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The arachidonic acid epoxygenase is a component of the signaling mechanisms responsible for VEGF-stimulated angiogenesis ☆

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Abstract

Cultured lung endothelial cells (LEC) respond to VEGF or arachidonic acid with increases in cell proliferation, the formation of tube-like structures, and the activation of Akt and ERK1/2 mediated growth pathways. LECs express a VEGF inducible Cyp2c44 epoxygenase and its 11,12- and 14,15-EET metabolites increase cell proliferation, tubulogenic activity, and the phosphorylation states of the ERK1/2 and Akt kinases. Ketoconazole, an epoxygenase inhibitor, blocks the cellular responses to VEGF. LECs expressing a Cyp2c44 epoxygenase small interference RNA show reductions in Cyp2c44 mRNA levels, and in their VEGF-stimulated proliferative and tubulogenic capacities; effects that are associated with decreases in VEGF-induced phosphorylation of the ERK1/2 and Akt kinases. We conclude that the Cyp2c44 arachidonic acid epoxygenase is a component of the signaling pathways associated with VEGF-stimulated angiogenesis, and suggest a role for EETs in the growth factor-induced changes in the activation states of the ERK1/2 and Akt kinase pathways.

Keywords

Arachidonic acid epoxygenase; EETs; Angiogenesis; Mitogen activated kinases; Cytochrome P450; EETs and angiogenesis; Vascular endothelial growth factor; Cell signaling

Introduction

Angiogenesis and *de novo* vascularization are key components of physiological responses to inflammation and wound healing, and play also important roles during organ recovery from injury [1–4]. Due its recognized roles in tumor vascularization [5–8], angiogenesis has become an attractive candidate for the development of anti-cancer therapies aimed to reduce tumor access to blood derived oxygen, nutrients and hormones [5,6]. Consequently, the identification of novel angiogenic factors, and the analysis of their mechanisms of action has become an active area of research, since it holds a promise for more effective and potentially less invasive approaches to cancer treatment. Current efforts to develop anti-angiogenic approaches for cancer treatment are primarily directed towards disrupting the interactions

☆During the preparation of this paper, Webler et al. published an article in Am. J. Cell Physiol. 295 (2008) C1202–C1301, containing results supporting a role for the EETs in VEGF signaling and pro-angiogenic responses.

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between VEGF and its receptors [6–13]. Angiogenesis is a complex process that requires the coordinated contributions of endothelial cell proliferation and migration, leading to the formation of nascent capillary structures and, ultimately, functional vessels. These processes, initiated upon VEGF binding to its receptors involve among other things, the activation of kinase cascades such as those of the PI3K/Akt and MAKP/ERK1/2 signaling pathways [7,8]. In addition to VEGF, several products of the oxidative metabolism of arachidonic acid (AA)[1] have been shown to have pro-angiogenic properties, including prostanoids, [14,15], *cis–trans* conjugated hydroxyeicosatetraenoic acids [16], as well as products of the cytochrome P450 (P450) AA epoxygenase and ω-hydroxylase pathways [17–25].

The P450 epoxygenase metabolizes endogenous pools of AA to 5,6-, 8,9-, 11,12-, and 14,15-EET [26–29], in reactions that are predominantly catalyzed by members of the CYP2C gene subfamily [26,29]. Among CYP2C isoforms, human CYP2C8 and CYP2C9, and mouse Cyp2c44 are known to be expressed in endothelial cells and to catalyze EET formation [25,30–32]. The EETs are enzymatically hydrated to 5,6-, 8,9-, 11,12-, or 14,15-dihydroxyeicosatrienoic acid (DHET) by cytosolic epoxide hydrolase [26]. A role for EETs in cell proliferation was first identified in 1990 when it was demonstrated that exogenously added 14,15-EET induced the proliferation of cultured rat glomerular mesangial cells [33]. Subsequently, 14,15-EET was shown to mediate the proliferative responses to EGF and HB-EGF, and to promote activation of the PI3K/Akt and MAPK/ERK1/2 pathways [34,35]. Since those early studies, 11,12- and 14,15-EET have been characterized a potent mitogens in cells obtained from kidney, brain, and endothelium [17,22–25], and 20-hydroxyeicosatetraenoic acid has been identified as pro-angiogenic [17–20]. Moreover, 5,6-, 8,9-, and 11,12-EET were shown to stimulate endothelial cell proliferation [25], to activate endothelial cell PI3K, MAPK pathways [25,36], and to be potent *in vitro* and *in vivo* angiogenic factors [25].

Recent studies identified PPARα and its ligands as effective inhibitors of tumor angiogenesis and growth [37–41]. Thus, PPARα ligands such Wyeth 14,643 and Fenofibrate inhibit tumor angiogenesis and growth by interfering with the proliferative activity of the host endothelial cells in a PPARα-dependent fashion [40,41]. A role for the murine Cyp2c epoxygenases in the anti-angiogenic, anti-tumor effects of Wyeth 14,643 was indicated by the demonstration that the ligand down-regulates endothelial cell Cyp2c44 expression and reduces the concentrations of plasma circulating pro-angiogenic EETs [40]. On the other hand, except for a postulated role for prostanoids and a VEGF receptor in EET-mediated mitogenesis [24], the mechanism of action and the role of the Cyp2c epoxygenase metabolites in VEGF-mediated angiogenesis remains uncertain. As part of ongoing efforts to understand the relevance of the AA epoxygenase to the angiogenic activities associated with tumor vascularization and growth, we have utilized cultured mouse pulmonary endothelial cells for studies of the role of the Cyp2c44 AA epoxygenase in VEGF signaling. Here, we provide evidence that the Cyp2c44 arachidonic acid epoxygenase and 11,12-EET are components of the metabolic responses elicited during VEGF signaling and angiogenic activity. Inasmuch as VEGF is a current target of efforts to develop novel anti-tumor strategies, these studies identify the Cyp2c epoxygenases as potentially useful targets for the development of new anti-angiogenic approaches for cancer treatment.

[1] *Abbreviations used:* AA, arachidonic acid; EET, epoxyeicosatrienoic acid; DHET, dihydroxyeicosatrienoic acid; PPAR, peroxisomal proliferator activated nuclear receptor; Cyp2c and Cyp4A, members of the cytochrome P450 2c and 4a gene subfamilies, respectively; GC, gas liquid chromatography; MS, mass spectrometry; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; LEC, immortalized mouse lung endothelial cells; EGF, epidermal growth factors; HB-EGF, heparin binding-EGF; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; Akt, protein kinase B; ERK1/2, extra cellular signal-regulated protein kinase; MAPK, mitogen activated protein kinase; PI3K, phosphoinositide 3-kinase.

Materials and methods

Cell culture and siRNA transfections

Pulmonary micro vascular endothelial cells (LEC) were isolated from the lungs of transgenic mice expressing a temperature sensitive large tumor (T) antigen (Tag) under the control of a mouse histocompatibility H-2 Kb interferon responsive promoter [42–44]. Cells were propagated at 33° in EBM2 media (EBM2, Cambrex Bio Science Inc.) containing interferon (10 units/ml), 5% fetal calf serum and a mixture of hydrocortisone, hFGF-B, VEGF, IGF-1, hEGF, and ascorbic acid (the concentrations of these components is treated by the EBM2 media supplier as confidential information) (Clonetics CC-3202, Cambrex Bio Science Inc.), and discarded after 6 passages. For experiments, cells were cultured at 37° in interferon free EBM2, and used between 2 and 5 passages. LEC cultured at 37° display a typical cobblestone shape and express cd31 [45,46], as well as endothelial cells markers such as ICAM-1, VACM-1, and E-selecting [43,44]. Furthermore, these cells show VEGF-stimulated growth, and form tubule-like structures when plated in Matrigel [40,43–46].

