

## 20-Hydroxyeicosatetraenoic Acid Stimulates Nuclear Factor- $\kappa$ B Activation and the Production of Inflammatory Cytokines in Human Endothelial Cells

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### ABSTRACT

Endothelial dysfunction is associated with endothelial cell activation, i.e., up-regulation of surface cell adhesion molecules and the release of proinflammatory cytokines. 20-Hydroxyeicosatetraenoic acid (HETE), a major vasoactive eicosanoid in the microcirculation, has been implicated in the regulation of endothelial cell function through its angiogenic and pro-oxidative properties. We examined the effects of 20-HETE on endothelial cell activation *in vitro*. Cells transduced with adenovirus containing either CYP4A1 or CYP4A2 produced higher levels of 20-HETE, and they demonstrated increased expression levels of the adhesion molecule intercellular adhesion molecule (ICAM) (4–7-fold) and the oxidative stress marker 3-nitrotyrosine (2–3-fold) compared with cells transduced with control adenovirus. Treatment of cells with 20-HETE markedly increased levels of prostaglandin (PG) E<sub>2</sub> and 8-epi-isoprostane PGF<sub>2 $\alpha$</sub> , commonly

used markers of activation and oxidative stress, and most prominently, interleukin-8, a potent neutrophil chemotactic factor whose overproduction by the endothelium is a key feature of vascular injury. 20-HETE at nanomolar concentrations increased inhibitor of nuclear factor- $\kappa$ B phosphorylation by 2 to 5-fold within 5 min, which was followed with increased nuclear translocation of nuclear factor- $\kappa$ B (NF- $\kappa$ B). Likewise, 20-HETE activated the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway by stimulating phosphorylation of ERK1/2. Inhibition of NF- $\kappa$ B activation and inhibition of ERK1/2 phosphorylation inhibited 20-HETE-induced ICAM expression. It seems that 20-HETE triggers NF- $\kappa$ B and MAPK/ERK activation and that both signaling pathways participate in the cellular mechanisms by which 20-HETE activates vascular endothelial cells.

The synthesis of 20-hydroxyeicosatetraenoic acid (HETE), the  $\omega$ -hydroxylation product of arachidonic acid, is catalyzed by enzymes of the cytochrome P450 (P450) 4 gene family. This family encodes multiple structurally and functionally similar P450 proteins (4A, 4B, and 4F) that are controlled by factors such as age, sex hormones, and dietary lipids (Okita and Okita, 2001; Kroetz and Xu, 2005). The importance of this metabolic pathway emerged with the seminal observa-

tion 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 (Sarkis and Roman, 2004). Its role in promoting prohypertensive mechanisms and in 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 (Imig et al., 1996; Gebremedhin et al., 2000). Its synthesis within the vascular wall is primarily localized to the smooth muscle cells; increases with decreased vessel diameter; is stimulated by vasoactive hormones such as an-

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**ABBREVIATIONS:** HETE, hydroxyeicosatetraenoic acid; P450, cytochrome P450; eNOS, endothelial nitric-oxide synthase; ICAM, intracellular adhesion molecule; IL, interleukin; NF- $\kappa$ B, nuclear factor- $\kappa$ B; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; I $\kappa$ B, inhibitor of nuclear factor- $\kappa$ B; MAP, mitogen-activated protein; PDTC, pyrrolidinedithiocarbamate; PD98059, 2'-amino-3'-methoxyflavone; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; PBS, phosphate-buffered saline; 8-isoP, 8-epi-isoprostane prostaglandin F<sub>2 $\alpha$</sub> ; PG, prostaglandin; Adv, adenovirus; GFP, green fluorescent protein; ROS, reactive oxygen species; SN50, H<sub>2</sub>N-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Val-Gln-Arg-Lys-Arg-Gln-Lys-Leu-Met-Pro-OH.

giotensin II, endothelin, and norepinephrine; and is inhibited by nitric oxide (Miyata and Roman, 2005). It elicits vasoconstriction largely via inhibition of the smooth muscle cell large conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channel, leading to depolarization and elevation in cytosolic  $[\text{Ca}^{2+}]$  (Harder et al., 1994), and in some blood vessels via a Rho-kinase phosphorylation of MLC20 and the sensitization of the contractile apparatus to  $\text{Ca}^{2+}$  (Randriamboavonjy et al., 2003). Besides its vasoactivity, 20-HETE has been shown to stimulate smooth muscle cell migration and proliferation (Muthalif et al., 1998; Stec et al., 2007).

Although endothelial synthesis of 20-HETE is questionable in most vascular beds, with the exception of 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 (Amaral et al., 2003; Jiang et al., 2004; Chen et al., 2005) 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). It is noteworthy that changes in the production of 20-HETE have been observed in numerous pathological conditions, including ischemic cerebrovascular diseases, cardiac ischemia-reperfusion injury, 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.

In recent studies, we demonstrated that increased vascular synthesis of 20-HETE results in endothelial dysfunction, i.e., reduced acetylcholine-induced relaxation, oxidative stress, and hypertension (Wang et al., 2006; Singh et al., 2007). Endothelial dysfunction is associated with endothelial cell activation, a phenotype of inflammatory tissue that promotes leukocyte adhesion and increases vascular permeability via up-regulation of surface cell adhesion molecules [e.g., intercellular adhesion molecule (ICAM), vascular cell adhesion molecule, and E-selectin] and the release of cytokines (e.g., IL-8). Endothelial cell activation may be the cause or the consequence of endothelial dysfunction. In all, endothelial dysfunction is implicated in the pathogenesis of diseases such as atherosclerosis, hypertension, diabetes, and heart failure, many of which show changes in 20-HETE levels.

