Arachidonic Acid Epoxide Metabolites Stimulate Endothelial Cell Growth and Angiogenesis via Mitogen-Activated Protein Kinase and Phosphatidylinositol 3-Kinase/Akt Signaling Pathways

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ABSTRACT
Cytochrome P450 arachidonic acid (AA) epoxide metabolites, the epoxyeicosatrienoic acids (EETs), dilate arteries via hyperpolarization of smooth muscle cells and also have non-vasodilatory effects within the vasculature. The present study investigated the angiogenic effects of endogenous and exogenous EETs and the relevant signaling mechanisms involved. Bovine aortic endothelial cells (BAECs) were incubated with synthetic EETs or infected with recombinant adeno-associated viruses (rAAVs) containing CYP2C11-NADPH-cytochrome P450 oxidoreductase (CYPOR), CYP2J2, or CYP102 F87V mutant to increase endogenous levels of EETs. The following endpoints were measured: BAEC proliferation, migration, capillary formation, and in vivo angiogenesis. The effects of EETs on proliferation, migration, and capillary tubule formation were attenuated by inhibitors of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 (PI3)-kinase/Akt pathways and partially attenuated by an endothelial nitric-oxide synthase (eNOS) inhibitor but not by a protein kinase C inhibitor. In a rat ischemic hind limb model, rAAV-mediated AA epoxygenase transfection induced angiogenesis. We conclude that AA epoxygenase metabolites can promote angiogenesis, which may provide protection to ischemic tissues. The results also suggest that the angiogenic effects of EETs involve the MAPK and PI3-kinase/Akt signaling pathways, and to some extent, the eNOS pathway.

Arachidonic acid (AA) is esterified to cell membrane glycerophospholipids and liberated by phospholipase A₂ in response to various stimuli. AA can be activated by three different enzyme pathways: the cyclooxygenase, the lipoxygenase, and the cytochrome P450 (P450) monoxygenase.
Epoxygenases and Angiogenesis

pathways (Müller, 1991). Whereas the cyclooxygenase and lipoxygenase pathways have been extensively studied, much less is known about the P450 pathway. Metabolism of AA via the P450 pathway produces two major groups of metabolites: the epoxyeicosatrienoic acids (EETs) formed by P450 epoxygenases and ω-terminal hydroxyeicosatetraenoic acids formed by P450 ω-oxidases (Campbell et al., 1991). P450 epoxygenases produce four different EET regioisomers; 5,6-, 8,9-, 11,12-, and 14,15-EET.

Over the last decade, accumulating evidence has suggested that EETs play crucial and diverse roles in cardiovascular homeostasis (Node et al., 1999; Fleming, 2001; Chen et al., 2003). EETs activate vascular smooth muscle cell large conductance Ca2+-activated K+ channels leading to hyperpolarization of the resting membrane potential and resulting in vasorelaxation and lowering of blood pressure. Indeed, 11,12-EET has been proposed to be identical to endothelium-derived hyperpolarizing factor (Baurersachs et al., 1997; Bolz et al., 2000; Matoba et al., 2000, 2002, 2003; Hamilton et al., 2001; Lacza et al., 2002; Matoba and Shimokawa, 2003; Miura et al., 2003; Morikawa et al., 2003; Tanaka et al., 2003; Yada et al., 2003). Exogenous application of EETs inhibits vascular smooth muscle cell migration, platelet aggregation, nuclear factor-κB activation, and vascular cell adhesion molecule-1 expression (Node et al., 1999; Fleming et al., 2001; Sun et al., 2002; Krotz et al., 2004), suggesting an overall beneficial role for EETs within the vasculature and a protective role in the development of atherosclerosis.

Endothelial cells play a central role in the cardiovascular system through regulation of blood circulation and fluidity, vascular tone, coagulation, inflammatory responses, and angiogenesis. The role of EETs in mediating endothelial cell functions has been a subject of particular interest in the cardiovascular field within the last decade. Addition of EETs or overexpression of the AA epoxygenase CYP2J2 in endothelial cells decreased cytokine-induced endothelial cell adhesion molecule expression and prevented leukocyte adhesion to the vascular wall via inhibition of nuclear factor-κB and inhibitor κB kinase. These studies also demonstrated that these effects of EETs were independent of their membrane-hyperpolarizing effects, suggesting an important non-vasodilatory role for EETs within the vasculature (Node et al., 1999). We previously demonstrated that EETs or overexpression of AA epoxygenases significantly up-regulated endothelial nitric-oxide synthase (eNOS) expression and enhanced eNOS phosphorylation through activation of extra-cellular signal-regulated kinase (ERK) and protein kinase C (PKC) pathways (Wang et al., 2003b). Furthermore, we found that transfection of three different epoxygenase cDNAs protected endothelial cells from apoptosis induced by tumor necrosis factor-α, an effect that was related to activation of the ERK and PI3-kinase/Akt signaling pathways, but independent of NO production in endothelial cells (Wang et al., 2002).