SureSilencing plasmids encoding a neomycin/Gentamycin resistance cassette and either a Cyp2c44 specific small interference RNA (5′-CCAGAAGGGACATGGAATTGT-3′) (Cyp2c44siRNA) or a control sequence (5′-GAGGTGCAAGCCAAAGTTCAT-3′) (mock) were purchased from SuperArray Bio Science Co. For cell transfections, LEC were cultured at 37° in EBM2 and 30 min before transfection, the media was replaced with serum free DMEM media, and the cells transfected with the Cyp2c44siRNA or the control plasmid using modified cationic liposomes (GenJet; SinGen Laboratories). Four hours after, the media was replaced with EMB2 and the cells cultured for an extra 24 h at 37°, prior to the addition of Gentamycin (5 μg/ml). Resistant cells were selected after a 2–4 weeks of culture in Gentamycin supplemented EMB2 (with changes every 48 h). Gentamycin resistant cells were plated in Gentamycin free EMB2 and used after 48 h. For analysis of Cyp2c expression, total RNAs were isolated by the Trizol reagent method (InVitrogen) and reversed-transcribed using a SuperScript II Kit (InVitrogen). The resulting cDNAs were submitted to quantitative PCR analysis using a TaqMan® Gene Expression Assays Kit (Applied Biosystems), and Cyp2c44 or β-acting specific primers (the sequence of these Applied Biosystems designed primers is treated by the company as confidential information). Expression levels were normalized using β-actin as reference cDNA.

Cell proliferation and tubulogenesis studies

Rates of LEC proliferation were estimated by cell counting and by measurements of [^{3}H]thymidine incorporation into the cellular DNA [25,40]. Transfected and non-transfected LEC (60% confluent) were cultured for 48 h in serum free DMEM and at 37°, prior to the addition of either individual EETs (between 0.01 and 5 μM final concentration, each), AA (1 μM, final concentration), VEGF (50 ng/ml), the epoxygenase inhibitor ketoconazole (10 μM, final concentration), ethanol vehicle when appropriate (to ≤0.1% final concentration) or after replacing the media with EBM2. Immediately thereafter, [^{3}H]thymidine (74 Ci/mmol; 2.5 μCi/ml) was added and 48 h after the media was removed, the cells washed twice with cold PBS, exposed for 10 min to 10% trichloroacetic acid, washed twice with PBS, and the cellular material solubilized in 100 μl of 0.1% SDS. The incorporation of [^{3}H]thymidine was quantified by liquid scintillation counting of the solubilized LECs [25].

The formation of capillary-like structures by LECs was analyzed as described [25,40]. Briefly, 96 well plates were coated with 50 μl/well of an 83% solution of Matrigel (BD Biosciences). Transfected and non-transfected LEC in serum free DMEM were added to the Matrigel coated plates (1.5×10^4 cells/well) and, after the addition of test compounds, the plates were incubated at 37° in serum free DMEM media for 6 h, and the cells then fixed

using formaldehyde (4%, final concentration). The presence of capillary-like structures was recorded using digital photography of randomly selected microscopic fields and quantified by counting cellular nodes. Cellular nodes were defined as junctions linking at least 3 cells [25,40]. Experiments were done in triplicate, and a total of 12 images were analyzed for each experiment. The test compounds used and their final concentrations were: 11,12-EET, 14,15-EET, or AA; 1 μM each, and VEGF, 50 ng/ml.

Western blot analysis of kinase activation

Semi confluent transfected and non transfected LECs were cultured at 37° in serum free DMEM media for 48 h, prior to the addition of either the ethanol vehicle (≤0.1% v/v final concentration), 11,12-, 14,15-EET, AA (1 μM final concentration each), or VEGF (50 ng/ml). At the indicated time points (see legends to Figs. 2, 5 and 9), the cells were washed thrice with cold PBS, suspended in a 50 mM Tris–Cl pH 7.5 lysis buffer containing: 10 mM $MgCl_2$, 200 mM NaCl, 1% NP-40, 5% Glycerol, 0.5% Triton X-100, 1 mM EDTA, a Protease Inhibitor Cocktail I, and a Phosphatase Inhibitor Cocktail II (both from BD Biosciences), and pulse sonicated for 20 s. (50% active cycle, 40% power) (Cole Parmer Instruments Co.). The cell lysates were centrifuged (20,000*g*, 15 min), the supernatants submitted to SDS/PAGE (20–40 μg protein/well; 10% acrylamide gels), and the resolved proteins transferred to Immobilon-P membranes (Millipore). Membranes were incubated with rabbit anti-phospho-ERK1/2 or -Akt antibodies (all from Cell Signaling Technology). Immunoreactive proteins were detected by chemio-luminescence using a peroxidase conjugated goat anti-rabbit antibody (Sigma Chemical Co.) and an ECL kit (Pierce). Protein loadings were checked by first stripping the membranes for 1 h at 55° in 50 mM Tris–Cl pH 6.5, containing 2% SDS and 0.4% β-mercaptoethanol, and then re-probing them using rabbit anti-ERK1/2 or anti-Akt antibodies (Cell Signaling Technology). To remove excess Gentamycin, transfected cells were cultured in Gentamycin free EBM2 for 48 h prior to replacing their media with serum free DMEM.

Analysis of EETs biosynthesis

The capacity of LEC to biosynthesize EETs was analyzed in cells transferred from EBM2 to serum free DMEM media at approximately 50% confluence. After 48 h in serum free DMEM, the media was replaced with EBM2 or with DMEM containing either AA, VEGF, or a mixture of VEGF and AA (1 μM, and 50 ng/ml each, respectively). 24, 48, or 72 h after these media changes, the cell media was collected and saved, and the attached cells washed with PBS, suspended in fresh PBS, and collected by centrifugation. Cell pellets were extracted in the presence of synthetic [$^2H_3$]8,9-, 11,12-, and 14,15-EET (5 ng each) with an acidified mixture of $CHCl_3$:CH_3OH, (2:1), and the resulting lipid extracts purified and analyzed by GC/MS as described [47,48]. For the quantification of cell-excreted DHETs, the cell media was extracted with acidified ethyl acetate containing a mixture of [$^2H_8$]5,6-, 8,9-, 11,12-, and 14,15-DHET (10 ng each), and the DHETs present in the organic extracts purified and analyzed by GC/MS as described [47]. Endogenous EET and DHETs were quantified using the ion current signal intensities of selected diagnostic ions and stable isotope dilution techniques [47,48].

Statistical analyses were done with Microsoft Excel, and a two tail Student's *t*-test was used for in between group comparisons, with $p < 0.05$considered as statistically significant.

Results and discussion

The isolation of endothelial cells from mice and the establishment of primary cultures of these cells in numbers and purity sufficient for biochemical analysis, is a difficult process that has hindered studies of the roles played by lipid mediators in growth factor signaling

[25,40]. To obviate some of these complications, we utilized as subrogates cultures of immortalized mouse lung endothelial cells or LEC. These cells express phenotypes similar to those of freshly isolated primary cultures of endothelial cells, including cd31 [45,46,49], VE-cadherin, ICAM-1 and VCAM-1 expression [43,44,50].