In the current study, we examined whether 20-HETE causes endothelial cell activation, and we examined possible cellular mechanisms that may account for its effect. We showed that 20-HETE stimulates the production of inflammatory molecules in endothelial cells via a mechanism that includes activation of NF- $\kappa$ B and MAPK/ERK signaling pathways, two major cellular circuits for the production of inflammatory molecules and the activation of the vascular endothelium.

## Materials and Methods

**Cell Culture.** The human umbilical vascular endothelial cell-derived EA.hy926 cell line was used (Edgell et al., 1983). Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and hypoxanthine-aminopterin-thymidine supplement (Sigma-Aldrich, St. Louis, MO). Cells (80–90% confluent) were incubated in serum-free medium for 24 h followed by the addition of 20-HETE (0.1–10 nM) for the indicated times. Cells were harvested and processed for the various measurements. In some experiments, cells (60–70% confluent) were infected with the adenoviral constructs pAd5CMV-NpA-eGFP (Adv-GFP) or

pAd5CMV-NpA-CYP4A2 (Adv-CYP4A2) as described previously (Wang et al., 2006). CYP4A2 protein expression was determined by Western blot analysis, and 20-HETE levels measured by gas chromatography/mass spectrometry as described previously (Wang et al., 2006).

**Nuclear Extract Preparation.** Nuclear proteins were extracted using the CellLytic NuCLEAR extraction kit (Sigma-Aldrich) according to the manufacturer's instructions. In brief, cells were grown to 80% confluence, treated with 20-HETE (0.1–10 nM) for the indicated time, washed, and collected into a hypotonic lysis buffer containing 1 mM dithiothreitol and protease inhibitor cocktail. After a 15-min incubation at 4°C, 10% Igepal CA-630 was added to a final concentration of 0.6%, and cells were centrifuged immediately for 30 s at 10,000g. The crude nuclei pellet was resuspended in extraction buffer containing dithiothreitol and protease inhibitor cocktail, mixed well, and centrifuged for 5 min at 20,000g. Aliquots of the supernatant were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use.

**Western Blot Analysis.** Cellular proteins were subjected to 12% SDS-polyacrylamide gel electrophoresis. The following antibodies were used: goat anti-rat CYP4A1 polyclonal antibody (Daiichi Chemical Co., Toyama, Japan); rabbit polyclonal ICAM-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit nitrotyrosine polyclonal antibody (Cayman Chemical, Ann Arbor, MI); and rabbit polyclonal NF- $\kappa$ B p65 antibody, rabbit anti-inhibitor of nuclear factor- $\kappa$ B (I $\kappa$ B) $\alpha$  polyclonal antibody, rabbit anti-phospho-I $\kappa$ B $\alpha$  (Ser32) monoclonal antibody, rabbit polyclonal phospho-p44/42 (ERK1/ERK2) MAP kinase (Thr202/Tyr204) antibody, and rabbit polyclonal p44/42 MAP kinase (ERK1/ERK2) antibody (Cell Signaling Technology Inc., Beverly, MA). Immunoreactive proteins were detected using the ECL Plus detection system (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. Anti  $\beta$ -actin was used to normalize for loading variations. Densitometry analysis was performed using Gel-Pro analyzer 3.1, Media Cybernetics).

**Immunofluorescence.** Cells were grown on four-chamber glass-bottomed culture dishes (Nalge Nunc International, Rochester, NY) at a density of 10,000 cells/well. Quiesced cells (70% confluence) were treated with 0.1 to 10 nM 20-HETE for 5 to 60 min in the presence and absence of the NF- $\kappa$ B inhibitor ammonium pyrrolidinedithiocarbamate (PDTC) at 100  $\mu\text{M}$  or SN50 at 5  $\mu\text{M}$ , a cell-permeable inhibitor peptide of NF- $\kappa$ B translocation. In some experiments, the MAPK/ERK kinase inhibitors PD98059 (10  $\mu\text{M}$ ) and U0126 (10  $\mu\text{M}$ ) were used. Cells were washed three times with PBS, fixed with 3.7% paraformaldehyde for 30 min, and permeabilized with 0.1% Triton X-100 on ice for 10 min. Cells were washed three times with PBS, incubated with 10% normal goat serum (Vector Laboratories, Burlingame, CA) for 20 min at room temperature followed by additional 1-h incubation with rabbit anti-NF- $\kappa$ B subunit p65 antibody (1:50; Santa Cruz Biotechnology, Inc.). Cells were washed with PBS, incubated with Cy3-conjugated goat anti-rabbit IgG (1:200; Jackson Immuno-Research Laboratories Inc., West Grove, PA) for 1 h, and counterstained for nuclei with 4,6-diamidino-2-phenylindole for 5 min. Immunofluorescence was visualized using an Axioplan-2 fluorescent microscope (Carl Zeiss, Gottingen, Germany). Cell images were captured and analyzed using AxioVision 2 multichannel image processing software (Carl Zeiss). The number of NF- $\kappa$ B-positive cells versus total number of cells was measured in four randomly chosen fields for each experiment. The results were based on three independent analyses.