Angiogenesis is a neovascularization process that is essential for the successful repair of wounds and tissues damaged by ischemia and is also important for tumor growth and metastasis. Endothelial cells play an important role in angiogenesis, but the role that epoxygenases and EETs play in this process remains enigmatic. Hence, the purpose of the present study was to investigate whether addition of synthetic EETs and/or overexpression of AA epoxygenase cDNAs effects endothelial proliferation, migration of vascular endothelial cells, and neovascularization and to begin to investigate the signaling mechanisms involved.

Materials and Methods

Experimental Reagents. All cell culture reagents were obtained from Invitrogen (Carlsbad, CA), including Dulbecco’s modified Eagle’s medium (DMEM), trypsin and fetal bovine serum (FBS). PD98059, apigenin, and H-7 were supplied by Calbiochem Novabiochem (Darmstadt, Germany). Rabbit anti-PI3-kinase antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit anti-ERK1/2 (also called p42/44 MAPK) and anti-phospho-ERK1/2 antibodies were purchased from New England Biolabs (Beverly, MA). Anti-CYP2C11 and anti-CYP102 antibodies were a generous gift from Dr. Jorge Capdevila (Vanderbilt University). Anti-CYP2J2 antibodies were prepared as described previously (Wu et al., 1996). Enhanced chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate) was purchased from Pierce Chemical (Rockford, IL). Hybriol solution was purchased from Intergen (Purchase, NY). Polyvinylidene difluoride and nylon membranes were purchased from Schleicher & Schuell (Dassel, Germany). Rabbit polyclonal antibodies specific for epidermal growth factor receptor (EGFR) and phosphorylated EGFR were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Vascular endothelial growth factor was from Collaborative Research (Bedford, MA; 8.9, 11,12-EET, 14,15-EET, HEPES, PD98059, LY294002, Tween 20, phenylmethylsulfonyl fluoride, aprotinin, 17-octadecynoic acid (17-ODYA), Nω-methyl-1-arginine, 2-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl tetrazolium bromide (MTT), and collagen IV were obtained from Sigma-Aldrich (St. Louis, MO). Matrigel was purchased from B&D Biosciences (Heidelberg, Germany). All other chemicals and reagents were purchased from Sigma-Aldrich unless otherwise specified.

Construction and Preparation of Recombinant Adeno-Associated Virus. The recombinant adeno-associated virus (rAAV) vector pXXUF1, packaging plasmid pXXv, adeno-virus helper plasmid pXX6, and a rAAV plasmid containing the GFP cDNA (GFP-pUF1) were from Dr. Xiao Xiao (University of Pittsburgh, Pittsburgh, PA). The CYP102 F87V mutant cDNA and a cDNA encoding rat CYP2C11 fused to rat NADPH-cytochrome P450 reductase (CYPOR) (Helvig and Capdevila, 2000) were kindly provided by Dr. Jorge Capdevila. The CYP2J2 cDNA was from Dr. Darryl Zeldin (National Institute of Environmental Health Sciences, Research Triangle Park, NC). These epoxygenase cDNAs or GFP were subcloned into the rAAV vector pXXUF1 downstream of the cytomegalovirus promoter and rAAV-CYP102 F87V, rAAV-CYP2C11-CYPOR, rAAV-CYP2J2, and rAAV-GFP were packed in human 293 cells (American Type Culture Collection, Manassas, VA) and purified by a single-step gravity-flow column purification method as described previously (Xiao et al., 1996; Xiao et al., 1998; Wang et al., 2003b). Purified rAAV viruses were titered using a dot blot method (Wang et al., 2004).

Isolation and Culture of Endothelial Cells. BAECs were isolated and cultured as described previously (Wang et al., 2003b). Briefly, fresh bovine thoracic aortas were obtained from a local slaughterhouse, and BAECs were harvested using trypsin (0.25%) and grown to confluence in a growth medium containing DMEM supplemented with 5 mM 1-glutamine, 10% FBS, and an antibiotic mixture of 100 units/ml penicillin and 100 μg/ml streptomycin. Purity of the BAEC preparation was determined by cell morphology using phase-contrast microscopy and by immunofluorescent staining for CD31. All passages were performed using 0.05% trypsin and 0.02% EDTA. Only the cells passaged less than five times were used for experiments.