VEGF and growth factor supplemented media stimulate LEC proliferation and tubulogenesis

We initiated these studies with a characterization of the growth and tubulogenic responses of cultured LECs to VEGF and AA; the substrate of the P450 epoxygenases. Compared to cells cultured in serum free media, the addition of media containing VEGF or AA caused significant increases in LEC proliferation, as measured by the incorporation of [^{3}H]thymidine into cellular DNA (Fig. 1A) or cell counting (1.5 ± 0.1, 4.6 ± 0.2, 4.9 ± 0.1, and 6.7 ± 0.4 cells × 10^4/ml; for serum free media, and media containing VEGF, AA, or VEGF and AA, respectively. Significantly different from serum free, $p < 10^{-3}$, 10^{-5}, and 10^{-3}, respectively for VEGF, AA, and AA plus VEGF. Significantly different from AA plus VEGF for AA, $p < 0.01$. The effects of AA were not significantly different to those of VEGF, $p > 0.05$). The growth effects of VEGF were magnified when added in combination with AA (Fig. 1A), indicating that the availability of non-esterified AA and/or its enzymatic release from phospholipids stores represented a limiting step during VEGF-stimulated LEC proliferation. Studies with primary cultures of mouse lung endothelial cells showed that the availability of free AA is also a limiting factor for cellular EET biosynthesis [25,40]. Control experiments showed that LEC proliferation was markedly increased when the cells were cultured in growth factor enriched EBM2 media containing 5% fetal calf serum (7.1 ± 0.2-fold increase, compared to cells cultured in the DMEM media; $n = 4$) [25,40] and that, in this case, added AA had only limited effects since fatty acids are known components of serum. A feature of primary endothelial cells platted in a Matrigel support in the presence of EBM2 or VEGF, is their ability to migrate and associate into tubule-like structures, reminiscent of mature capillaries [25]. Similarly, and as shown in Fig. 1B and C, LECs suspended in serum free media and plated on Matrigel supports, respond to the addition of VEGF by forming well defined microtubule-like structures. As with proliferation, the formation of these tubule-like structures was stimulated by AA although in this case, the fatty acid was less effective than VEGF. However, AA amplified the tubulogenic response of LECs to the growth factor (Fig. 1B), suggesting that, as is with proliferation, the availability of AA is a limiting step during VEGF-stimulated tubulogenesis. We interpret these results as indicating that AA is but one component of the overall proliferative and tubulogenic responses induced by VEGF. Finally, to minimize effects attributable to changes in cell proliferation, all tubulogenesis experiments were done under conditions in which mitogenesis was minimal, *i.e.*, we used serum starved cells that were plated in serum free DMEM media and all measurements were done within the first 6 h of plating onto Matrigel supports [25].

The role of MAPK pathways in serum or VEGF-stimulated endothelial cell proliferation and tubulogenesis has been reported for cultured endothelial cells of human, bovine, or mouse origin [25,50–53]. Among these, the ERK1/2, p38 MAPK, and PI3/Akt pathways have been linked to the growth and tubulogenic responses of serum stimulated mouse lung endothelial cells in primary culture [25]. Similarly, the VEGF-induced proliferative and tubulogenic responses of LEC appear to be also associated with transient increases in Akt and ERK1/2 phosphorylation (Fig. 2), reflecting the activation of the PI3 and MEK kinase pathways. Moreover, and as shown in Fig. 2, ERK1/2 and Akt are components of the acute responses to this growth factor and are maximally activated within the first 5 min of VEGF addition and rapidly deactivated thereafter, presumably via phosphatase mediated de-phosphorylation (Fig. 2). The apparent increases in ERK1/2 and Akt phosphorylation seen 40 min after

VEGF stimulation, are likely caused by differences in sample loading (Fig. 2). In Summary, these studies: (a) show that LEC respond to VEGF and AA stimulation in a manner similar to what has been reported for primary cultures of mouse lung endothelial cells treated with EETs [25,40], and (b) suggest that the observed LEC responses could involve an epoxygenase catalyzed conversion of AA to EETs.

Cultured LEC express a growth factor sensitive Cyp2c44 epoxygenase

We and others have demonstrated the presence of AA epoxygenases [22,25,28,53], and of a PPARα regulated Cyp2c44 epoxygenase in primary cultures of mouse endothelial cells [40]. To determine whether LEC express a VEGF sensitive epoxygenase, we isolated RNAs from cells that were serum starved for 48 h, and then cultured for 8 or 24 h in serum free media in the presence or absence of VEGF. The RNAs were reversed transcribed, and analyzed for Cyp2c44 expression using real time quantitative PCR as described in Methods. Within the first 8 h of addition, VEGF-induced significant increases in the level of Cyp2c44 mRNA transcripts, and they remained increased for the next 24 h (Fig. 3A). Importantly, Western blot analysis of microsomal fractions isolated from LEC cultured for 24 h in the presence of VEGF showed that they expresses a VEGF-inducible Cyp2c44 AA epoxygenase (Fig. 3B). The expression of a Cyp2c44 epoxygenase in LECs cultured in EBM2 media has been reported [40]. To determine whether LECs were capable of AA epoxidation, cells were cultured in serum free media in the presence or absence of VEGF or in growth factor enriched EBM2 media. After 72 h, the cells were collected by centrifugation, and the concentrations of EETs and DHETs present in the cell pellets and the culture media were quantified by mass spectroscopy as described in Methods. Only in LECs cultured in EBM2 we were able to detect the presence of cellular epoxygenase products (73 ± 15 pg of total epoxygenase products/mg of cell protein; $n = 4$) (EETs plus DHETs); with 11,12- and 14,15-EET, and the corresponding DHETs accounting for ≥90% of the total products. In these cells, the extent of EET hydration to DHETs was highly variable but, as reported, while the EETs were found associated with cells, the DHETs were almost completely excreted into the cultured media [25]. Overall, the levels of epoxygenase metabolites present in EBM2 cultured LEC are lower than those reported for cultures of freshly isolated mouse lung endothelial cells [25,40], perhaps a reflection of known reductions in P450 expression that occur following culture. Due to limitations in detection sensitivity we were unable to reliably quantify epoxygenase metabolites in LEC cultured in serum free media with or without VEGF. The presence of CYP2C epoxygenases, including human CYP2C8 and Cyp2C9, and mouse Cyp2c44 and Cy2c29 in freshly isolated and cultured endothelial cells has been reported [31,32,40,53], as it is the biosynthesis of EETs and DHETs from endogenous and exogenously added AA [25,28,40]. Finally, the above results show that: (a) as is with primary endothelial cell cultures [25], LEC biosynthesize EETs when cultured in serum and growth factor containing EBM2 media, and (b) cells of endothelial origin possess a VEGF-inducible Cyp2c epoxygenase.