**Inflammatory Mediators.** Cells were incubated with 1 nM 20-HETE for 1 to 24 h after which medium was collected. Cytokines were measured in medium using a custom multiplex sandwich enzyme-linked immunosorbent assay for protein analysis (SearchLight human microarray; Pierce Biotechnology, Woburn, MA). Levels of 8-epi-isoprostane PGF $_{2\alpha}$  (8-isoP) and prostaglandin (PG)E $_2$  were de-

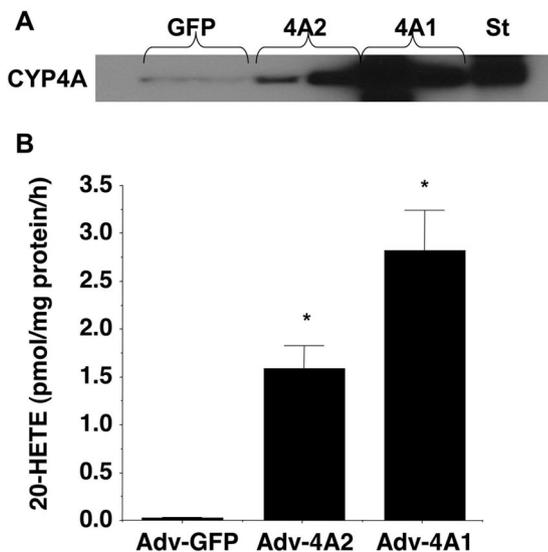
terminated in the media using enzyme-linked immunoassays (Cayman Chemical) following the instructions provided by the manufacturer.

**Statistical Analysis.** Data were analyzed by analysis of variance using Dunnett's multiple comparison test, and they are expressed as mean  $\pm$  S.E.

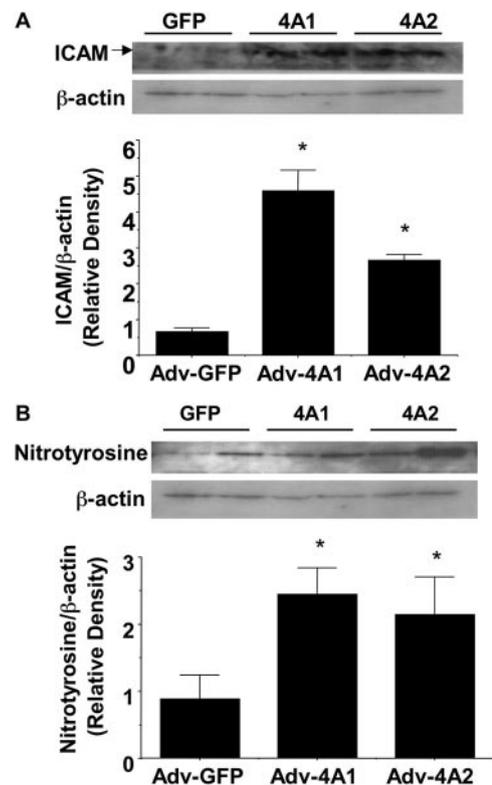
## Results

**CYP4A Overexpression or Treatment with 20-HETE Brings about Endothelial Activation.** The expression levels of CYP4A-immunoreactive proteins are hardly detectable in human umbilical vein endothelial cell-derived EA.hy926 cells. However, 48 h after infection with adenovirus constructs containing the CYP4A1 or CYP4A2 cDNA (Adv-4A1 and Adv-4A2, respectively), CYP4A-immunoreactive proteins were highly expressed, and these cells produced 20-HETE. As seen in Fig. 1A, Western blot analysis of cells transduced with Adv-4A1 or Adv-4A2 showed a strong CYP4A immunoreactivity signal, which was hardly visible in cells transduced with the control adenoviral construct Adv-GFP. Moreover, Adv-4A1- or Adv-4A2-transduced cells produced 20-HETE at rates of  $2.82 \pm 0.37$  and  $1.59 \pm 0.27$  pmol/mg/h, respectively, whereas 20-HETE levels were barely detectable in Adv-GFP-transduced cells (Fig. 1B). Transduction of either Adv-4A1 or Adv-4A2 increased ICAM-1 protein levels by 7- and 4-fold, respectively (Fig. 2A). Likewise, the levels of nitrotyrosine protein expression increased by 2 to 2.5-fold in cells transduced with Adv-4A1 or Adv-4A2 (Fig. 2B). Endothelial cell activation at 48 h after transduction with CYP4A1 or CYP4A2 adenovirus was also evident by increased levels of  $\text{PGE}_2$  and 8-isoP, a commonly used marker of oxidative stress.  $\text{PGE}_2$  levels increased from  $5.3 \pm 0.6$  in GFP-transduced cells to  $7.1 \pm 0.3$  pg/mg protein in 4A-transduced cells ( $n = 3$ ;  $p < 0.05$ ). 8-isoP increased from  $0.05 \pm 0.02$  to  $0.29 \pm 0.04$  pg/mg ( $n = 3$ ;  $p < 0.05$ ).