Protein Extraction and Western Blotting. BAEC protein was extracted as described previously (Wang et al., 2003b). Briefly, the
Fig. 1. EETs and AA epoxygenase transfection promote proliferation of BAECs. A and C, BAECs incubated with individual EETs (50 nM) and the AA epoxygenase inhibitor 17-ODYA (10 μM) in the presence or absence of the eNOS inhibitor L-NMMA (100 μM) for 24 h. VEGF (1 μg/ml) was used as a positive control. A and C represent results from direct cell counts and MTT assays, respectively. B and D, BAECs stimulated with different concentrations of individual EETs (10, 50, and 250 nM) for 24 h to show dose-dependent effects on cell proliferation. B and D represent the results of direct cell counts and MTT assays, respectively. E, Western blots showing AA epoxygenase levels in BAECs infected with rAAV-CYP102 F87V, 524 Wang et al. at ASPET Journals on December 23, 2017 jpet.aspetjournals.org Downloaded from
media in six-well plate was discarded, and cells were gently washed three times with cooled PBS. Lysis buffer (500 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% Nonidet P-40, and 0.5% sodium deoxycholate) was added to the cells (0.25 ml/well). After incubation on ice for 30 min, the lysate was centrifuged at 12,000g at 4°C for 10 min. The protein concentration of the supernatant was determined using the Bradford method. Lysates (25 μg protein/lane) were resolved by SDS-polyacrylamide gel electrophoresis (12%), transferred to nitrocellulose membranes, and blocked with 5% nonfat dry milk in 10 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20. The membranes were then incubated with the primary rabbit antibody (1:150 dilution) overnight at 4°C, followed by peroxidase-conjugated secondary antibody for 2 to 3 h. The ECL product was solubilized with dimethyl sulfoxide. Absorbance was measured at 570 nm for each well using a microplate reader (Bio-Tek Instruments, Winooski, VT) according to the manufacturer’s protocol. To confirm a linear relationship between optical density and cell number, we performed correlational analysis. To study the effects of AA epoxygenase overexpression on BAEC proliferation, BAECs were seeded in triplicate 24-well plates (1 × 10^4 cells/well), placed in reduced serum (0.5%) media for 12 h after cell attachment, and then stimulated with different concentrations of EETs (10, 50, and 250 nM) at 0 h and again at 12 h. The second dose at 12 h was added to minimize reduction in levels of EETs due to autooxidation. After 24 h, the cells were washed with PBS, trypsinized, and counted. For the MTT assays, BAECs were seeded in triplicate 96-well plates (1 × 10^4 cells/well) and treated in an identical manner. Twenty-four hours after treatment with EETs, viable cell numbers were estimated by the MTT assay as described previously (Law et al., 1996). Briefly, medium was removed and replaced with medium containing 5 mg/ml MTT and incubated for 4 h. The medium was then aspirated, and the product was solubilized with dimethyl sulfoxide. Absorbance was measured at 570 nm for each well using a microplate reader (Bio-Tek Instruments, Winooski, VT) according to the manufacturer’s protocol. To confirm a linear relationship between optical density and cell number, we performed correlational analysis. Flow Cytometry Analysis. To further examine the proliferation-stimulating potential of EETs and AA epoxygenases in endothelial cells, we analyzed cell cycle distribution after treatment with EETs and after transfection with different AA epoxygenases cDNAs. Cells (1.5 × 10^6 cells) were cultured as described above, and 12 h later, they were fixed with 70% ethanol and incubated with 4 mM phosphate-citrate buffer (4 mM citric acid and 192 mM Na2HPO4) for 30 min at room temperature. After centrifugation, cell pellets were resuspended in PBS containing propidium iodide/RNase (10 μg/ml each) and incubated for 20 min at room temperature. Quantification and ratio of sub-G1 DNA content was determined using the CELLQuest program in a FACStar-Plus flow cytometer (BD Biosciences, San Jose, CA).