EETs induce LEC proliferation and tubulogenesis

Since 11,12- and 14,15-EET are products of the Cyp2c44 AA epoxygenase [54] and are biosynthesized by LEC, we cultured these cells in serum free media in the presence or absence of synthetic 11,12-EET or 14,15-EET (1 μM, final concentration), and determined their effects on proliferation and the formation of tubule-like structures. As shown in Fig. 4A, both EET regioisomers were almost equipotent in stimulating LEC proliferation, and their effects on cell growth were similar in magnitude to those elicited by VEGF (Fig. 1A). To determine the angiogenic potential of the epoxygenase metabolites, LECs were platted on Matrigel in serum free media and in the presence or absence of 11,12- or 14,15-EET, and their capacity to form tubule-like structures estimated based on branching activity. As shown

in Fig. 4B and C, compared to untreated controls, 11,12- and 14,15-EET (1 μMfinal concentration each) induced significant increases in tubular structure formation.

To explore mechanisms involved in the above functional responses of LECs to EET stimulation, we characterized the effects of these lipids on the activation state of the ERK1/2 and Akt pathways, both known components of the VEGF signaling cascade [7,8,25,51,52]. As shown in Fig. 5, the addition of 11,12- or 14,15-EET (1 μM final concentration) to LECs cultured in serum free media, increased their levels of phospho-ERK1/2 and phospho-Akt; known targets of the MAPK and PI3K signaling pathways that, as shown in Fig. 2, are activated by VEGF [25]. However, at difference with VEGF, the effects of the EETs on the phosphorylation state of these kinases were less intense and reach their maximum within the first 20 min of addition (Figs. 2 and 5). In this and other experiments, the activation of Akt by 14,15-EET appears biphasic, and to reach a minimum within approximately 10 min of VEGF stimulation (Fig. 5). The reasons for this biphasic response to 14,15-EET stimulation are presently unknown.

Despite extensive efforts to identify plasma membrane bound EET-receptors, capable of trans-membrane signaling, evidence for such receptors is limited to a few studies using membrane preparations isolated from cultured U937 cells [55,56]. Furthermore, none of the so far reported EET effects on mitogen activated kinases have been linked to an EET-receptor mediated amplification cascade [22–29,33,34]. It is therefore of interest that, in the absence of a documented signal amplification cascade, the metabolically unstable EETs [26–29] more or less mimic the functional effects of growth factors such as VEGF or EGF [33,34]. In summary, the results presented above show that 11,12- or 14,14-EET can behave as VEGF mimetics by increasing cell growth and tubulogenic activity, and indicate that they do so by activating the cellular MAPK and PIP3K pathways. These studies point to a role for these lipid mediators in VEGF angiogenic signaling. As mentioned, and in support of this proposal, several published studies have described EET mitogenic activities [23,25,32,53], and 11,12- and 14,15-EET were shown to promote tubular formation in studies in which their effects were analyzed within either 18 h [22] or 14 days [23] of cell plating and EET treatment. Both the mitogenic and tubulogenic properties of the EETs have been linked to the activation of MAPK pathways [22,25,52]. Since 11,12-EET is a major product of the Cyp2c44 epoxygenase [54], and it is formed by LEC it was selected for further studies.

VEGF signaling is sensitive to epoxygenase inhibition

Ketoconazole is an effective epoxygenase inhibitor that has been previously used for studies of growth factor and EET induced cell proliferation [25,57]. To explore the role of the AA epoxygenase and the EETs in VEGF signaling and angiogenic activity, we analyzed the effects of Ketoconazole on VEGF-stimulated LEC growth and tubulogenesis. As shown in Fig. 6A, Ketoconazole (10 μM, final concentration) almost completely abolished the proliferative responses of LEC to VEGF stimulation. Importantly, 11,12-EET, when added in conjunction with ketoconazole and VEGF, rescued cell proliferation from inhibition, albeit to levels lower than those achieved with VEGF or 11,12-EET alone (Fig. 6A). Similarly, Ketoconazole blunted the tubulogenic effects of VEGF (Fig. 6B and C) and again, 11,12-EET was able to partially rescued the cells from the effects of the epoxygenase inhibitor, although in this case, tubulogenesis was restored to levels comparable to those elicited by 11,12-EET (Fig. 6B). These data indicate that, as it has been observed with serum stimulated mouse lung endothelial cells in primary culture [25], a portion of the LECs functional responses to VEGF are epoxygenase mediated and that its 11,12-EET metabolite is a component of these responses. On the other hand, the inability of 11,12-EET to fully rescue the cells from the effects of Ketoconazole on VEGF-stimulated proliferation could reflect yet to be defined side effects of the inhibitor. In this regard, the growth responses to

the 11,12-EET were also significantly reduced when the EET was added in the presence of Ketoconazole (not shown).

The Cyp2c44 epoxygenase is a component of the VEGF signaling pathways

While the studies summarized provided compelling evidence for an epoxygenase role in VEGF-stimulated LEC proliferation and tubulogenesis, their interpretation was complicated by the potential for additional, non-epoxygenase, effects of Ketoconazole. As mentioned, Cyp2c44 is an active EET synthase [54], is present in LECs (Fig. 3A and B), and its expression is sensitive to VEGF stimulation (Fig. 3A and B). To better define the role of this epoxygenase in VEGF-stimulated LEC proliferation, cells were transfected with either a non coding (mock) plasmid, or with a plasmid coding for a Cyp2c44 selective silencing RNA (Cyp2c44 siRNA). Plasmid bearing cells were then obtained by standard G-418 selection techniques, and the expression of Cyp2c44 analyzed by real time quantitative RT-PCR. As shown in Fig. 7A, and compared to mock transfected cells, the Cyp2c44 siRNA transfected LECs show marked reductions in their levels of Cyp2c44 mRNA transcripts (Fig. 7A). However, due to the low amounts of microsomal protein that could be isolated from siRNA transfected cells, we were unable to characterize the effects of the siRNA on Cyp2c44 protein levels.

The functional consequences of the siRNA induced reductions in Cyp2c44 mRNA levels are illustrated in Fig. 7B and C. Thus, compared to mock transfected cells, siRNA expressing LECs show significant reductions in their basal and VEGF-stimulated growth as determined by [^{3}H]thymidine DNA incorporation (Fig. 7B and C). Reductions in LEC growth rates associated with the Cyp2c44 siRNA plasmid were also observed during the antibiotic selection and subsequent plating of Cyp2c44 siRNA expressing LECs. Thus, when seeded at similar densities in EBM2 media, mock transfected cells reached confluence in approximately half the time of those expressing the Cyp2c44 siRNA (not shown). As with Ketoconazole inhibition, the effects of the Cyp2c44 siRNA on VEGF-stimulated cell proliferation were partially restored by the addition of 11,12-EET (Fig. 7C), suggesting again that while the Cyp2c44 epoxygenase is required for the full growth effects of VEGF, its 11,12-EET metabolite is but one of the components of the signaling cascades elicited upon VEGF stimulation. Of interest, mock and Cyp2c44 siRNA transfected LECs responded to mixtures of 11,12-EET and VEGF with increased proliferation and, in both cases, the combined effects of VEGF and 11,12-EET were not that different from those elicited by 11,12-EET (not shown). Taken together, these results indicate: (a) a regulatory and/or functional redundancy for the epoxygenase branch of the AA cascade, a feature common to several intracellular signaling cascades, and (b) the presence of additional LEC epoxygenase enzyme(s) [40], that could partially compensate for the reductions in Cyp2c44 levels brought about by the Cyp2c44 siRNA. As illustrated in Fig. 7B and C, even when cultured in serum free media, Cyp2c44 siRNA transfected LECs maintain a basal level of growth, indicating that either: (a) the siRNA expressing cells express levels of residual Cyp2c44 that are sufficient to sustain basal growth, (b) the presence of yet to be identified epoxygenase isoforms involved in VEGF-independent proliferative activity, or (c) that different, EET-independent, mechanisms could be responsible for non-stimulated LEC growth and proliferation. In summary, the studies show that the Cyp2c44 epoxygenase is required to attain maximal basal and VEGF stimulation proliferation, and that, as with Ketoconazole inhibition, exogenously added 11,12-EET partially restores the growth properties of Cyp2c44 siRNA transfected cells.