Similar results were obtained when 20-HETE was added



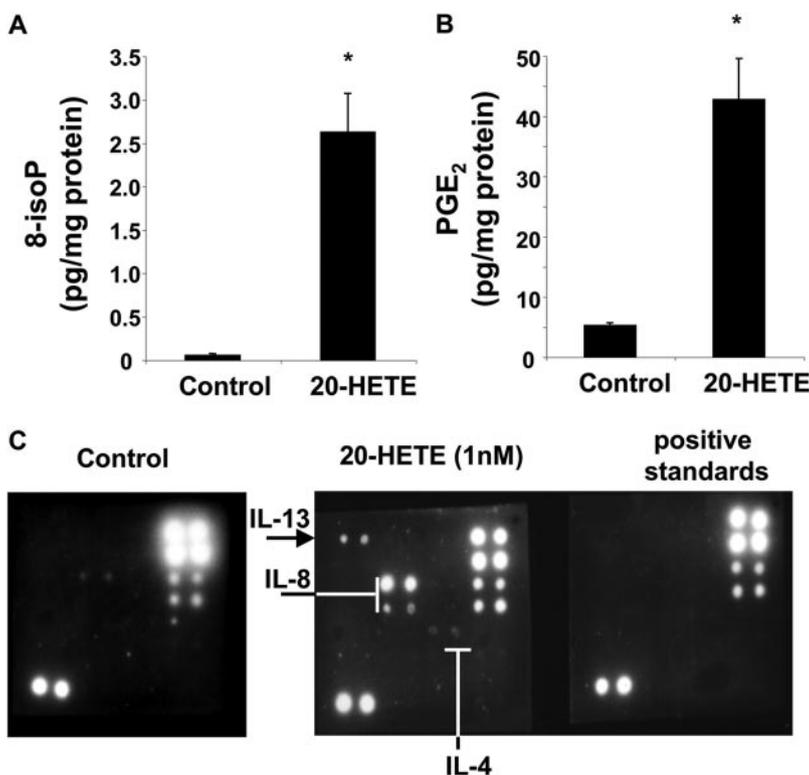
**Fig. 1.** P450 protein expression (A) and CYP4A catalytic activity (20-HETE production) (B) in adenovirus-treated EA.hy926 cells. Cells (70% confluent) were infected with adenoviruses (Adv-GFP, Adv-4A1, and Adv-4A2;  $10^6$  cells of  $1$  to  $1.2 \times 10^{12}$  plaque-forming units/ml) for 48 h, and they were processed for Western blot analysis with anti-CYP4A antibody and for measurements of 20-HETE levels by gas chromatography/mass spectrometry. Results are mean  $\pm$  S.E. ( $n = 3$ ). \*,  $p < 0.01$  from Adv-GFP. St, positive controls for CYP4A proteins.



**Fig. 2.** ICAM and nitrotyrosine immunoreactivity in EA.hy926 cells transduced with Adv-GFP, Adv-4A1, and Adv-4A2. Cells were harvested 48 h after infection, and lysates were processed for Western blot analysis. Shown are representative blots and densitometry analysis as mean  $\pm$  S.E. ( $n = 3$ ). \*,  $p < 0.05$  from Adv-GFP-infected cells.

exogenously to endothelial cells. Hence, treatment of endothelial cells with 1 nM 20-HETE for 1 h markedly increased 8-isoP levels (from  $0.05 \pm 0.02$  to  $2.61 \pm 0.32$  pg/mg protein) and  $\text{PGE}_2$  levels (from  $5.2 \pm 0.4$  to  $42.70 \pm 6.80$  pg/mg protein) in the media (Fig. 3, A and B). Moreover, cytokine array analysis of cells treated with 1 nM 20-HETE for 24 h revealed a marked increase in the expression of inflammatory cytokines/chemokines, including IL-8, IL-13, and IL-4 (Fig. 3C), suggesting that 20-HETE is a potent endothelial cell activator.

**20-HETE Stimulates NF- $\kappa$ B Activation.** Because NF- $\kappa$ B activation underlies the cellular mechanisms by which many inflammatory pathways relevant to endothelial cell activation are induced, we evaluated whether it also constitutes the mechanism of 20-HETE actions. Addition of 20-HETE to endothelial cells evoked a time- and concentration-dependent phosphorylation of I $\kappa$ B $\alpha$ . As seen in Fig. 4A, 20-HETE at 1 nM increased I $\kappa$ B $\alpha$  phosphorylation by 2.5- and 2-fold, 2 and 5 min, respectively, after its addition. Moreover, this effect was concentration-dependent; I $\kappa$ B $\alpha$  phosphorylation was evident at concentrations of 20-HETE as low as 0.1 nM and maximal at 5 nM (Fig. 4B). NF- $\kappa$ B activation was further determined by measuring nuclear translocation of NF- $\kappa$ B p65 subunit by Western blot analysis and immunofluorescence. As seen in Fig. 5, NF- $\kappa$ B p65 immunoreactivity increased in the cytoplasmic fraction of cells treated as early as 5 min after the addition of 1 nM 20-HETE, and then it gradually decreased to basal levels after 60 min. Concurrently, NF- $\kappa$ B immunoreactivity increased in the nuclear fraction, and it was maximal at 30 min (Fig. 5). Immunofluorescence studies concurred with the Western blot analysis



**Fig. 3.** Effect of 20-HETE on levels of 8-isoP (A), PGE<sub>2</sub> (B), and on the expression of inflammatory cytokines (C). Endothelial cells were treated with 1 nM 20-HETE or the vehicle control for 1 h. The medium was collected, and levels of 8-isoP and PGE<sub>2</sub> were measured by enzyme-linked immunosorbent assay. Results are mean  $\pm$  S.E. ( $n = 3$ ). \*,  $p < 0.01$ . Expression levels of inflammatory cytokines in culture media of cells treated with 1 nM 20-HETE for 24 h were measured by cytokine Searchlight arrays (Pierce Biotechnology). The four dots indicate IL-8 different concentrations of the capture antibody.

and demonstrated a maximal NF- $\kappa$ B p65 nuclear localization 30 min after addition of 20-HETE (Fig. 6). Moreover, known inhibitors of NF- $\kappa$ B activation, including PDTC and SN50, abolished 20-HETE-induced NF- $\kappa$ B nuclear translocation (Fig. 6).