Cell Migration. Migration was assessed by a cell-wounding assay. Briefly, BAECs were grown to confluence in 60-mm-diameter dishes and synchronized in 0.5% FBS for 6 h. Round cell-free areas were made by abrasion with a sterile pipet tip, and cells were then stimulated with different EETs (100 nM) for 36 h. Cells that migrated into the cell-free area were visualized using a Nikon TE2000 microscope, and cell-free surface area was calculated using a Scion Image Analysis System (NIH Image; Scion Corporation, Frederick, MD). Each experiment was performed in triplicate for each EET, and experiments were repeated four times. Data are presented as a ratio of cell-covered surface area to initial cell-free surface area. In addition, cell migration was assayed using a modified Boyden chamber technique (Transwell analysis). Porous filters (8-μm pores) were coated with type IV collagen on the both sides via passive absorption by incubating with 10 μg/ml collagen in coating buffer for 24 h. Serum-free medium containing individual EETs (100 μM) was added into the lower chamber as a chemoattractant, and cells (1 × 10^4) were plated in the upper chamber and allowed to migrate for 24 h. Nonmigrating cells were removed from the upper chamber with a cotton swab, filters were stained with Diff-Quik stain, and migrating cells adherent to the underside of the filter were enumerated using an ocular micrometer and by counting a minimum of 10 high-powered fields (HPFs). Data are presented as relative migration (number of cells/HPF) and represent mean ± standard error of quadruplicate experiments.

Tube Formation. In vitro formation of capillary-like tube structures was examined on Matrigel. Matrigel (0.5 ml) was polymerized on 24-well plates, and 5 × 10^4 cells were then plated in full-growth media for 1 h. Once the cells were seeded, the media was replaced with media containing 0.5% serum with or without individual EETs. Tube formation was visualized using an inverted microscope (Nikon TE2000) equipped with digital imaging. For each treatment, 10 HPF images were captured, and the area of endothelial tubes and networks formed was quantified using the Scion Image analysis system with background subtraction. To examine effects of AA epoxygenase transfection on tube formation, BAECs were first infected with different rAAV viruses, and 4 days later, they were plated in 24-well plates with Matrigel followed by tube formation analysis as described above.

Chicken Embryo Chorioallantoic Membrane Assay. Fertilized chicken eggs were incubated at 37°C in an 80% humidified atmosphere. On day 6 of development, a window was made in the eggshell on the large side of the egg, and a small piece (2 × 2 mm²) of nitrocellulose membrane containing rAAV (5 × 10^9 virions/membrane) was put on the CAM and then the window was sealed with sterile plastic tape. Incubation of the eggs continued for 9 days, after which the tape was removed and the CAM around nitrocellulose membrane was fixed in 4% paraformaldehyde for 30 min at room temperature. The area containing the nitrocellulose membrane was then removed for further analysis. Photos of each CAM were taken under a stereomicroscope (Nikon SMZ800) using a digital camera (Nikon Coolpix 950). Two observers quantified the small vessels (first- and second-order). The result was taken as the mean number from the two observers. A minimum of six eggs was used for each treatment, and the experiments were repeated at least twice.

Evaluation of Signaling Pathways. To examine the signaling mechanisms through which EETs and AA epoxygenases enhanced endothelial cell proliferation, migration, and angiogenesis, inhibitors of ERK (also called MAPK) (apigenin), MEK (PD98059), PI3-kinase (LY294002), PKC (H-7), eNOS (l-NMMa), and AA epoxygenases (17-ODYA) were added to cultured BAECs, and their effects on cell proliferation, cell cycle, cell migration, and tube formation were observed.

In Vivo Study in Rat Ischemic Hind Limb Model. This study was approved by the Institutional Animal Research Committee of Tongji Medical Center. Animals were cared for according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For these experiments, thirty 12-week-old male normotensive Wistar rats were used. The right femoral arteries were