As shown in Fig. 8, compared to mock transfected cells, the siRNA induced decreases in Cyp2c44 epoxygenase expression were accompanied by significant reductions in LEC basal and VEGF-stimulated tubulogenic activity (Fig. 8A, B, C, and D). Furthermore, as shown in Fig. 8B and D, the Cyp2c44 siRNA transfected LEC responded to the addition of 11,12-EET

with a more than doubling in their capacity to generate tubule-like structures and, at difference with cell growth (Fig. 7B and C), the EET is significantly better than VEGF in stimulating tubulogenesis in siRNA transfected cells (Fig. 8B). Finally, and to explore the mechanistic basis of the growth and tubulogenic effects associated with the Cyp2c44 siRNA transfection, we studied the phosphorylation state of the Akt and ERK1/2 kinase present in cells transfected with mock or siRNA plasmids, before and after cell stimulation with VEGF, AA, mixtures of AA and VEGF, or 11,12-EET. As shown by the immunoblots in Fig. 9, mock transfected cells in serum free media, responded to VEGF, AA, a combination of AA and VEGF, or 11,12-EET with significantly increases in ERK1/2 and Akt phosphorylation (Fig. 9). In contrast, the effects of VEGF and AA, added individually or in combination, were markedly attenuated in Cyp2c44 siRNA expressing LECs (Fig. 9). As observed during the proliferation and tubulogenesis studies, 11,12-EET was able to partially restore ERK1/2 and Akt phosphorylation in cells expressing the Cyp2c44 siRNA plasmid (Fig. 9). Furthermore, we consistently observed small but, detectable levels of ERK1/2 and Akt activation after the addition of mixtures of VEGF and AA to the Cyp2c44 siRNA transfected cells (Fig. 9), suggesting again that the LECs contain additional Cyp2c epoxygenases and that these may be upregulated to compensate for the effects of the siRNA on Cyp2c44 protein levels. In previous studies with primary cultures of mouse lung endothelial cells, we noted significant differences between the four EET regioisomers in their ability to stimulated either cell growth or cell migration, a reflection of their differential effects on the activation of the ERK1/2, P38 MAPK or PIK3 pathways [25]. These results, as well as those obtained using Ketoconazole to inhibit epoxygenase activity support a role for the AA epoxygenase, Cyp2c44 in particular, as a component of the signaling cascade that mediates VEGF-stimulated LEC proliferation and tubulogenesis. Furthermore, they identify ERK1/2 and Akt as targets of the EET-mediated proliferative responses induced upon VEGF stimulation.

The characterization of a role for the AA epoxygenases in endothelial cell proliferation [25,32,40,53] and VEGF signaling, as well as previous studies of its roles in EGF-induced mitogenesis and signaling [33–35] point to the EETs as regulators of intracellular MAPK and PI3K cascades. Several published studies have documented a role for ion channels, K^+ channels in particular, in the proliferative responses associated with growth factor induced MAPK activation [58–63]. The studies reported here, as well as abundant published data identifying the EETs as mediators of EGF signaling [33–35], and regulators of K^+, Na^+, and Ca^{++} channel activity [26–29], are suggestive of a more general role for these eicosanoids as intracellular signaling lipids, and provide a potential logical link between the seemingly unrelated biological activities of these compounds as both vasoactive and mitogenic mediators [26–29]. Finally, in addition to their physiological implications, these studies raise important questions regarding the potential for pathophysiological consequences of experimental and/or genetically induced changes in AA epoxygenase expression or EET levels. Thus, for example, reductions in endothelial cell Cyp2c expression and systemic EET levels were recently associated with the anti-angiogenic and anti-tumorogenic effects of PPARα ligand activation [40].

In summary, the studies presented here identify: (a) the Cyp2c44 AA epoxygenase as a gene target for VEGF-mediated regulation, (b) the EETs as a components of the proliferative and angiogenic responses of cultured endothelial cells to VEGF stimulation, and (c) the PI3/Akt and MAPK/ERK1/2 as intracellular signaling targets for the EET-mediated, VEGF-induced changes in endothelial cells growth and tubulogenic activities. Since the EETs have been shown to be effective *in vivo* angiogens [25], these results suggest a role for the endothelial epoxygenases in VEGF-mediated physiological and/or pathophysiological processes that led to increases *in vivo* vascularization. It is apparent that defining the mechanism(s) by which the EETs contribute to VEGF hormonal signaling should lead to a better understanding of

basic vascular biology and have a positive impact in effort to develop new approaches for cancer treatment. The latter could be of special relevance to ongoing efforts designed to inhibit VEGF-dependent tumor vascularization and thus, prevent or reduced tumor growth [5–8].

Acknowledgments

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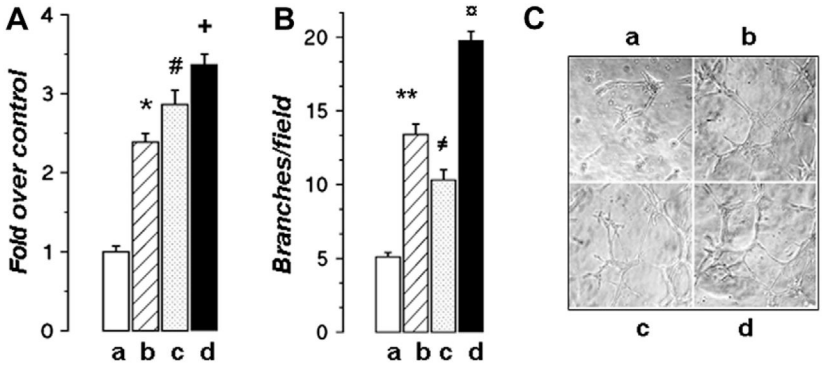

Fig. 1.