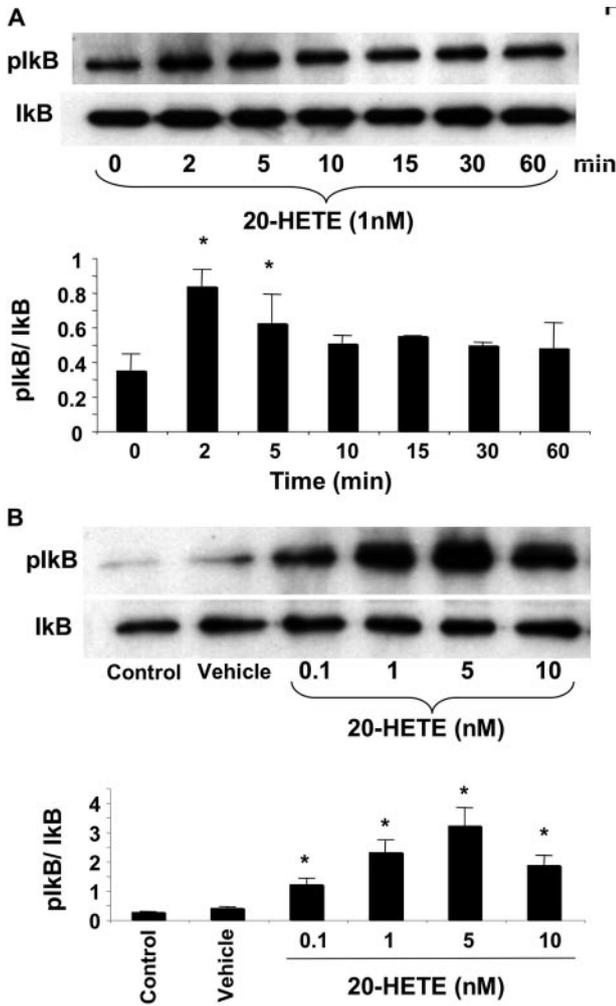
**20-HETE Activates the MAPK/ERK Pathway.** The MAPK/ERK kinase pathway also constitutes a major signaling route for endothelial cell activation. We measured the effect of 20-HETE on the phosphorylation of the ERK1/2 (p44/p42) kinase. 20-HETE rapidly and potently stimulated phosphorylation of ERK 1/2 in concentration- and time-dependent manners. As seen in Fig. 7A, both 0.1 and 1 nM 20-HETE induced a significant increase of ERK1/2 phosphorylation after 10 min of treatment. 20-HETE (0.1 nM) activated ERK1/2 phosphorylation as soon as 5 min, and it reached maximum at 10 min (Fig. 7A). Interestingly, 20-HETE-induced NF- $\kappa$ B translocation, measured by the fluorescence assay, was inhibited by 48 and 42% following treatment with the MAPK/ERK kinase inhibitors PD98059 ( $p = 0.06$ ) and U0126 ( $p < 0.05$ ), respectively (Fig. 7B), suggesting that NF- $\kappa$ B activation by 20-HETE is linked to MAPK/ERK activation.

**20-HETE-Induced ICAM-1 Expression Is Blocked by Inhibitors of NF- $\kappa$ B and MAPK Activation.** ICAM-1 is readily expressed on the surface of endothelial cells following exposure to inflammatory stimuli, and our data indicate that its levels are increased in endothelial cells overexpressing the CYP4A proteins (Fig. 2). Here, we further show that the addition of 20-HETE, the putative mediator of CYP4A actions in the endothelium, increased ICAM-1 expression by approximately 2.5-fold (Fig. 8). More importantly, inhibition of NF- $\kappa$ B activation and translocation, as well as inhibition of MAPK activation, abrogated 20-HETE-induced ICAM-1 expression, suggesting that both signaling pathways are part of the cellular mechanism by which 20-HETE causes endothelial cell activation.