rAAV-CYP2C11-CYPOR, rAAV-CYP2J2, and rAAV-GFP (50 virions/cell) for 1 week. F, MTT assay results 7 days after transfection with AA epoxygenases or GFP in the presence or absence of the epoxygenase inhibitor 17-ODYA (10 μM). G, standard curve showing linear relationship between cell number and optical density values in the MTT assay. Each experiment was performed at least in triplicate. *, p < 0.05 versus control group. #, p < 0.05 versus no l-NMMa or no 17-ODYA group.
Fig. 2. EETs and AA epoxygenase transfection promote endothelial cell proliferation via PI3-kinase, MAPK, and EGFR signaling pathways, but not via PKC. A and B, MTT assays showing effects of treatment with inhibitors of ERK or MAPK (apigenin, 25 μM), PKC (H-7, 12 μM), PI3-kinase (LY294002, 15 μM), and MEK (PD98059, 20 μM) on EET- (50 nM) or AA epoxygenase-induced BAEC proliferation. C–E, effects of EETs (50 nM) or AA epoxygenase inhibitor (17-ODYA, 10 μM) on levels of phospho-ERK, PI3-kinase, and phospho-EGFR, respectively, in BAECs. Top, representative blots; bottom, densitometric analysis. Blots represent three separate experiments. F, effects of eNOS inhibitor L-NMMA (100 μM) on EET-induced increase in PI3-kinase. G, effects of eNOS inhibitor L-NMMA (100 μM) on EET-induced increase in phospho-ERK. Data represent three separate experiments. *, p < 0.05 versus control group. #, p < 0.05 versus no signaling pathway inhibitor group.
occluded using a 3-0 silk suture under pentobarbital anesthesia (50 mg/kg, intraperitoneal injection). The ligature was placed on the femoral artery 0.5 cm proximal to the bifurcation of the saphenous and popliteal arteries. A total volume of 200 μl of saline (containing 3 × 10^11 virions of rAAV-CYP2J2, rAAV-CYP102 F87V, rAAV-CYP2C11-CYPOR, or rAAV-GFP) was injected at four to five sites in the adductor and surrounding muscles 6 days after surgery. Six weeks after gene delivery, microangiographic analysis was completed by assessment of capillary density as described previously by Silvestre et al. (2000). Briefly animals were sacrificed under anesthesia, and their hind limb skeletal muscles were excised and fixed in formalin. After paraffin embedding, 3-μm-thick sections were cut from each sample with the muscle fibers were oriented in a transverse direction and immunostained with an antibody specific for platelet endothelial cell adhesion molecule. The number of capillaries was counted at 400× magnification (mean number of capillaries per square millimeter) in 20 randomly chosen fields in a blinded manner. To ensure that analysis of capillary density was not overestimated due to muscle atrophy, capillary density was also evaluated as a function of the number of muscle fibers in the histological section (capillary-to-fiber ratio).

**Statistical Analysis.** Continuous data were expressed as mean ± S.E. Comparisons between groups were performed by a Student’s paired two-tailed t test. Two-way analysis of variance was used to examine differences in response to treatments and between groups, with post hoc analyses performed by Student-Newman-Keuls method. p values of <0.05 were considered statistically significant.

**Results**

**Effects of EETs and AA Epoxide Transfection on BAEC Proliferation.** To determine the effects of AA epoxide products on BAEC proliferation, a necessary process for angiogenesis, we conducted experiments involving either direct addition of synthetic EETs or production of endogenous EETs via overexpression of AA epoxygenases and then determined proliferative effects using cell counting.

![Flow cytometry analysis showing that EETs and AA epoxide transfection enhance cell cycle progression in BAECs](image-url)

Fig. 3. Flow cytometry analysis showing that EETs and AA epoxide transfection enhance cell cycle progression in BAECs. A and C, representative histograms of flow cytometry analysis for rAAV-mediated AA epoxide gene-transfected BAECs and EET-treated (50 nM) BAECs, respectively. The x- and y-axes represent the intensity of propidium iodide fluorescence and cell number, respectively. B and D, proportion of BAECs in S and G2/M phases from A and B, respectively. Each experiment was performed in triplicate. *p < 0.01 versus control group.
Fig. 4. EETs and AA epoxygenase transfection enhance migration of BAECs. A, BAECs were cultured to confluence and treated with individual EETs (50 nM), VEGF (1 μg/ml), or vehicle in the presence or absence of L-NMMA (100 μM), and the monolayer was then wounded by means of a pipet tip. Cellular wounding healing was observed under a microscope. B, summary of cellular wound healing results showing the ratio of cell-covered surface area to initial cell-free area (AS/SS). C, BAECs stimulated with different concentrations of individual EETs (10, 50, and 250 nM) to show dose-dependent effects on wound healing. D, transfection with AA epoxygenases enhanced cellular wound healing of BAECs, an effect that was inhibited by incubation with 17-ODYA (10 μM). E and F, effects of EETs (100 nM), 17-ODYA (10 μM), and VEGF (1 μg/ml) in the presence or absence of L-NMMA (100 μM) on BAEC migration through the membrane filters in a Transwell analysis. E, representative photomicrograph of filters. F, migrated cell number per HPF from five individual experiments. G, dose-dependent increase in the number of migrated BAECs through the membrane filter in response to treatment with different concentrations of EETs (10, 50, and 250 nM) for 24 h. H, effects of inhibitors of MAPK or ERK (apigenin, 25 μM), PKC (H-7, 12 μM), PI3-kinase (LY294002, 15 μM), and MEK (PD98059, 20 μM) on EET enhancement of wound healing in cellular wound assay. I, effects of signaling pathway inhibitors on EET enhancement of migration of BAECs through membrane filter. Original magnification, 40× (A), 200× (E). * p < 0.05 versus control group. #, p < 0.05 versus no L-NMMA, no 17-ODYA, or no signaling pathway inhibitor group.
and MTT viability assays. Since EETs and overexpression of AA epoxygenases up-regulate eNOS at the mRNA, protein, and enzyme activity levels in BAECs (Wang et al., 2003b), we also stimulated BAECs with EETs in the presence or absence of l-NMMA to study whether NO was involved in the effect of EETs on BAEC proliferation. Addition of individual EETs significantly promoted proliferation of endothelial cells as assessed by cell counts, and this effect was significantly inhibited by l-NMMA (Fig. 1A). Furthermore, the effects of EETs occurred at concentrations as low as 10 nM and were concentration-dependent up to 250 nM (Fig. 1B). MTT assays gave similar results (Fig. 1, C and D). Addition of the AA epoxygenase inhibitor 17-ODYA (10 μM) into the cultures significantly inhibited BAEC proliferation, measured by both cell counts (Fig. 1A) and MTT assay (Fig. 1C), lending further support to the concept that AA epoxygenase products promote proliferation of endothelial cells.

