CHARACTERIZATION OF 5,6- AND 8,9-EPOXYEICOSATRIENOIC ACIDS (5,6- AND 8,9-EET) AS POTENT IN VIVO ANGIogenic LIPIDS *

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Running Title: 5,6- and 8,9-EET are angiogenic lipids

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The cytochrome P450 arachidonic acid epoxygenase metabolites, the epoxyeicosatrienoic acids (EETs) are powerful, non regio-selective, stimulators of cell proliferation. In this study we compared the ability of the four EETs (5,6-, 8,9-, 11,12- and 14,15-EETs) to regulate endothelial cell proliferation in vitro and angiogenesis in vivo and determined the molecular mechanism by which EETs control these events. Inhibition of the epoxygenase blocked serum induced endothelial cell proliferation, and exogenously added EETs rescued cell proliferation from epoxygenase inhibition. Studies with selective ERK, p38 MAPK, or PI3K inhibitors revealed that while activation of p38 MAPK is required for the proliferative responses to 8,9- and 11,12-EET, activation of PI3K is necessary for the cell proliferation induced by 5,6- and 14,15-EET.

Among the four EETs, only 5,6- and 8,9-EET are capable of promoting endothelial cell migration and the formation of capillary like structures, events that are dependent on EET mediated activation of ERK and PI3K. Using subcutaneous sponge models, we showed that 5,6- and 8,9-EET are pro-angiogenic in mice and that their neo-vascularization effects are enhanced by the co-administration of an inhibitor of EET enzymatic hydration, presumably due to reduced EET metabolism and inactivation.

These studies identify 5,6- and 8,9-EET as powerful and selective angiogenic lipids; provide a functional link between the EET proliferative, chemotactic properties and their angiogenic activity, and suggest a physiological role for them in angiogenesis and de novo vascularization.

Interest in the biochemical mechanism of angiogenesis, the de novo formation of blood vessels from pre-existing vessels, stems from the critical roles played by this process in the pathophysiology of inflammation, cancer, and cardiovascular diseases. Neo-vascularization requires the coordinated contributions of endothelial cell proliferation and migration leading to the formation of nascent capillary structures and finally, to new functional vessels. The eicosanoids prostaglandin E₂, prostacyclin (1,2), HETEs (3-7), and the epoxyeicosatrienoic acids (EET) (8-11) have been characterized as pro-angiogenic molecules. In addition, the roles of cyclooxygenases 1 and 2, as well as cytochrome P450 (P450) 4A and 2C isoforms in these processes have been suggested (4-11). However, the individual and/or combined contribution of these lipids to in vivo
angiogenic responses remains to be determined.

The P450 arachidonic acid epoxygenases catalyze the metabolism of endogenous pools of arachidonic acid to 5,6-, 8,9-, 11,12-, and 14,15-EET (12-17), a reaction catalyzed predominantly by members of the P450 CYP2C gene subfamily (12). Furthermore, CYP2C8, CYP2C23, and Cyp2c44 have been identified as predominant stereoselective epoxygenases in several human, rat, and mouse organ tissues (12,13,17,18). Since the original description of the mitogenic properties of 14,15-EET (12,19), several studies have characterized 11,12- and 14,15-EET as powerful mitogens using cultured cells derived from kidney, brain, and endothelium (4,8-12,18-21), and a role for 14,15-EET in mediating the mitogenic responses to EGF and HB-EGF has been thoroughly documented in cultured LLCPk cells (20,21). More recently, 11,12-EET was identified as an angiogenic molecule, and in vitro roles for cyclooxygenase-2 and EGF receptor identified (10,11). Furthermore 11,12-EET has also been shown to activate PI3K and tyrosine kinases (10,22). However, only a few studies have addressed whether activation of these downstream pathways are indeed required to mediate these EET-induced cell responses. While the mitogenic activity of 11,12- and 14,15- EET, and the signaling pathways for 14,15-EET are well documented, less is known regarding the proliferative and/or angiogenic properties of 5,6-EET and 8,9-EET and their potential mechanisms of action. The biosynthesis of 8,9-EET from endogenous fatty acid pools is well documented (12), and that of the labile 5,6-EET has been inferred from the identification of its hydration product, 5,6-dihydroxyeicosatrienoic acid (5,6-DHET), in several biological samples (12,17). Furthermore, these EETs are known to circulate, associated with plasma lipoproteins, in rat and human blood (12).

We report here i) the characterization of 5,6-and 8,9-EET as powerful mitogens, and selective chemotactic lipids for primary cultures of mouse pulmonary endothelial cells; ii) the identification of the intracellular signaling pathways associated with their proliferative and chemotactic activities; and iii) the demonstration of a role for these two EETs in promoting de novo angiogenesis in vivo. The observation that inhibition of enzymatic EET hydration potentiates the angiogenic activities of 5,6-and 8,9-EET suggests that inhibitors of epoxide hydrolase(s) could serve to promote angiogenesis in pathophysiological conditions in which de novo vascularization is compromised.