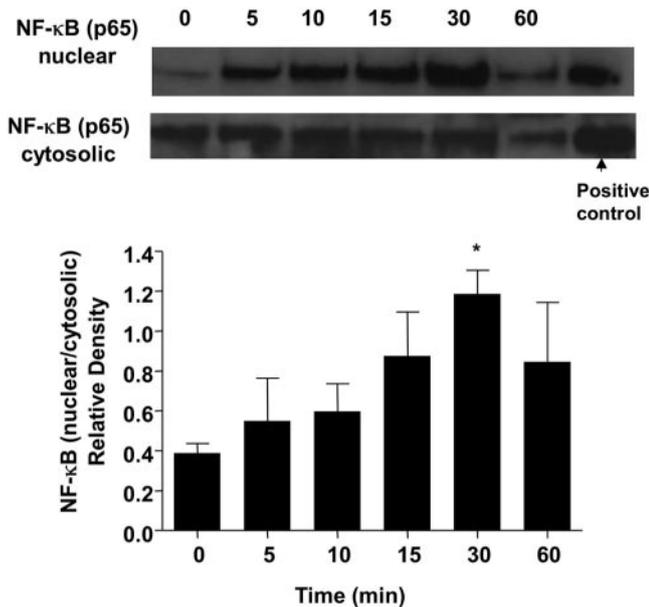
## Discussion

Endothelial activation is characterized by enhanced production of cytokines and increased expression of adhesion molecules leading to increased leukocyte adhesion and transmigration into the intima of the vessel wall (De Caterina et al., 2000). Recent studies demonstrated that the endothelial cell is a site of production and action of 20-HETE, and they implicated its involvement in the regulation of endothelial cell function primarily with regard to eNOS activation, superoxide production, and angiogenesis (Amaral et al., 2003; Jiang et al., 2004; Chen et al., 2006; Wang et al., 2006; Guo et al., 2007). To this end, two recent reports provided substantial evidence for a causative link between 20-HETE and endothelial dysfunction (Wang et al., 2006; Singh et al., 2007). These reports, the prominent presence of 20-HETE in the microcirculation (Miyata and Roman, 2005), its potent bioactions on endothelial function, and the fact that endothelial activation is associated with endothelial dysfunction prompted the current study. Hence, we examined whether 20-HETE acts as a proinflammatory molecule, and we discerned possible mechanisms that transduce its action. The results of this study clearly indicate that 20-HETE is an endothelial cell activator with potent proinflammatory actions that are mediated via activation of the NF- $\kappa$ B and MAPK/ERK kinase signaling pathways.

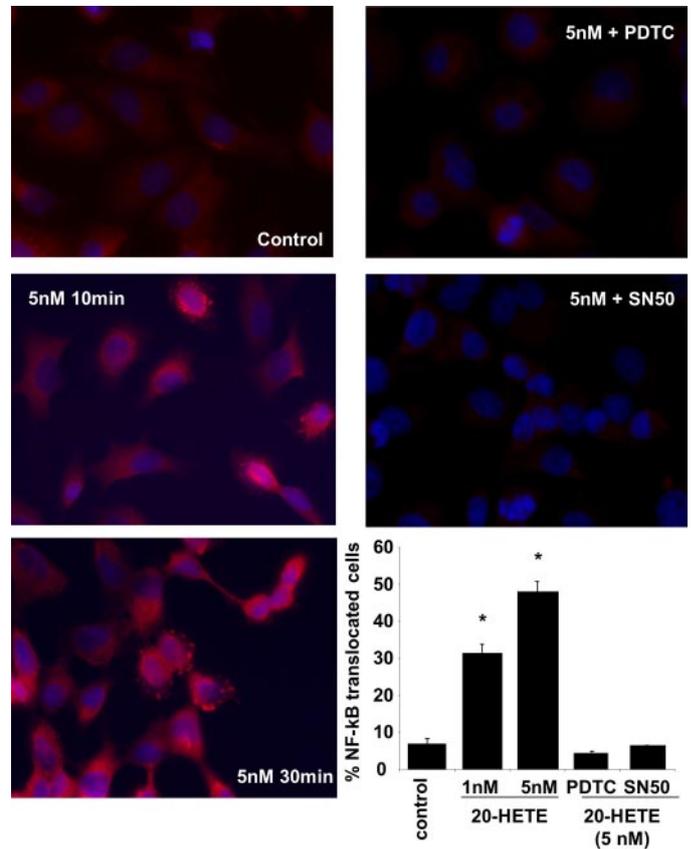
Since the endothelium has been shown to express CYP4A and to produce 20-HETE (Zhu et al., 2002), the impact of expressing the CYP4A1 or CYP4A2 cDNAs, which are both active arachidonic acid  $\omega$ -hydroxylases (Nguyen et al., 1999), or adding exogenous 20-HETE on markers of endothelial cell activation, were evaluated. Endothelial activation is marked by increased oxidative stress, i.e., increased production of reactive oxygen species and their associated targets. Expression levels of 3-nitrotyrosine have been readily used to mea-



**Fig. 4.** Time- (A) and concentration-dependent (B) phosphorylation of IκBα by 20-HETE. Results are mean  $\pm$  S.E. ( $n = 3$ ). \*,  $p < 0.05$  from vehicle (0.1% ethanol)-treated cells.

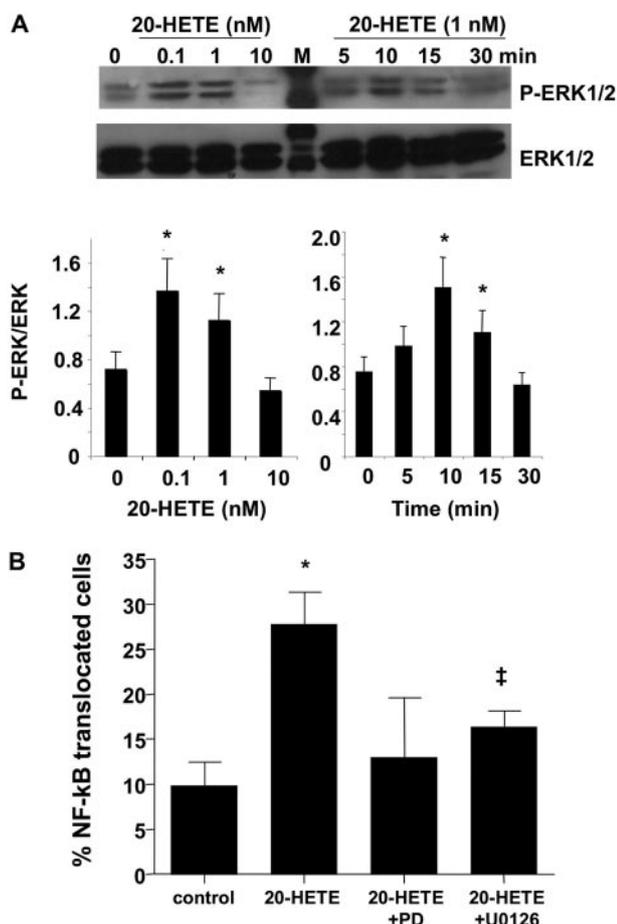


**Fig. 5.** Time-dependent NF-κB (p65) nuclear translocation by 20-HETE. Cells were treated with 1 nM 20-HETE for 0 to 60 min. NF-κB levels were determined in nuclear and cytosolic fractions by Western blot analysis. Results are mean  $\pm$  S.E. ( $n = 3$ ). \*,  $p < 0.05$  from time 0.