Transfection of BAECs with rAAV-CYP102 F87V, rAAV-CYP2C11-CYPOR, or rAAV-CYP2J2 resulted in expression of the corresponding epoxygenase proteins (Fig. 1E) and significantly enhanced BAEC proliferation compared with transfection with the control rAAV-GFP (Fig. 1F). The proliferative effects of AA epoxygenase transfection were significantly inhibited by addition of 17-ODYA (Fig. 1F). To verify the reliability of the MTT assay, we examined the relationship between cell count and optical density values; results demonstrated that these parameters were linearly related (Fig. 1G).

To investigate the signaling mechanisms through which EETs and AA epoxygenases promote proliferation of endothelial cells, we applied various signaling pathway inhibitors to BAEC cultures in the presence of EETs or after transfection with AA epoxygenases. Inhibitors of ERK or MAPK (apigenin), MEK (PD90859), and PI3-kinase (LY294002) inhibited proliferation induced by EETs and by AA epoxygenase transfection, whereas the PKC inhibitor H-7 did not (Fig. 2, A and B). This suggests the involvement of the MAPK and PI3-kinase/Akt signaling pathways in EET-mediated proliferation of BAECs. Immunoblot analysis revealed that EETs significantly increased phosphorylation of ERK (Fig. 2C) and the level of PI3-kinase (Fig. 2D) and also increased the phosphorylation level of EGFR (Fig. 2E). Treatment with the AA epoxygenase inhibitor 17-ODYA resulted in reduced phosphorylation of ERK, decreased levels of PI3-kinase, and reduced phosphorylation of EGFR (Fig. 2, C–E).

To determine the role of the eNOS pathway in mediating the effects of EETs, we determined levels of phosphorylated ERK and PI3-kinase in BAECs in the presence and absence of l-NMMA. Inhibition of eNOS attenuated the EET-induced increase in PI3-kinase (Fig. 2F), suggesting a role for NO in EET-mediated activation of this pathway. In contrast, inhibition of eNOS had no effect on the EET-induced increase in phosphorylated ERK (Fig. 2G).

**Flow Cytometry Analysis.** Cell cycle analysis by flow cytometry was performed 1 wk after infection of BAECs with rAAV-CYP2J2, rAAV-CYP2C11-CYPOR, rAAV-CYP102 F87V, or rAAV-GFP, or after treatment with synthetic EETs for 12 h. Infection with rAAV-CYP2J2, rAAV-CYP2C11-CYPOR, or rAAV-CYP102 F87V resulted in a significant increase in the proportion of cells in S/G2/M phase (45.67 ± 0.22, 49.17 ± 0.17, and 51.04 ± 1.34%, respectively) compared with noninfected and rAAV-GFP-infected cells (26.83 ± 1.65 and 26.61 ± 0.25%, respectively; p < 0.01) (Fig. 3, A and B). In contrast, infection with rAAV-CYP2J2, rAAV-CYP2C11-CYPOR, or rAAV-CYP102 F87V dramatically reduced the proportion of cells in G0/G1 phase (54.45 ± 0.22, 51.00 ± 0.98, and 49.09 ± 1.35%, respectively) compared with noninfected and rAAV-GFP-infected groups (73.32 ± 1.67 and 73.52 ± 0.26%, respectively; p < 0.01). Treatment with individual EETs produced similar results (Fig. 3, C and D). Combined, these data provide further evidence that P450 epoxygenases and their eicosanoid products promote proliferation of endothelial cells.