**MATERIALS AND METHODS**

**Synthesis of EETs by endothelial cells.** Pulmonary murine microvascular endothelial cells were isolated and cultured as previously described (23). Cells at early passages (1-3) were used for these studies since the expression of CYP2Cs in cultured endothelial cells decreases with increased cell passage (24). To study cellular EET synthase activity, semi-confluent endothelial cells (passage 3) were cultured in serum free medium in the presence or absence of arachidonic acid (10 µM). After 4 or 24 hours, the cells and the media were removed from the plates, and the suspension centrifuged to obtain a cell pellet and a media containing supernatant. The media was mixed with 10 ng each of [1-14C] labeled DHETs (55 µCi/µmol; 95 atom % enrichment in 14C) or [1-14C] labeled EETs (55 µCi/µmol) and extracted with ethyl acetate. The EETs and DHETs present in the culture media were purified by reversed phase HPLC (25), converted to the corresponding pentafluorobenzylesters (EETs) or
pentafluorobenzylester-trimethylsilylether derivatives and quantified by NICI/GC/MS exactly as described (26). The cell pellets were suspended in PBS, mixed with 10 ng each of \([1^{-13}C]\) labeled EETs and DHETs (55 \(\muCi/\mumol\), and extracted with CHCl\(_3\)/CH\(_3\)OH (2:1). The organic extracts were submitted to alkaline hydrolysis, and EETs and DHETs purified and quantified by NICI/GC/MS exactly as described (26). Prior to use, synthetic EETs and DHETs were purified by reversed phase HPLC (25). The labile 5,6-EET was purified by normal phase HPLC on a \(\mu\)Sorb silica column (250 x 4.6 mm; 5\(\mu\)), using hexane containing 0.5% HOAc and 2% 2-propanol as mobile phase at 2 ml/min.

**Proliferation assays.** Endothelial cells (5x10\(^3\)/96-well plates) were plated in EGM-2-MV (Clonetics) containing 5% FCS with or without different concentrations of synthetic 5,6-, 8,9-, 11,12-, and 14,15-EET or their corresponding DHETs. In some experiments cells were incubated in 5% FCS in the absence or presence of various concentrations of the EET synthase inhibitors \(N\)-methylsulphonyl-6-(2-proparglyoxyphenyl)hexanamide (MS-PPOH) and ketoconazole (17,27), or adamantyl-cyclohexyl-urea (ACU), an inhibitor of cytosolic epoxide hydrolase (17). Two days after, the medium was replaced with fresh media containing \(^{3}H\) thymidine (1 \(\muCi/well\), the cells incubated for another 48 hours, and their levels of \(^{3}H\) thymidine incorporation determined as described (23). In some experiments endothelial cells were plated as above and serum starved for 24 hours. The cells were then cultured in serum free medium containing \(^{3}H\) thymidine (1 \(\muCi/well\)) with or without 5,6-, 8,9-, 11,12-, and 14,15-EET or their corresponding DHETs. Some cells were also treated with 10 \(\muM\) MS-PPOH, ketoconazole or ACU added alone or in combination with each EET (1 \(\muM\), each). Twenty-four hours later cells were collected and proliferation determined as above.

To determine the pathways involved in the EET-induced cell proliferation, 24 hour serum starved cells were incubated in serum free medium containing \([^{3}H]\) thymidine (10 \(\muCi/ml\)) with or without the individual EET regioisomers (1 \(\muM\)), and in the presence or absence of either a MEK1 inhibitor (PD98059) (10 \(\muM\), a P38 MAPK inhibitor (10 \(\muM\) (cat. number 506126) or a PI3K inhibitor (Wortmannin) (0.1 \(\muM\)) (all from Calbiochem). After 24 hours, the cells were collected and \(^{3}H\) thymidine incorporation was determined as above. At least three independent experiments with quadruplicate samples were performed for each set of experiments described above. The \(^{3}H\)thymidine incorporation assay was also corroborated by manual cell counting (not shown).

**Migration assays.** Cell migration was assayed in transwell plates fitted with 8 \(\mum\) membrane filters (CorningWare). Lower wells were coated by an overnight incubation with collagen type I (10 \(\mug/ml\)) at 4\(^\circ\)C, and then incubated one hour at 37\(^\circ\)C with BSA (1% in PBS) to inhibit non-specific cell migration. Serum-free medium with or without 1 \(\muM\) EETs was then added to the lower wells, and 24 hour serum starved endothelial cells (5x10\(^4\) cells in 300 \(\mul\) serum-free medium containing 0.1 % BSA) to the upper wells. To determine the contribution of ERK, p38 and PI3K in EET-induced migration, serum starved cells were allowed to migrate as indicated above with the difference that PD98059 (10 \(\muM\)), P38 MAPK inhibitor (10 \(\muM\), or wortmannin (100 \(nM\)) were added to both upper and lower wells. After 12 hours at 37\(^\circ\)C, cells on the top of the filter were removed by wiping, and the filters were then fixed in 4% formaldehyde in PBS. Migrating cells were stained with 1% crystal violet, and five randomly chosen fields from triplicate wells were counted at 400\(\times\).
magnification. Three independent experiments were performed in duplicate.

**Matrigel-based capillary formation assay.** Capillary-like formation was analyzed as described (28). Briefly, 96 well plates were coated with 50 µl of Matrigel, and incubated 30 min at 37°C. Serum starved endothelial cells (1 × 10⁴) were plated over solidified Matrigel in 200 µl serum free medium with or without EETs (1 uM). To determine the contribution of EET synthases in capillary-like formation, endothelial cells were plated on Matrigel in 200 µl complete medium in the presence or absence of different concentrations of MS-PPOH or ketoconazole. Capillary-like structures were recorded (3 images per gel per treatment) hourly for a period of 10 hours, and representative images taken 3 hours after plating are shown. To quantify capillary-like network formation, cellular nodes were defined as junctions linking at least three cells, and they were counted from digital images. Four independent experiments were performed with a total of 12 images analyzed per treatment.