VEGF, arachidonic acid, and growth factor supplemented media (EBM2 media stimulate LEC proliferation (A) and tubulogenesis (B and C): (A) LEC cells (5×10^3 cell/well; 96 well plate) were serum starved for 48 h prior to changing the media to either serum free DMEM (a), or serum free DMEM containing either VEGF (b), (50 ng/ml, final concentration), arachidonic acid (c), (1 μM, final concentration), or a mixture of VEGF and arachidonic acid (d), (50 ng/ml and 1 μM final concentrations, respectively). Immediately after, [^{3}H]thymidine (0.5 μCi/well) was added, the cells cultured for an additional 48 h, and the extent of thymidine incorporation into cellular DNA determined as described in Methods. Values shown are changes relative to cells cultured in serum free media and treated with vehicle (EtOH), and are averages ± SEM calculated from four different experiments each done in triplicate. Significantly different from vehicle controls, $^{*}p < 2 \times 10^{-6}$; $^{\#}p < 3 \times 10^{-7}$; $^{+}p < 10^{-9}$. Significantly different from VEGF, $^{+}p < 2 \times 10^{-3}$. The differences between cells treated with arachidonic acid *vs* VEGF, or with the fatty acid and VEGF *vs* VEGF were non significant, $p > 0.05$. (B) LECs were cultured in serum free DMEM for 24 h and then plated onto Matrigel (1.5×10^4 cells/well; 96 well plates) in the presence or absence of either vehicle (a), VEGF (b), arachidonic acid (c), or a mixture of arachidonic acid and VEGF (d), all added at the concentrations used in A. After 6 h at 37°, the cells were fixed and tubule-like structures and branching nodules recorded and quantified as described in Methods. Shown are values calculated from three different experiments each done in triplicate. Significantly different from vehicle controls, $^{**}p < 10^{-9}$; $^{\neq}p < 5 \times 10^{-11}$; $^{\hat{E}}p < 4 \times 10^{-11}$. Significantly different from VEGF, $^{\neq}p < 3 \times 10^{-6}$. Significantly different from arachidonic acid, $^{\hat{E}}p < 4 \times 10^{-7}$. (C) Representative digital images of cells fixed after a 6 h treatment with either vehicle (a), VEGF (b), arachidonic acid (c), VEGF and arachidonic acid (d), (added at the same concentrations as above). Shown are images obtained at 40× magnification.

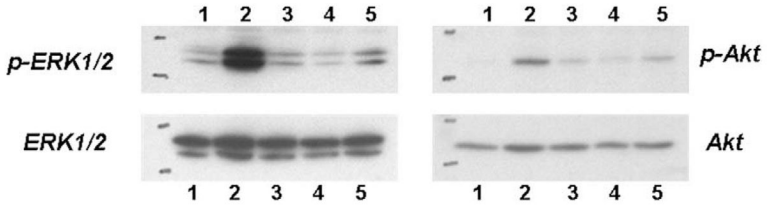

Fig. 2.

LEC cells respond to growth stimulation with increases in the phosphorylation states of ERK1/2 and Akt: Semi-confluent cultures of LEC (2.5×10^5 cells/100 mm dish) were cultured for 48 h in serum free DMEM, prior to the addition of VEGF (50 ng/ml final concentration). After 5, 10, 20, or 40 min of adding VEGF, the cells were suspended in ice cold PBS, collected by centrifugation, lyzed, and submitted to SDS–PAGE (20–40 μg of protein/lane) and Western blot analysis using antibodies raised against either phospho-ERK1/2 (p-ERK), phospho-Akt (p-Akt), ERK1/2 or Akt and as described in Methods. Loadings were normalized based on immuno-reactivities towards anti-ERK1/2 and anti-Akt antibodies. Lane 1 contained lysates obtained from cells exposed to vehicle for 40 min, while lanes 2, 3, 4, and 5 lysates obtained 5, 10, 20, and 40 min after VEGF addition, respectively. The electrophoretic mobilities of 50 and 37 kD (ERK1/2K panel) or 75 and 50 kD (Akt panel) protein standards are marked at the left edge of each panel.

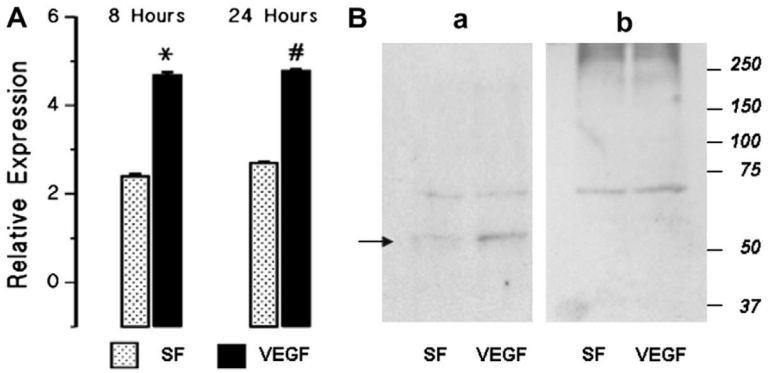

Fig. 3.

LEC express a VEGF-inducible Cyp2c44 arachidonic acid epoxygenase: LECs were cultured in serum free DMEM for 48 h prior to the addition of VEGF (50 ng/ml final concentration) and 8 or 24 h after, the cells were removed with ice cold PBS, collected by centrifugation, and the cell pellets used for either RNA extraction or immunoelectrophoresis as described in Methods. (A) Real time Quantitative PCR analysis of Cyp2c44 expression. Samples of cell RNAs were reversed transcribed and analyzed for β-actin and Cyp2c44 expression as described in Methods. Cyp2c44 expression levels were normalized using β-actin mRNAs as reference. Shown are averages ± SEM calculated 2 different experiments, each performed in triplicates. Significantly different from 8 h controls, $^{*}p < 4 \times 10^{-6}$. Significantly different from 24 h controls, $^{\#}p < 3 \times 10^{-6}$. (B) Immunoelectrophoresis analysis of Cyp2c44 levels. Microsomal fractions from LECs cultured for 24 h in serum free DMEM or VEGF containing serum free DMEM were isolated by differential centrifugation and proteins resolved by 10% SDS–PAGE (40 μg cell protein/lane). Western blot analyses (panel a) were done using a Cyp2c44 immunoreactive rabbit anti-rat CYP2C23 antibody (Cyp2c44 and CYP2C23 share 89% amino-acid identity) as described in Methods. Equal protein loadings were confirmed by Ponceau staining of the membranes used for Western blots (panel b). The electrophoretic mobilities of selected protein standards are shown.

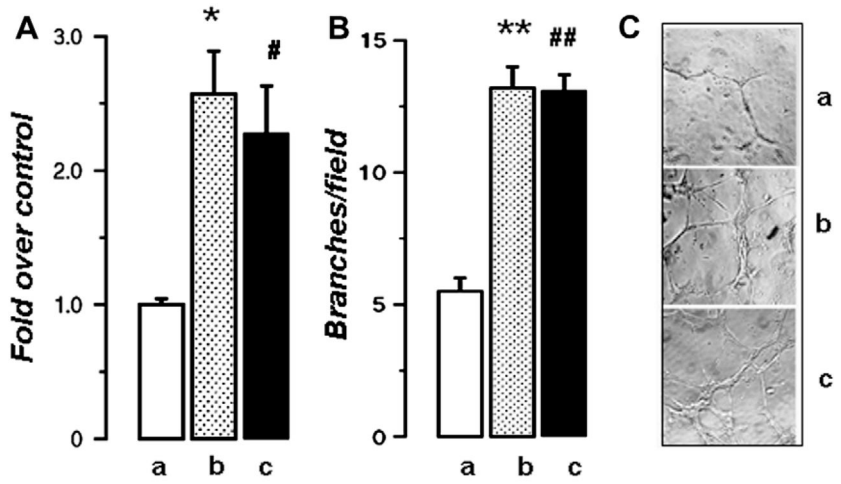

Fig. 4.

