced relaxations via tyrosine kinase-, MAPK/ERK- and IKK-dependent mechanisms that involve the dissociation and association of HSP90 to eNOS and IKK β , respectively. We also provide evidence to suggest that 20-HETE-stimulated endothelial activation is the consequence of NF- κ B activation through IKK-dependent and independent I κ B phosphorylation. Figure 8 depicts the postulated mechanism by which 20-HETE mediates endothelial dysfunction and stimulates endothelial activation.

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Address correspondence to: Dr. Michal Laniado-Schwartzman, Department of Pharmacology, New York Medical College, Valhalla, NY 10595. E-mail: michal_schwartzman@nymc.edu