**Western blot and RT-PCR analyses.** To evaluate the effects of EETs on ERK, p38 and Akt phosphorylation, semi confluent endothelial cells were serum starved for 24 hours and then treated with the EET (1 µM each) for 0, 10 and 40 minutes. The cells were washed with PBS, collected, suspended in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, and centrifuged for 10 min at 14,000 rpm. Cell lysates were resolved by SDS/PAGE (10% gels; 50 µg total protein/lane) and transferred to Immobilon-P membranes (Millipore, Billerica, MA). Membranes were incubated with a rabbit anti-phospho ERK, anti-phospho p38, or anti-phospho Akt antibody (all from Cell Signaling Technology). Immunoreactive proteins were visualized using a peroxidase-conjugated goat anti-rabbit and an ECL kit (Pierce). Total ERK, p38 and Akt content was verified by stripping the membranes in 50 mM Tris HCl (pH 6.5) containing 2% SDS and 0.4% β-mercaptoethanol for 1 hour at 55°C, and re-probing with a rabbit anti-ERK, anti-p38, or anti-Akt antibody (Cell Signaling Technology). EET synthase expression in endothelial cell lysates (50 µg protein/lane) was analyzed as above using a rabbit anti-CYP2C23 or anti-CYP2C11 (14) antibody, cross reactive against the murine Cyp2c44 and Cyp2c38 EET synthase, respectively. Purified cyp2c44 or cyp2c38 (20ng/lane) was used as positive controls.

Total RNA was purified from primary pulmonary endothelial cells (passage 2) using TRIzol reagent (Invitrogen). RNA samples were reverse-transcribed using a SuperScript II™ kit and oligo(dT) (12-18 bp), and cDNAs amplified using the following mouse Cyp2c isoform specific PCR primers: Cyp2c38 (300 bp) sense, 5'-tttgtgaatggattaattgc-3', antisense, 5'-tgccggtgaagtgtattc-3'; Cyp2c44 (700 bp) sense, 5'-ttggatcctggcctaccgtg -3', antisense, 5'-tgctctgtgctgctgccgtaa-3'. These primers are cDNA specific in that they amplify exons that are separated by intronic sequences in the genomic DNA. The following primers for β-actin (800 bp) were used as positive control: sense, 5'-ccagagcaagagaggtatcctgac -3', anti-sense, 5'-aatctccttctgctgtacctgag -3'.

**Immunofluorescence.** Frozen sections (7 µm each) of tumors derived from human non-small cell lung cancer cells grown subcutaneously for three weeks in athymic nude mice were co-stained with biotinylated rabbit anti-rat CYP2C23 (1:300) and rat anti-mouse CD31 (1:100, Pharmingen) followed by FITC-conjugated streptavidin (1:200 Sigma), RITC-conjugated goat anti-rat IgG (1:200, Jackson) and DAPI (2 ng/ml, Sigma) to visualize cell nuclei. Fluorescence emissions were with an epifluorescence microscope equipped with a triple filter channel.
In vivo angiogenesis. The subcutaneous sponge model was used to determine the effects of EETs on in vivo angiogenesis (29). Sterile polyvinyl acetal CF-50 round sponges (8 x 3 mm, a gift from Dr. J.M. Davidson, Vanderbilt University) were implanted under the dorsal skin of 129 Sv/J male mice (6 weeks of age, 20 gr body weight, n=5/treatment). The sponges were then injected every second day for 14 days with 50 µl of either vehicle (corn oil), EETs (50 µM), ACU (250 µM) or a mixture of EET and ACU (50 and 250 µM, respectively). Ten minutes before sacrifice, mice were injected intravenously with 50 µl Rhodamine-Dextran (M_r 65, 2% in PBS, Sigma) to label blood vessels (30), and the sponges were subsequently collected and analyzed under an epifluorescence microscope. Rhodamine-Dextran positive structures were imaged, the color images converted to black and white pictures using Photoshop (Adobe) and processed as described (23). Vascularity within sponges was expressed as a percentage of area occupied by Rhodamine-Dextran positive structures per microscopic field. Three images/sponge with a total of 15 images per treatment were used for analysis.

Statistical analysis. We used the t-test for comparisons between two groups, and analysis of variance using Sigma-Stat software for statistical differences between multiple groups. P ≤0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Microvascular endothelial cells express CYP2C isoforms and biosynthesize EETs. To establish the presence of a functional epoxygenase in mouse lung endothelial cells, we quantified cellular EETs and DHETs by the isotope ratio GC/MS method (26). Lung endothelial cells generate epoxygenase metabolites from endogenous precursors. While EETs were found only in the cell pellet (∼ 0.37 ng/mg total proteins), their hydrated products, the DHETs, were mostly secreted into the culture media (∼ 0.65 ng/mg). Incubation of the cells with exogenous arachidonic acid for 4 or 24 hours, significantly increased the yield of epoxygenase products. Interestingly, the amount of total cell epoxygenase products (EETs + DHETs) was higher at 4 hours than at 24 hours (36 vs. 24 ng/mg, respectively), suggesting that depletion of the precursor arachidonic acid and further metabolism (i.e. β-oxidation) could account for these changes (17). Furthermore, within the first 4 hours, approximately 80% of the EETs (~3.6 ng/mg) and >95% of the DHETs (~30 ng/mg) were secreted into the media. In contrast, after 24 hours the EETs were found exclusively in the cells (~1.3 ng/mg), while most of the DHETs formed (95% of the total; ~20 ng/mg) were secreted into the culture media. These results identify the release of arachidonic acid from glycerophospholipid stores, catalyzed by phospholipases A2 and epoxide hydrolase(s) (31) mediated hydration as two key components of the cellular mechanisms controlling EET biosynthesis, disposition, intracellular concentrations and, ultimately, biological activity (12,17). The biosynthesis of EETs by cultured and freshly isolated endothelial cells, and the role of hydration in cellular EET metabolism has been reported (17).