LEC proliferation and tubulogenesis are stimulated by the addition of 11,12-EET or 14,15-EET: (A) LEC cells (5×10^3 cell/well; 96 well plate) were serum starved for 48 h prior to the addition serum free DMEM (a) or serum free DMEM containing 11,12-EET (b) or 14,15-EET (c) (1 μM final concentration each). Immediately after, [^{3}H]thymidine (0.5 μCi/well) was added, the cells cultured for an additional 48 h, and the incorporation of thymidine into cellular DNA determined described in Methods. Values shown are changes relative to cells cultured in serum free media and treated with vehicle (EtOH) (a), and are averages ± SEM calculated from three different experiments each done in triplicate. Significantly different from vehicle controls, $^{*}p < 2 \times 10^{-4}$; $^{\#}p < 2 \times 10^{-3}$. (B and C) LECs in serum free DMEM containing either the EtOH vehicle (a), 11,12-EET (b), or 14,15-EET (c) at the concentrations listed above, were plated onto Matrigel (1.5×10^4 cells/well; 96 well plate) and, after 6 h at 37°, the formation of tubule-like structures was recorded by light microscopy and quantified as describe in Methods and Fig. 1B and C. Shown are averages ± SEM, calculate from 3 different experiments each done in triplicate (B), and images of fixed cells (40× magnification) (C). Significantly different from vehicle controls, $^{**}p < 0.03$; $^{\#\#}p < 0.01$.

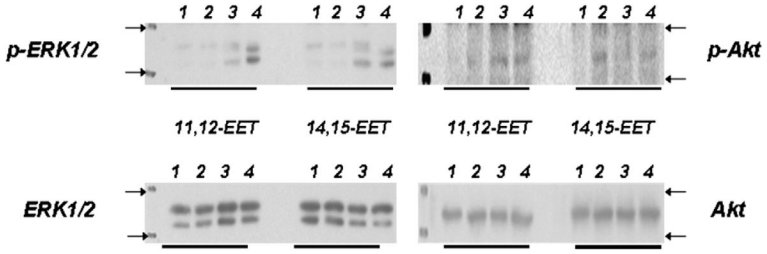

Fig. 5.

LEC cells respond to the addition of 11,12-or 14,15-EET by increasing the phosphorylation states of ERK1/2 and Akt: Semi-confluent cultures of LEC (2.5×10^5 cells/100 mm dish) were cultured for 48 h in serum free DMEM, prior to the addition of either 11,12-EET or 14,15-EET (1 μM each, final concentration). 20 min after the addition of the vehicle (lane 1) or 5, 10, or 20 min after the EET addition (lanes 2, 3, and 4, respectively) the cells were suspended in ice cold PBS and process and analyzed as detailed in Methods and the legend to Fig. 2. The arrows indicate the electrophoretic mobilities of 50 and 37 kD (ERK1/2K panel) or 75 and 50 kD (Akt panel) protein standards.

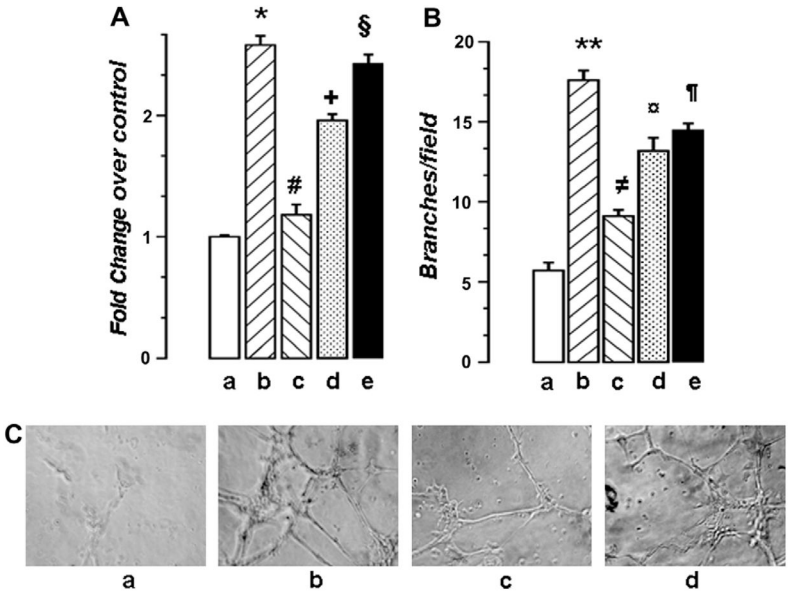

Fig. 6.

The VEGF stimulation of LEC proliferation and tubulogenesis is sensitive to epoxygenase inhibition: LECs were serum starved for 48 h prior to replacing the media with DMEM containing either vehicle (EtOH) (a); VEGF (b); a mixture of Ketoconazole and VEGF (c); a mixture of Ketoconazole, VEGF, and 11,12-EET (d) or only 11,12-EET (e);. For all treatments, VEGF, 11,12-EET, and Ketoconazole were added at 50 ng/ml; 1 and 10 μM, final concentrations, respectively. Measurements of cell proliferation (A) were done as detailed in Methods and the legend to Fig. 1, and are expressed as changes relative to cells cultured in serum free media and treated with vehicle (EtOH). Shown are averages ± SEM, calculate from 3 different experiments each done in triplicate. Significantly different from vehicle controls, $p < 5 \times 10^{-7}$ for *, #, +, and §. Significantly different from VEGF, $^{\#}p < 3 \times 10^{-6;}$ $^{+}p < 4 \times 10^{-3;}$ $^{\S}p < 0.05$. Significantly different from 11,12-EET, $^{+}p < 0.01$. For analysis of tubulogenic activity (B and C) the LECs in serum free DMEM were plated onto Matrigel (1.5×10^4 cells/well; 96 well plate), and the formation of tubule-like structures recorded by light microscopy, and quantified as described in Methods and the legend to Fig. 1. Show are averages ± SEM, calculated from 3 different experiments each done in triplicate (B), and images of fixed cells (40× magnification) (C). Significantly different from vehicle controls, $^{**}p < 1 \times 10^{-9;}$ $^{\neq}p < 2 \times 10^{-8}$; Ê $p < 1 \times 10^{-11;}$ $^{¶}p < 1 \times 10^{-7}$. Significantly different from VEGF, $^{\neq}p < 2 \times 10^{-7;}$ $^{\hat{E}}p < 2 \times 10^{-3}$. Significantly different from 11,12-EET, $^{\neq}p < 4 \times 10^{-3}$. The differences between d and e, and between b and e were not statistically significant ($p > 0.05$).

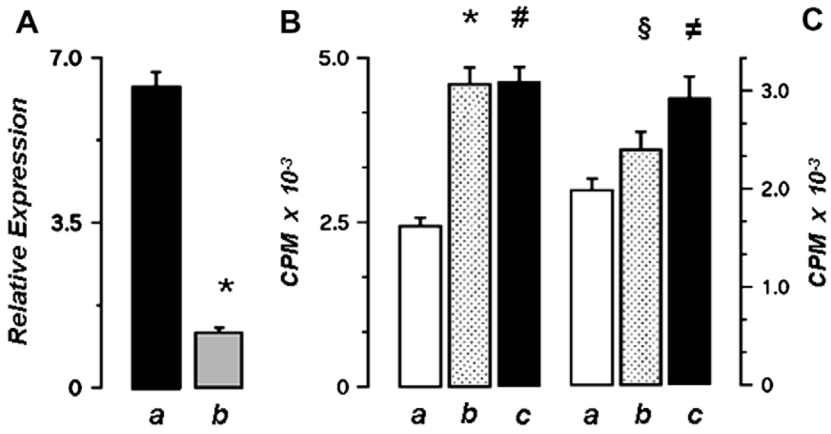

Fig. 7.