**Effect of EETs and AA Epoxygenase Transfection on Migration of BAECs.** Migration of endothelial cells is an important process in angiogenesis and vessel sprouting. We determined the effects of EETs and rAAV-mediated AA epoxygenase gene transfection using wound healing and Boyden chamber assays. In wound healing studies, EETs markedly enhanced BAEC migration, an effect that was significantly attenuated by inhibition of eNOS with l-NMMA (Fig. 4, A and B). The effects of EETs were dose-dependent and occurred at concentrations as low as 10 nM (Fig. 4C). Similarly, infection of rAAV-CYP102 F87V, rAAV-CYP2C11-CYPOR, or rAAV-CYP2J2 stimulated BAEC migration, an effect that was attenuated by the epoxygenase inhibitor 17-ODYA (Fig. 4D). In Transwell Boyden chamber assays, EETs increased cell migration (Fig. 4, E and F), and this effect was concentration-dependent (Fig. 4G). Importantly, addition of l-NMMA also attenuated the EET-induced migration of BAECs in Boyden chamber assays (Fig. 4, E and F).

Similar to their effects on EET-induced BAEC proliferation, inhibitors of ERK or MAPK (apigenin), MEK (PD98059), and PI3-kinase (LY294002) markedly attenuated the EET-stimulated migration of BAECs and delayed wound repair, whereas the PKC inhibitor H-7 did not (Fig. 4, H and I). These data suggest that the ERK and PI3-kinase/Akt pathways are involved in EET-induced BAEC proliferation.
pathways play important roles in EET-stimulated migration of BAECs.

**Effects of EETs and AA Epoxygenase Transfection on Angiogenesis.** The possibility that EETs could promote angiogenesis was assessed using a variety of experimental approaches. First, we used the CAM assay, which allows for investigation of the ongoing angiogenic process in vivo. Infection with rAAV-CYP102 F87V, rAAV-CYP2C11-CYPOR, or rAAV-CYP2J2 dramatically increased capillary formation and novel capillary branch number relative to that observed in noninfected or rAAV-GFP infected groups ($p < 0.01$) (Fig. 5, A and B).

We next examined whether EETs or AA epoxygenase transfection promoted tubule formation. Matrigel tests demonstrated that treatment with EETs significantly increased tubule formation (Fig. 6, A and B) and that this effect was attenuated by inhibitors of ERK or MAPK (apigenin), MEK (PD89059), PI3-kinase (LY294002), and eNOS (L-NMMA), but not PKC (H-7) (Fig. 6C). rAAV-mediated AA epoxygenase gene transfection also increased tubule formation. Importantly, 17-ODYA inhibited tubule formation in this model (Fig. 6D). These data suggest that EETs and AA epoxygenases enhance capillary tubule formation through mechanisms involving the MAPK, PI3-kinase/Akt, and eNOS signaling pathways.

Finally, an ischemic rat hind limb skeletal muscle model was used to determine the effect of AA epoxygenase transfection on angiogenesis in vivo. Skeletal muscle sections were immunostained with an antibody against CD-31 to quantify capillary formation. Muscle capillary density was significantly increased after AA epoxygenase transfection; capillary numbers in rAAV-CYP102 F87V, rAAV-CYP2C11-CYPOR, and rAAV-CYP2J2-infected groups were $1260 \pm 62$, $1096 \pm 53$, and $905 \pm 43/\text{mm}^2$, respectively. In comparison, capillary number in the rAAV-GFP-transfected group was $706 \pm 24/\text{mm}^2$ ($p < 0.01$).