Several members of the CYP2C P450 gene subfamily have been identified as the predominant arachidonic acid epoxygenases in several organ tissues (12), and CY2C23, 2C11, 2C8 and 2C9 are characterized as renal and vascular epoxygenases in rat and human tissues (12-14,17,18,32,33). The murine homologues of rat CYP2C23 and 2C11, Cyp2c44 and 2c38, are cloned and the recombinant proteins were shown to catalyze arachidonic acid epoxidation (15,16). Amplification by RT-PCR of pulmonary
endothelial cell RNAs using Cyp2c38 and 2c44 specific PCR primers, showed the presence of Cyp2c44 transcripts, but the absence of detectable Cp2c38 mRNAs (Fig. 1A). Western blots using anti-CYP2C23 or -2C11 antibodies, cross reactive towards Cyp2c44 and 2c38, respectively, demonstrated the presence of Cyp2c44 (Fig. 1B), but not Cyp2c38 (not shown), in cell lysates. We conclude that Cyp2c44 is the predominant Cyp2c mouse EET synthase present in the cultured lung endothelial cells, and the enzyme responsible for most of cellular EET biosynthesis.

**EETs are mitogenic for microvascular endothelial cells.** To determine EET mitogenic activity, we incubated primary cultures of pulmonary murine endothelial cells with different amount of each EET regioisomer, and estimated their effects on cell proliferation by measurements of ³H-thymidine incorporation. All four EETs induced significant increases in cell proliferation whether the cells were incubated with the EETs in the absence (Fig 2A) or presence (Fig 2B) of serum. In the absence of serum significant increased proliferation over control cells (cells incubated with vehicle, ethanol, only) was observed when EETs were used at 0.5 µM. Maximum induction was observed when cells were incubated with 1µM EETs and this was independent from the cell culture conditions. The most effective regioisomer, 5,6-EET, induced 2 or 2.5 fold increases in proliferation in cells cultured in the presence or absence of serum, respectively (Figs. 2A and 2B).

Since the DHETs are formed and secreted by endothelial cells (see above), and since they have been reported to modulate endothelial cell functions (34), we determined their ability to stimulate endothelial cells proliferation. As shown in Figure 2, none of the DHETs stimulated endothelial cell proliferation, independently of their dose or culture conditions (Figs. 2C and 2D).

While the functional properties of 11,12-, and 14,15-EET have been extensively characterized (12,13,17), significantly less is known regarding the biological activities of 5,6- and 8,9-EET. The proximity of the epoxide functionality to the carboxylic acid group in 5,6-EET, facilitates oxido ring opening, and the formation of the δ-lactone of 5,6-DHET (12) and thus requiring extra precautions during handling and purification immediately prior to use. Furthermore, most of the published studies dealing with the mitogenic activity of EETs in endothelial cells, have either used synthetic 11,12- and 14,15-EET or cells transfected with viral vectors coding for CYP2C9 cDNA (9-11), an epoxygenase expressed in the human liver, kidney, and vascular tissues (12,17,18). While the effects of these CYP2C9 transfected cells have been attributed to the enzyme-mediated generation of 11,12-EET, recombinant CYP2C9 generate mixtures of 8,9-, 11,12-, and 14,15-EET (18).

To determine the contribution of the endogenous EETs to endothelial cell proliferation, cells were cultured in complete medium containing different concentrations of the EET synthase inhibitors MS-PPOH or ketoconazole. Compared with controls (cells incubated with ethanol only), the epoxygenase inhibitors decreased endothelial cell proliferation in a dose-dependent manner (Fig. 3A). Maximal inhibition was obtained at 20 µM MS-PPOH or ketoconazole, concentrations shown to block by nearly 90% microsomal epoxygenase activity (12,27). To test the capacity of exogenously added EETs to rescue cell proliferation from EET synthase inhibition, endothelial cells were treated with MS-PPOH or ketoconazole in the presence or the absence of each EET (1µM). The EETs were here used at 1 µM since, within a range of 0 to 5 µM, this was the concentration...
inducing maximal proliferation (Fig. 2). As shown in Fig. 3B, both inhibitors (10 \(\mu\)M each) significantly decreased endothelial cell proliferation, and co-incubation with EETs rescued their proliferation to levels comparable or slightly higher to those observed in cells treated with vehicle (ethanol) only.