**Fig. 6.** NF-κB nuclear translocation by immunofluorescence. Cells were treated with 1 or 5 nM 20-HETE for 10 or 30 min in presence or absence of the NF-κB inhibitors, PDTC, and SN50. Representative images showing positive cells (merged NF-κB fluorescence with 4,6-diamidino-2-phenylindole nuclear staining) and negative cells are displayed. The number of activated NF-κB-positive cells in cells treated for 30 min was calculated (bottom right). Results are expressed as the percentage of nuclear NF-κB-positive cells to total cells, and are values are mean  $\pm$  S.E. ( $n = 4$ ). \*,  $p < 0.05$  from control, vehicle-treated cells.

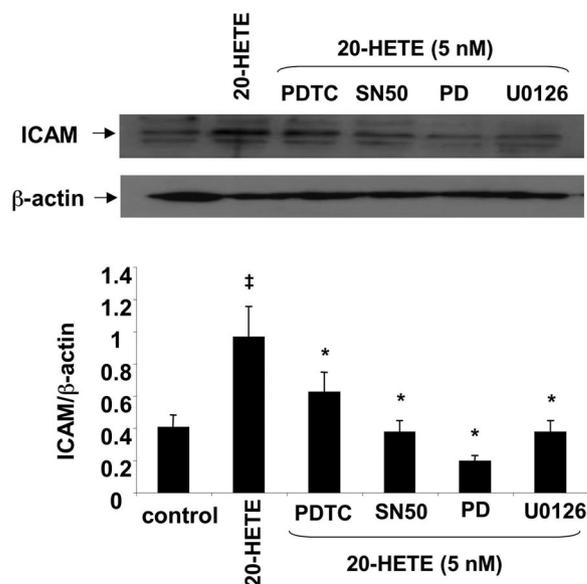
sure the degree of oxidative stress. Cells overexpressing CYP4A1 or CYP4A2 produced large amounts of 20-HETE, and they expressed higher levels of 3-nitrotyrosine. Likewise, addition of 20-HETE to endothelial cells greatly stimulated the production of 8-isoP, a frequently used lipid biomarker for oxidative stress (Montuschi et al., 2004). The mechanisms by which 20-HETE increased oxidative stress are largely unknown. Recent reports have suggested that increased expression of components of the NADPH oxidase system underlies the mechanisms by which CYP4A overexpression leads to increased vascular production of superoxide (Wang et al., 2006; Singh et al., 2007). This type of regulation may not apply to the rapid generation of superoxide following addition of 20-HETE to endothelial cells, as demonstrated by Guo et al. (2007). These investigators reported that 20-HETE-stimulated superoxide production in endothelial cells is mediated by mechanisms other than NADPH oxidase. Conversely, the rapid increases in superoxide levels in response to 20-HETE may be the consequence of eNOS uncoupling (Wang et al., 2006). Taken together, the results of this study and published reports to date implicate the CYP4A-20-HETE pathway in the development of oxidative stress. It is noteworthy that there are two reports (Ward et al., 2005; Barden et al., 2007) demonstrating a direct correlation between urinary levels of isoprostane and 20-HETE in hypertensive hu-



**Fig. 7.** A, concentration- and time-dependent phosphorylation of ERK1/2 MAP kinase by 20-HETE in endothelial cells. Cells were incubated with 0.1 to 10 nM 20-HETE for the indicated times and immunoblotting was performed with antibodies against phosphorylated and nonphosphorylated ERK1/2 (p42/p44). Representative Western blots and densitometry analysis are given. Results are the mean  $\pm$  S.E. ( $n = 3$ ). \*,  $p < 0.05$  versus the respective control; M, kilodalton markers. B, inhibition of 20-HETE-induced NF- $\kappa$ B translocation by MAPK/ERK inhibitors. Cells were incubated with 10  $\mu$ M PD98059 and 10  $\mu$ M U0126 15 min before the addition of 5 nM 20-HETE for an additional 30 min. Cells were processed for measurements of nuclear NF- $\kappa$ B by immunofluorescence. Results are expressed as the percentage of nuclear NF- $\kappa$ B-positive cells to total cells, and values are mean  $\pm$  S.E. ( $n = 4$ ). \*,  $p < 0.05$  from control; ‡,  $p < 0.05$  from 20-HETE-treated cells.

man subjects and as a function of alcohol consumption in healthy individuals. These authors concluded that increased oxidative stress and 20-HETE production may be linked, at least in part, to the pathogenesis of hypertension and alcohol-related hypertension.