**Discussion**

Mounting evidence supports the concept that EETs possess a variety of activities in the vasculature, in addition to their recognized ability to relax smooth muscle cells via activation of Ca$^{2+}$-sensitive K$^+$ channels. These nonvasodilatory activities affect the function and viability of vascular endothelial cells and include up-regulation of eNOS at the levels of mRNA, protein, posttranslational modification, protection from apoptosis induced by tumor necrosis factor-$\alpha$, and reduction of expression of cytokine-induced endothelial cell adhesion molecules, thereby preventing leukocyte adhesion to the vascular wall (Fisslthaler et al., 1999; Node et al., 1999; Wang et al., 2003a,b). Recently, astrocyte-derived...
EETs were shown to induce cerebral capillary endothelial cell mitogenesis and tube formation (Munzenmaier and Harder, 2000), and adenovirus-mediated CYP2C9 gene transfection and exogenous 14,15-EET were shown to exert similar effects in human lung endothelial cell lines (Medhora et al., 2003). In addition, CYP2C9 overexpression in endothelial cells was shown to induce endothelial tube formation via stimulating cyclooxygenase-2 expression and prostacyclin production (Michaelis et al., 2005). Collectively, these data suggest that P450-derived EETs may represent a class of important endogenous angiogenic factors. In the current study, we used both in vitro and in vivo approaches to demonstrate that both exogenous and endogenously formed EETs enhance endothelial cell proliferation, migration, and capillary tube formation, and stimulate angiogenesis.

Vascular endothelial growth factor (VEGF) is an angiogenic factor that has been successfully used to treat severe myocardial ischemia and occlusive peripheral artery disease (Kliche and Waltenberger, 2001; Bliznakov, 2002). In addition, fibroblast growth factor and NO exert angiogenic effects by increasing proliferation and migration of endothelial cells and capillary tube formation (Cooke, 2003; Penny and Hammond, 2004). NO and endothelium-derived hyperpolarizing factor represent the two major endothelial autacoids involved in the local control of vascular tone (Bauersachs et al., 1997). Importantly, the angiogenic effects of NO have been shown to be mediated by activation of the PI3-kinase/Akt pathway (Kawasaki et al., 2003).

The present study demonstrated that addition of synthetic EETs or overexpression of AA epoxygenases stimulated proliferation and migration of BAECs and angiogenesis, similar to the effects of VEGF. We previously reported that EETs or overexpression of AA epoxygenases up-regulated eNOS at the levels of mRNA, protein, and enzyme activity in BAECs via activation of MAPK (Wang et al., 2003b). To determine whether the effects of EETs and AA epoxygenases observed in the present study were related to up-regulation of eNOS, we examined the influence of the eNOS inhibitor l-NMMA. Addition of l-NMMA to cultures of BAECs attenuated the EET- and AA epoxygenase-induced migration and proliferation of these cells, suggesting that eNOS up-regulation may mediate, at least in part, the angiogenic effects of EETs. We also demonstrated that inhibitors of the PI3-kinase/Akt pathway significantly attenuated the EET-mediated angiogenic effects, consistent with the concept that the PI3-kinase/Akt pathway may mediate the angiogenic effects of NO (Kawasaki et al., 2003).

The present study also revealed that EETs or epoxygenase gene transfection activate the MAPK pathway in BAECs, suggesting that EET-stimulated angiogenic processes may be only partially dependent on NO (Wang et al., 2003b). Our previous studies demonstrated that EETs and AA epoxygenase overexpression enhance phosphorylation of EGFR and activate MAPK in LLC-PK1 pig kidney epithelial cells (Burns et al., 1995; Chen et al., 1999), indicating that EETs function as second messengers of EGFR. Here, we found that EETs induced proliferation and migration of BAECs and promote angiogenesis and that these effects were reduced by inhibitors of MAPK and MEK. Furthermore, both endogenous and exogenous EETs promoted phosphorylation of EGFR. Together, these data indicate that the EET-mediated effects on BAEC proliferation, migration, and angiogenesis likely occur via NO-dependent mechanisms as well as via activation of PI3-kinase/Akt, MAPK, and possibly EGFR pathways as well. Interestingly, a recent study indicated that EETs inhibit vascular smooth muscle cell migration but not proliferation and that this effect is mediated by an EET-induced increase in intracellular cAMP and activation of the PKA signaling pathway (Sun et al., 2002). The differential response to EETs in vascular smooth muscle cells and endothelial cells may be due to tissue specificity of EET activity. Indeed, cAMP is elevated in the former cell types whereas cGMP is elevated in the latter cell types due to G protein stimulation and activation of MAPK and Akt (Kawasaki et al., 2003).

Together, our data demonstrate that EETs and AA epoxygenase overexpression promote an angiogenic phenotype, including endothelial cell proliferation, migration, and capillary tube formation. All of these processes are requisite steps in new vessel formation. The mechanisms underlying these effects may include activation of the MAPK pathway and of the PI3-kinase/Akt pathway, with NO formation contributing as well. Our observations also suggest the possibil-
ity that AA epoxyenone products may promote development of collateral circulation in ischemic tissues.

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References