The EETs are enzymatically hydrated to DHETs by epoxide hydrolases, particularly, cytosolic epoxide hydrolase (31). As mentioned above, hydration does contribute to cellular EET disposition, and the administration of inhibitors of this enzyme to rats has been associated with reduction in the urinary excretion of DHETs (35). To determine whether the inhibition of cytosolic epoxide hydrolase alters cell proliferation, endothelial cells were cultured as indicated above in the absence or presence of different concentrations of the hydrolase inhibitor ACU (17). As shown in Figure 3A, ACU increased cell proliferation in a dose-dependent manner, with a maximal stimulation obtained between 10 and 40 \(\mu\)M (Fig. 3A). This data suggests that preventing endogenous EET hydration enhances endothelial cell proliferation, in agreement with the finding that only exogenously added EETs, but not DHETs, stimulate endothelial cells proliferation (Fig. 2). Interestingly, co-administration of ACU (10\(\mu\)M) and EETs (1\(\mu\)M) further enhanced endothelial cell proliferation compared to EETs alone (Fig. 3B). We concluded from these experiments that experimental manipulation of cellular EET levels by either inhibiting their biosynthesis or their degradation has opposing anti- and pro-proliferative effects. This is consistent with a role for them as endogenous pro-mitogenic lipids. In addition to establishing a functional link between EET biosynthesis and cell proliferation, these studies expand the list of lipid-derived mitogens to include the 5,6- and 8,9-EETs.

5,6- and 8,9-EET promote microvascular endothelial cell migration and tubule-like structure formation. Endothelial cell migration is an important step in angiogenesis as it confers these cells with the capacity to form capillary-like structures both in vivo and in vitro. We therefore evaluated the ability of the EETs to induce endothelial cell migration using transwell plates. As shown in Figure 4A, 5,6- and 8,9-EET caused a significant increase in cell migration (~2 fold over cells treated with vehicle only), while 11,12- and 14,15-EET showed no detectable chemotactic activity. The fact that 11,12-EET has been previously characterized as a chemotactic EET in cultures of cerebral and umbilical endothelial cells (8,10,11), albeit at higher concentration, raises the interesting possibility of species and/or vascular bed EET-specific effects on cell migration.

We next determined whether the combined mitogenic and pro-migratory activities of 5,6- and 8,9-EET led to the formation of capillary-like structures. Endothelial cells were plated in serum free media on solidified Matrigel, and the ability of the EETs (1 \(\mu\)M each) to promote the formation of capillary-like structures was compared to that of cells treated with vehicle (ethanol) only. Within the first three hours of treatment, 5,6- and 8,9-EET induced capillary-like structures, as determined by the ability of endothelial cells to sprout, branch and form ring-like structures (Figs. 4B and 4C). In contrast, no significant changes were observed with 11,12- or 14,15-EET. Interestingly, at 0.5 \(\mu\)M 5,6- was the only EET able to induce capillary formation (not shown).

To explore the role of cellular EET synthase(s) in the formation of capillary-like structures, endothelial cells were plated on solidified Matrigel and cultured in serum containing medium to which we added MS-PPOH or ketoconazole. Between 2.5 and 5.0
µM, both epoxygenase inhibitors markedly reduced the formation of capillary-like structures (Figs. 5A and 5B), indicating that blocking endogenous EET biosynthesis impairs the capacity of endothelial cells to form these structures. Significant inhibition of tube formation was also observed in human endothelial cells treated with MS-PPOH or miconazole, although these effects were reached only at high concentration of inhibitors (9). In contrast to our results, 11,12- and 14,15-EET have been reported to promote the formation of capillary-like structures and angiogenesis by using either synthetic 11,12-EET (10), or as a consequence of overexpressed CYP2C9 directed 14,15-EET biosynthesis (9). However, in the CYP2C9 over-expression experiments, the spectrum of EET metabolites generated by CYP2C9 was not characterized. Furthermore, capillary like structures were examined only after 18 hours (9) or 14 days (10) from plating. Nevertheless, the effects described for these EETs give further support to the idea of EET isomers and/or vascular bed specific effects for the epoxygenase metabolites.

**EETs activate distinct signaling pathways in microvascular endothelial cells.** 11,12- and 14,15-EET have been shown to participate as second messengers in several signaling pathways, including growth factor-depend mitogenic cascades (10,20,36-38). Among the various mitogenic signaling pathways, the activation of ERK, p38 MAPK and PI3K/Akt play important roles in endothelial cell function. To determine whether EETs could activate these pathways in murine lung endothelial cells, serum starved endothelial cells were treated with each EET (1µM,) for 0, 10 and 40 minutes and ERK, p38 MAPK and Akt activation analyzed by Western blot. Within the first 10 min of addition, all four EETs caused a significant activation of Akt (Fig. 6A), however, only for 8,9- and 11,12-EET was the response long lasting and persisting for at least 30 min after EET addition (Fig. 6A). While all four EETs caused marked and transient increases in ERK activation (Fig 6A), 8,9- was the most potent EET in activating p38 MAPK (Fig. 6A). These results show that these eicosanoids are capable of activating mitogenic signaling cascades known to regulate endothelial cell functions, such as proliferation and migration.

Most of the intracellular pathways activated in endothelial cells have been analyzed following treatment with 11,12- and/or 14,15-EET, or by transfecting human endothelial cells with Cyp2c isoforms. In agreement with our data, treatment of human endothelial cells with 11,12-EET leads to transient Akt activation (38), and this event seems to be related to the ability of this eicosanoid to induce EGF receptor phosphorylation (10). Moreover, treatment of bovine artery endothelial cells with 14,15-EET activates ERK1/2 which then stimulates the phosphorylation and activation of endothelial nitric-oxide synthase (39). However, in most of the studies with endothelial cells, only one or few EET-signaling pathways have been analyzed, and the downstream signaling molecule(s) activated by the 5,6- and 8,9-EETs are yet to be identified. We now provide evidence that the three major pathways involved in endothelial cells function, namely PI3K/Akt, p38 MAPK and ERK1/2, can be activated by the four EETs, and that the 8,9-EETs is the most potent activator of p38 MAPK. Based on this finding we next determined whether activation of one or more of these pathways was important to modulated endothelial cell proliferation and migration.