The hallmark of endothelial cell activation is the increased production of adhesion molecules and cytokines that culminate with increased adhesion of leukocytes and the transformation of the endothelium to an inflammatory phenotype. The initial indication that 20-HETE may cause endothelial activation came from the demonstration that CYP4A overexpression increased levels of the adhesion molecule ICAM and that addition of 20-HETE at concentrations as low as 1 nM markedly increased production of the proinflammatory lipid mediator PGE<sub>2</sub> and stimulated the expression levels of distinct inflammatory cytokines by many fold, most prominently, IL-8 and IL-13. NF- $\kappa$ B is a ubiquitous transcription factor and a key regulator of the transcription of a number of



**Fig. 8.** Inhibition of 20-HETE-induced ICAM expression by NF- $\kappa$ B and MAPK/ERK inhibitors. Cells were treated with 5 nM 20-HETE with and without 100  $\mu$ M PDTC, 5  $\mu$ M SN50, 10  $\mu$ M PD98059 (PD), and 10  $\mu$ M U0126 for 24 h. ICAM protein levels were determined by Western blot and densitometry analysis. Results are mean  $\pm$  S.E. ( $n = 3$ ). ‡,  $p < 0.05$  from control; \*,  $p < 0.05$  from 20-HETE-treated cells.

proinflammatory genes, including those that lead to the expression of ICAM and IL-8 (Karin, 1999; Monaco and Paleolog, 2004; Häcker and Karin, 2006). Its activation constitutes a central mechanism for endothelial cell activation (Kinlay et al., 2001); therefore, it was considered to be a likely candidate for the transduction of bioactions of 20-HETE in the endothelium. Here, we showed for the first time that 20-HETE potently and rapidly induced NF- $\kappa$ B nuclear translocation in endothelial cells. 20-HETE-induced NF- $\kappa$ B nuclear translocation was preceded by a rapid phosphorylation of the inhibitory protein I $\kappa$ B $\alpha$ . The effect of 20-HETE was evident at concentrations as low as 0.1 nM, which suggests that it differs from the effects of other fatty acids on NF- $\kappa$ B activation (Hennig et al., 2000). It also indicates potency and relevancy because such concentrations are easily detected in vascular beds, including the renal bed (Marji et al., 2002; Singh et al., 2007) and cerebral bed (Harder et al., 1994). It is noteworthy that inhibition of NF- $\kappa$ B activation significantly attenuated 20-HETE-induced ICAM expression, suggesting that NF- $\kappa$ B activation underlies, in part, the mechanism by which 20-HETE stimulates the expression of this adhesion molecule and probably the expression of other inflammatory molecules (e.g., IL-8) that are under the control of NF- $\kappa$ B (Karin and Delhase, 2000).

The proximal signaling mechanisms of 20-HETE-induced activation of NF- $\kappa$ B may include generation of reactive oxygen species (ROS)/superoxide. It is well established that NF- $\kappa$ B is a redox-sensitive transcription factor (Kabe et al., 2005), and recent studies suggest that the NADPH oxidase system may be a proximal circuit for its activation (Anrather et al., 2006). Findings in this study clearly indicate that either up-regulation of the CYP4A-20-HETE system or addition of 20-HETE increase oxidative stress indices such as isoprostanes and 3-nitrosylated proteins. Other studies demonstrated that increased expression of the CYP4A-20-HETE system increases vascular levels of components of the

NADPH oxidase system, including gp91 and p47<sup>phox</sup> and the production of superoxide anion (Wang et al., 2006; Singh et al., 2007). Hence, superoxide anion may act as the mediator of 20-HETE-induced NF- $\kappa$ B activation. Conversely, NF- $\kappa$ B has been shown to induce the expression of gp91, and the existence of a positive feedback loop in which NF- $\kappa$ B activation by oxidative stress leads to further radical production via NADPH oxidase has been postulated (Anrather et al., 2006). 20-HETE may be the driving force of such an amplification process to maintain the state of endothelial activation. To this end, CYP2C9-derived ROS seem to be responsible for the increase in NF- $\kappa$ B activity and the subsequent induction of vascular cell adhesion molecule-1 in CYP2C9-expressing endothelial cells (Fleming et al., 2001).

We also showed that 20-HETE rapidly and potently stimulated phosphorylation of ERK1/2. Moreover, inhibition of ERK1/2 phosphorylation attenuated 20-HETE-induced ICAM expression, suggesting that the MAPK/ERK kinase pathway serves as a signaling circuit for actions of 20-HETE. 20-HETE has been shown to mediate norepinephrine-, angiotensin II-, and epidermal growth factor-induced mitogenic effects in smooth muscle cells (Muthalif et al., 1998, 2000a,b) and platelet-derived growth factor-induced migration (Stec et al., 2007) of smooth muscle cells by increasing MAPK/ERK kinase activity. In human endothelial cells, the MAPK/ERK pathway has been shown to mediate 20-HETE-induced vascular endothelial growth factor expression and cell proliferation (Guo et al., 2007). NF- $\kappa$ B has also been reported to be a downstream target of the MAPK/ERK pathway (Zhao and Lee, 1999; Kang et al., 2006). Consistent with these reports, our data showed that the specific inhibitor of MAPK/ERK pathway U0126 suppressed NF- $\kappa$ B activation in 20-HETE-stimulated endothelial cells, indicating that the MAPK/ERK pathway is important for NF- $\kappa$ B activation in our system. The finding that inhibitors of NF- $\kappa$ B activation as well as inhibitors of MAPK/ERK pathways inhibited 20-HETE-induced ICAM expression suggests that the MAPK/ERK is upstream of the NF- $\kappa$ B pathway. Moreover, that both pathways are regulated/activated by ROS (Thannickal and Fanburg, 2000) and that 20-HETE stimulates superoxide production suggest that ROS may be a common cellular messenger for the 20-HETE signaling pathways in endothelial cells. However, further study is required to substantiate this speculation.

In summary, NF- $\kappa$ B activation and MAPK phosphorylation are major signaling pathways involved in the transcriptional activation of inflammatory genes, and they may constitute the major cellular mechanisms by which overexpression of CYP4A and/or overproduction of 20-HETE bring about endothelial dysfunction and activation.

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