Cells expressing a Cyp2c44 epoxygenase selective siRNA show reductions in Cyp2c44 mRNA transcripts and in the proliferative response to VEGF stimulation. LECs were transfected with either a control (Mock) plasmid or a plasmid coding for a Cyp2c44 specific small interference RNA (siRNA), and selected for plasmid expression as described in Methods. (A) Total RNAs were extracted from Mock (a) and siRNA (b) transfected cells and reversed transcribed as detailed in Methods. The levels of Cyp2c44 mRNA transcript were estimated by Real time Quantitative PCR analysis, and expression levels normalized using the β-actin mRNAs as reference. Shown are averages ± SEM, calculated from 3 different experiments, each done in triplicates. Significantly different from mock transfected cells, $^{*}p < 3 \times 10^{-5}$. Mock (B) and Cyp2c44 siRNA (C) transfected LECs (5×10^3 cells/well, 96 well plates) were serum starved for 48, prior to replacing the media with serum free DMEM containing [^{3}H]Thymidine (0.5 μCi/well). Immediately after the cells received either the EtOH vehicle (a), VEGF (b), or 11,12-EET (c) (<0.1%; 50 ng/ml, or 1 μM, final concentrations each, respectively). After a 48 h culture, thymidine incorporation into cellular DNA was determined described in Methods. Shown are averages ± SEM calculated from three different experiments each done in triplicates. (B) Significantly different from mock vehicle controls, $^{*}p < 3 \times 10^{-7}$; $^{\#}p < 2 \times 10^{-8}$. The differences between the * and # bars were not statistically significant ($p > 0.05$). (C) Significantly different from vehicle treated siRNA cells, § $p < 9 \times 10^{-5}$; $^{\neq}p < 7 \times 10^{-6}$. Significantly different from VEGF treated siRNA cells, $^{\neq}p < 0.02$.

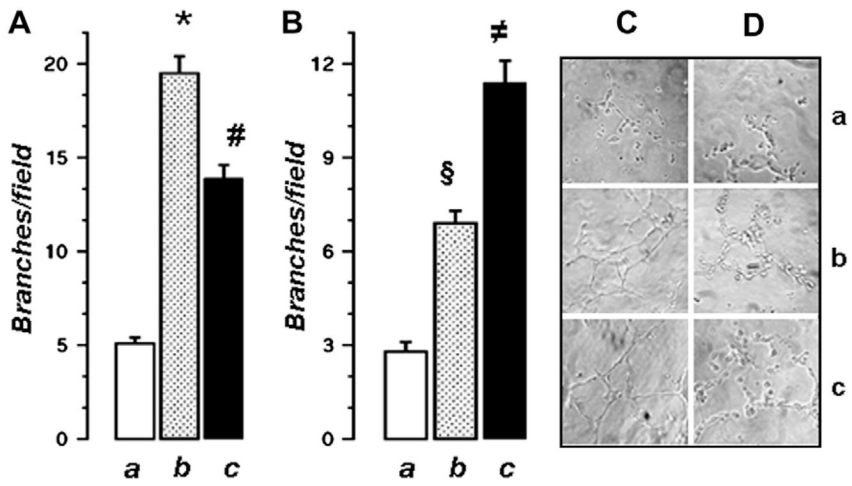

Fig. 8.

Cells expressing a Cyp2c44 epoxygenase selective siRNA show a reduced tubulogenic activities in response to the addition of VEGF or 11,12-EET: Serum starved (48 h) Mock or Cyp2c44 siRNA transfected LECs in serum free DMEM were plated onto Matrigel (1.5×10^4 cells/well; 96 well plate) in the absence (a) or presence of either VEGF (b) or 11,12-EET (c) (50 ng/ml and 1 μM final concentrations each, respectively), and the formation of tubule-like structures recorded by light microscopy and quantified as described in Methods and Fig. 1A and B. A and B show the number of branches/field generated by mock (A) or siRNA (B) transfected cells, expressed as averages ± SEM calculate from three different experiments each done in triplicate. (A) Significantly different from vehicle controls, $^{*}p < 2 \times 10^{-13;}$ $^{\#}p < 3 \times 10^{-14}$. Significantly different from VEGF, $^{\#}p < 0.001$. (B) Significantly different from vehicle controls $^{\S}p < 1 \times 10^{-9;}$ $^{\neq}p < 2 \times 10^{-13}$. Significantly different from VEGF, $^{\neq}p < 2 \times 10^{-6}$. C and D are images of mock (C) or siRNA (D) transfected LECs platted on Matrigel in the presence of DMEM containing vehicle (a), VEGF (b), or 11,12-EET (c), and fixed 6 h after platting (40× magnification).

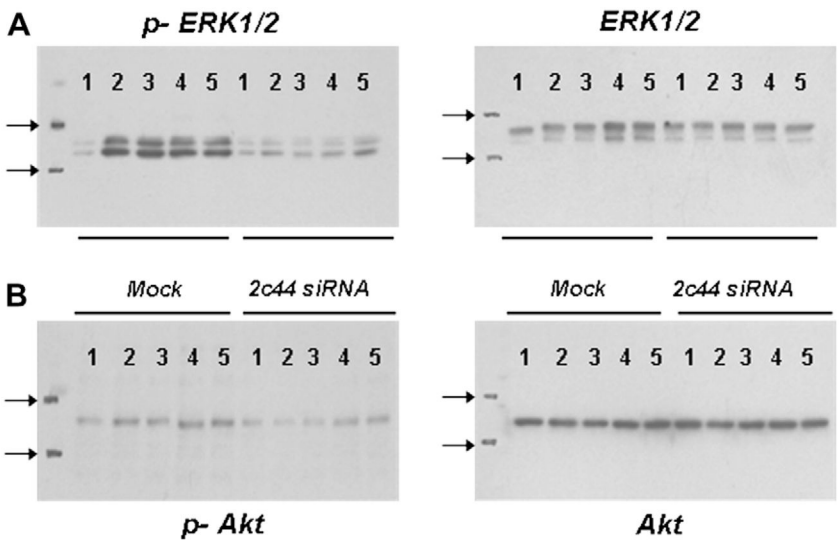

Fig. 9.

Cells expressing a Cyp2c44 epoxygenase selective siRNA show reductions in VEGF-induced ERK1/2 and Akt activation: Semi-confluent cultures of mock or Cyp2c4 siRNA transfected LECs (2.5×10^5 cells/100 mm dish) in serum free DMEM were cultured for 48 h, prior to replacing the media with DMEM containing the ethanol vehicle (lane 1), VEGF (lane 2), arachidonic acid (lane 3), a mixture of VEGF and arachidonic acid (lane 4), or 11,12-EET (lane 5) (final concentrations: ≤0.1% for ethanol, 50 ng/ml for VEGF, and 1 μM for AA and 11,12-EET). The cells were lyzed 10 min after stimulation, and the levels of phospho-ERK1/2 (p-ERK) and ERK1/2 (A) and of phospho-Akt (p-Akt) and Akt (B) analyzed by Western blot as described in Methods and in the Fig. 2 legend. The arrows show the electrophoretic mobilities of 50 and 37 kD (A) or 75 and 50 kD (B) protein standards.