**EETs promote microvascular endothelial cell proliferation by activating EET regioisomer-specific signaling pathways.** To determine the signaling pathway/s involved in EET-induced proliferation, serum starved endothelial cells
(to minimize endogenous Akt, ERK and p38 MAPK activity, see also Fig. 6A) were incubated with each EET (1 µM) in the presence or absence of either PD98059 (a MEK1/ERK inhibitor), wortmannin (a PI3K inhibitor) or a p38 MAPK inhibitor. After 24 hours, cell proliferation was evaluated by measurements of ³H-thymidine incorporation. As seen in Figure 6B, all four EETs rescued cell proliferation from PD98059 inhibition, suggesting that ERK is not involved in EET-mediated mitogenesis. In contrast, while only 5,6- and 14,15-EET rescued cell proliferation from p38 MAPK inhibition (Fig. 6B), 8,9- and 11,12-EET restored cell proliferation in PI3K/Akt inhibited cells. These data indicates that the proliferative response of endothelial cells following treatment with either 8,9- or 11,12-EET involves p38 MAPK activation, while the mitogenic activity of 5,6- and 14,15-EET are mediated by the PI3K/Akt kinase. Our results suggest that these eicosanoids are capable of stimulating the same cell function by activating quite different cellular pathways. In this regard, it has been shown that the ability of 11,12-EET to induce endothelial cell proliferation results from PI3/Akt-mediated down-regulation of the cyclin-dependent kinase inhibitor p27kip1 (38). Moreover, studies with CYP2C9 transfected endothelial cells suggested that the mitogenic activity of 11,12-EET, one of the EETs formed by the enzyme (18), results from activation of the EGF receptor and subsequent PI3/Akt-mediated activation (10). In contrast, our studies show that pulmonary endothelial cell proliferation is primarily mediated by 11,12-EET via activation of p38 MAPK. As mentioned above, this apparent discrepancy could reflect EET isomer specific and/or vascular bed specific effects. Finally, Zhang and colleagues have shown that cerebral capillary endothelial cell proliferation can be stimulated by all four EETs via activation of a tyrosine kinase receptor, with 8,9-EET being the most potent and 5,6-EET being the less active (8). On the other hand, our results identified 5,6- and 8,9-EETs as the most potent mitogens among the four EETs, and show that their mitogenic activity is dependent on PI3K/Akt or p38 MAPK activation, respectively. Again, these differences are not surprising considering that i) endothelial cells derived from different organs were used for the study, and ii) it is well established that mature endothelial cells have distinctive functional properties, depending on the tissue of origin (40). Finally, an important issue when comparing results is that of the chemical lability of 5,6-EET. All our studies were done with 5,6-EET stocks that were purified and characterized by HPLC immediately prior to use.

ERK and PI3K/Akt activation is required for 5,6- and 8,9-EET induced chemotaxis. As discussed, 5,6- and 8,9-EET induce significant endothelial cell migration (Fig 4A), a cellular function also known to involve several protein kinase signaling cascades. To determine the pathways involved in EET-induced endothelial cell migration, we evaluated the chemotactic activity of these EETs (1µM each) in cells that were cultured in the absence or the presence of PD98059, wortmannin or a p38 MAPK inhibitor. As shown in Figure 6C, neither 5,6- nor 8,9-EET rescued cell migration from ERK or PI3K/Akt inhibition. On the other hand, 5,6-EET, but not 8,9-EET, rescued endothelial cell migration from p38 kinase inhibition (Fig. 6C), suggesting that this kinase is not involved in 5,6-EET-dependent chemotaxis. To the best of our knowledge, this is the first study showing not only that 5,6- and 8,9-EETs enhance endothelial cell migration, but also that they achieve this effect by activating distinct intracellular pathways.

The Cyp2c44 epoxygenase is expressed in angiogenic blood vessels. The expression of CYP2C isoforms and the formation of
Epoxide metabolites by cultured endothelial cells has been reported [reviewed in (24) and present study]. Furthermore, isolated rat vascular tissues catalyze EET formation, and express a CYP2C23 epoxygenase (33). However, other than the demonstration of the EETs as endogenous constituents of human and rat plasma (12), there is scant evidence of vascular CYP2C expression in vivo (12,15). To determine whether the murine Cyp2c44 epoxygenase is expressed in angiogenic blood vessels, we performed immunofluorescence analysis on sections from tumors originated by human cancer cells grown into nude mice using: a) anti-mouse CD31 antibody to visualize vessels within the tumor, and b) anti-rat CYP2C23 antibody, cross reactive towards mouse Cyp2c44. A human tumor cell line was selected for these studies since it lacks proteins that are immunoreactive with CYP2C23 antibodies, thus facilitating the identification of mouse Cyp2c44 positive structures. Figure 7A illustrates the expression and co-localization of the Cyp2c44 epoxygenase with the CD31 endothelium marker, indicating that the mouse-derived tumor vessels express this epoxygenase, and that it could play a role in angiogenesis in vivo.

5,6- and 8,9-EET are potent angiogenic factors in vivo. Endothelial cell proliferation, migration and the development of capillary-like morphology, combined responses elicited only after treatment with 5,6- and 8,9-EET (Figs. 2 and 4), are essential components of angiogenesis. To test the angiogenic activity of 5,6- and 8,9-EET in vivo, we utilize a subcutaneous sponge model (29). Inert sponges, implanted subcutaneously in the back of adult mice, were injected every other day with either vehicle (oil), 5,6-EET, or 8,9-EET (50 µM each). After 14 days, we retrieved the sponges and compared their vessel density by direct observation and by analysis of vessel associated Rhodamine-Dextran fluorescence. Sponges injected with 5,6- or 8,9-EET show significantly increased vessel density compared to sponges injected with vehicle only, demonstrating clearly in vivo de novo vascularization (Figs. 7B-D).

To determine whether the potentiating effects of ACU on the EET-stimulated endothelial cell proliferation in vitro (Fig. 3) have functional significance to in vivo angiogenesis, the epoxide hydrolase inhibitor was injected either alone or a combination with either 5,6- or 8,9-EET into implanted sponges, and its effects on angiogenic activity determined as above. As shown in Figure 7, the pro-angiogenic activity of 5,6- and 8,9-EET was significantly enhanced when they were co-injected with ACU (Fig. 7B-D). Furthermore, the administration of ACU alone also caused significant increases in vascularization when compared to vehicle only, suggesting that inhibition of exogenous EET hydration increases angiogenic potential. Finally, analysis of EET and DHETs present in the sponges at 14 days indicted that ACU prevented by 40% EET hydration (not shown).

Several groups, using different models of angiogenesis, have documented the pro-angiogenic activities of synthetic 11,12- and 14,15-EET (8-11,17). However, this is the first study that characterizes the 5,6- and 8,9-EET as potent pro-angiogenic molecules, and that addresses the mechanisms responsible for their mitogenic and chemotactic activity. In addition, our in vivo angiogenesis assay not only shows the ability of 5,6- and 8,9-EETs to induce de novo vascularization, but also to promote the formation of functionally intact vessels, as determined by vessel-associated Rhodamine-Dextran fluorescence. Finally, we demonstrate that preventing 5,6- and 8,9-EET enzymatic hydration could be used to enhance the angiogenic activity of these endogenous lipids.
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**FOOTNOTES**

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1The abbreviations used are: P450, cytochrome P450; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; AA, arachidonic acid; MAPK, mitogen activated protein kinase; EET, epoxyeicosatrienoic acid; DHET, dihydroxyeicosatrienoic acid, MS-PPOH, N-methylsulphonyl-6-(2-proparglyoxyphenyl)hexanamide; ACU, adamantyl-cyclohexyl-urea.
**FIGURE LEGENDS**

**Fig. 1.** *Expression of cyp2c44 in primary endothelial cells.* (A) RT-PCR amplification of Cyp2c44 and Cyp2c38 isoforms. RT-PCR amplification of total RNA samples was performed using mouse Cyp2c44 and Cyp2c38 specific primers as described in Materials and Methods. (B) Endothelial cell lysates (50 µg/lane) were analyzed by Western blot using anti rat CYP2C23 specific antibodies as described in Materials and Methods. 20 ng/lane purified cyp2c44 was used as positive control.

**Fig. 2.** *EETs induce endothelial cell proliferation.* (A) Primary murine endothelial cells (5x10^3 cells/well) were plated onto 96-well plates in complete medium. After 12 hours the cells were serum starved for 24 hours and subsequently incubated with serum free medium containing [³H]thymidine (1 µCi/well) in the presence of 0, 0.5, 1, and 5 µM EETs. After 24 hours cell proliferation was evaluated as described in Materials and Methods. Values are changes relative to cells treated with vehicle (ethanol) only, and are averages calculated from 3 experiments. (B) Endothelial cells were plated as above in the presence of complete medium and in presence of 0, 0.5, 1, and 5 µM EETs. After 2 days, the medium was replaced, [³H]thymidine added (1 µCi/well) and cell proliferation evaluated. Values are as in (A). Differences between cells untreated and treated with EETs (*) were significant with p<0.05. (C, D) Endothelial cells were plated and treated as indicated in (A) and (B) respectively, with the difference that 0, 0.5, 1, and 5 µM DHETs were added to the wells. Values were then expressed as in (A).

**Fig. 3.** *Effect of EET synthase and epoxide hydrolase inhibitors on endothelial cell proliferation.* (A) Cells were plated in complete medium in the presence or absence of MS-PPOH, ketoconazole, ACU at the concentrations indicated. After 2 days, the medium was replaced, [³H]thymidine added (1 µCi/well) and cell proliferation evaluated. Values are changes relative to cells treated with vehicle (ethanol) only, and are averages calculated from 3 experiments. (B) Endothelial cells were plated as described in Fig. 2A with or without 1µM EETs alone or in combination with the inhibitors indicated (10 µM, each) and proliferation measured 24 hours after incubation. Differences between untreated and EET-treated cells (*), or inhibitor-treated and inhibitor + EET treated cells (**), or EET-treated and ACU + EET treated cells (δ) were significant with p<0.05.

**Fig. 4.** *EETs induce endothelial cell migration and capillary-like structure formation.* (A) Endothelial cells were plated in serum free medium in transwell dishes coated with 10 µg/ml collagen I and allowed to migrate for 12 hour at 37C towards serum free medium with or without EETs (1µM). Values are changes relative to cells migrating in the absence of EETs (“EtOH”) and are averages calculated from 3 experiments. (B) Endothelial cells were serum starved for 24 hours and then plated as described in Materials and Methods onto Matrigel in the absence or presence of EETs (1µM). Representative images of capillary-like structures taken 3 hours after plating are shown. (C) Capillary network formation was quantified as described in Materials and Methods. Values are the mean +/- SD calculated for 15 images per treatment. Note that cells incubated with 5,6- and 8,9-EETs form tubule-like structures more efficiently than control cells (treated with ethanol only) or cells treated with 11,12- and 14,15-EETs. (*) indicates significant differences (p<0.05) between untreated and EET-treated cells.
**Fig. 5.** EET synthase inhibitors prevent the formation of capillary-like structures. (A) Endothelial cells were plated as described in Fig. 3 onto Matrigel in complete medium in the absence or presence MS-PPOH or ketoconazole at the concentration indicated. Representative images of tubule-like structures taken 3 hours after plating are shown. (B) Capillary network formation was quantified and expressed as mean +/- SD calculated for 15 images per treatment. Note that cells incubated with 2.5-5 µM EET synthase inhibitors form capillary-like structures less efficiently than control cells (treated with ethanol, only) (* = <0.05).

**Fig. 6.** EET-induced cell proliferation and migration requires activation of specific downstream signaling molecules (A) Serum-starved endothelial cells were either kept untreated or treated for 10 and 40 minutes with the different EETs indicated (1 μM each). Levels of phosphorylated as well as total ERK, Akt and p38 were determined on total cell lysates (50 µg/lane) by Western blot analysis. (B) Endothelial cells were cultured as described in Fig. 2A with or without 1μM EETs alone or in combination with the kinase inhibitors indicated and proliferation measured 24 hours after. Values are changes relative to cells grown in the absence of EETs and kinase inhibitors (ethanol only was added) and are averages calculated from 3 experiments. (C) Endothelial cells were serum starved for 24 hours and then plated in transwell dishes coated with 10 µg/ml collagen I. Cells were then allowed to migrate for 12 hour at 37°C towards serum free medium with or without 1µM EETs alone or in combination with the kinase inhibitors indicated. Values are changes relative to cells migrating in the absence of EETs and kinase inhibitors (ethanol only added) and are averages calculated from 3 experiments. Differences between untreated and EET-treated cells (*), or cells treated with EETs alone or in combination with the kinase inhibitors indicated (**) were significant with p<0.05.

**Fig. 7.** 5,6- and 8,9 EETs are pro-angiogenic in vivo. (A) Frozen sections of tumors derived from human non-small cell lung cancer cells grown into nude mice were co-stained with biotinylated rabbit anti-rat CYP2C23 (left panel) and rat anti-mouse CD31 (middle panel) antibodies followed by incubation with FITC-conjugated streptavidin, RITC-conjugated goat anti-rat antibodies and DAPI. Note the high expression of the murine Cyp2c44 isoform in CD31 positive blood vessels as shown by the merging image on the right panel. Scale bar, 20 um. (B) Gross images of polyvinyl acetal CF-50 sponges implanted for 14 days into mice and injected with vehicle (“oil”), 5,6-EET or 8,9-EET in the presence or absence of ACU as described in Materials and Methods. Ten minutes prior sacrifice mice were injected intravenously with 50 µl Rhodamine-Dextran (2% in PBS). Sponges were then removed and placed under a fluorescence microscope to visualize vascularization. (C) Vascularity within sponges was determined by calculating the area occupied by Rhodamine positive structures per microscopic field as described in the Materials and Methods. Values indicate the mean and standard deviation calculated for 15 images per treatment. Differences between sponges untreated and EET-treated (*) or untreated and ACU-treated ,(**) or EET-treated and ACU+EET-treated (δ) were significant with p<0.05.
Pozzi et al., Fig. 1

A

| bp  | 2C44 | 2C38 | β-actin |
|-----|------|------|---------|
|     |      |      |         |

B

| kDa | ECs     | Cyp2c44 |
|-----|---------|---------|
| 36  |         |         |
| 55  |         |         |
Pozzi et al., Fig. 3

A

Fold changes over cells treated with EtOH

B

Fold changes over cells treated with EtOH
Pozzi et al., Fig. 5

A

| P450 inhibitors [µM] | 0.625 | 1.25 | 2.5 | 5 |
|----------------------|-------|------|-----|---|

EtOH

B

Number of branches/microscopic field

EtOH MS-PPOH ketoconazole

*
Pozzi et al., Fig. 6

(A) Protein expression levels at 10 min and 40 min after treatment with different EETs and EtOH.

(B) Graph showing fold changes over cells treated with EtOH for PD98059, p38 inh., and Wort.

(C) Graph showing fold changes over cells treated with EtOH for PD98059, p38 inh., and Wort.
Charaterization of 5,6-and 8,9-Epoxyeicosatrienoic acids (5,6- AND 8,9-EET) as potent in vivo angiogenic lipids
Ambra Pozzi, Ines Macias-Perez, Tristin Abair, Shouzuo Wey, Yan Su, Roy Zent, John R. Falk and Jorge H. Capdevila

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