Cytochrome P450 2C9-Induced Angiogenesis Is Dependent on EphB4

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Objective—Cytochrome P450 (CYP) epoxygenase-derived epoxyeicosatrienoic acids (EETs) are known to stimulate angiogenesis, but the mechanisms involved are incompletely understood. Because EphB4 is involved in vascular development, the aim of this study was to investigate whether, and to what extent, EphB4 is part of the signaling cascade that results in CYP2C9-mediated angiogenesis.

Methods and Results—CYP2C9 overexpression as well as stimulation with 11,12-EET (up to 48 hours) time-dependently increased EphB4 expression in endothelial cells. This effect and the activation of the EphB4 promoter were mediated by the phosphatidylinositol-3-kinase (PI3-K)/Akt pathway and sensitive to the PI3-K inhibitor LY 294002 as well as to simultaneous transfection with dominant-negative Akt. 11,12-EET treatment also increased EphB4 expression in isolated mouse mesenteric arteries as well as in the vessels that developed in 11,12-EET-impregnated Matrigel plugs. Moreover, the CYP2C9-stimulated formation of capillary-like structures in a modified spheroid assay was markedly attenuated by EphB4 downregulation (antisense oligonucleotides). Using a parallel approach in vivo, the inclusion of siRNA directed against EphB4 in EET-impregnated Matrigel plugs prevented endothelial cell invasion and vascularization.

Conclusions—Our data indicate that EphB4 is a critical component of the CYP2C9- and 11,12-EET-activated signaling cascade that promotes angiogenesis in vitro as well as in vivo. (Arterioscler Thromb Vasc Biol 2008;28:000-000.)

Key Words: ●●●

Cytochrome P450 (CYP) epoxygenases, of the CYP2B, 2C, and 2J subfamilies are expressed in endothelial cells, and metabolize arachidonic acid to different regioisomers of epoxyeicosatrienoic acid (EET; for recent reviews see1,2). Interest in the vascular actions of CYP enzymes, and consequently EETs, was initially linked to their identification as endothelium-derived hyperpolarizing factors (EDHFs).3,4 However, it is now generally appreciated that these compounds mediate a number of membrane potential-independent effects, including endothelial cell proliferation, migration, and angiogenesis.5 Relatively little is known about the molecular mechanisms underlying CYP epoxygenase/EET-induced angiogenesis. However, CYP epoxygenase-derived metabolites of arachidonic acid are reported to transactivate the EGF receptor by activating matrix metalloproteinas to release heparin-binding EGF-like growth factor (HB-EGF) from the cell membrane.6,7 The EET-mediated activation of the EGF receptor leads in turn to the activation of the kinase Akt and an enhanced expression of cyclin D1. Other signaling pathways also contribute to the increase in cyclin D1 expression including the MAP kinase phosphatase-1 which decreases JNK activity.8 Activation of Akt by EETs also induces the phosphorylation and therefore inhibition of the forkhead factors FoxO1 and FoxO3a and subsequently a decrease in the expression of the cyclin-dependent kinase inhibitor p27kip1.9 EphB4 receptor and its ligand ephrinB2 play an important role in neuronal patterning and vascular development during embryogenesis (for review see10). Although classically ephrinB2 was thought to be expressed in arterial endothelial cells whereas EphB4 was expressed preferentially in venous endothelial cells,11,12 more recent observations suggest that endothelial cells in newly developing vessels of the adult, at least in the cornea and remodeling rat mesenteric microvessels, express different members of the Eph/ephrin family of proteins, including ephrinB2 and EphB4.13,14

The aim of the present investigation was to assess the role of EphB4 in CYP2C-mediated angiogenesis in vitro as well as in vivo and to determine whether EETs contribute to angiogenic signaling pathways upstream of EphB4.

Materials and Methods

Materials
Matrigel was from BD Biosciences, 11,12-EET was from Cayman Chemicals and MSPOOH was kindly provided by John R. Falck

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The polyclonal antimouse EphB4 antibody was purchased from R&D Systems, and the monoclonal mouse anti-EphB4 antibody was from Zymed. The monoclonal anti-mouse PECAM-1 (clone: MEC 13.3) antibody was from Santa Cruz Biotechnology, and the antibodies directed against Akt and phospho-Ser473 Akt were from Cell Signaling. All other substances were from Sigma.

Cell Culture
Human umbilical vein, porcine aortic, and murine lung endothelial cells were isolated and cultured as described previously. First passage human umbilical vein endothelial cells were used throughout. Porcine aortic and murine lung endothelial cells were used up to passage 5.

Adenoviral Transduction
Endothelial cells (80% confluent) were serum-starved for 10 hours before infection with adenoviral vectors. Cells were incubated with recombinant adenoviruses (10 pfU/cell) expressing either CYP2C9 sense or antisense cDNA in medium for 4 hours and afterward left to recover in medium containing 2% FCS. As reported previously, infection efficiency was between 90% and 100%, and those cells infected with CYP2C9 sense adenovirus generated approximately 2-fold more 11,12- and 14,15-EET under basal conditions than cells infected with the control (CYP2C9 antisense) virus. This increase in EET production was sensitive to the CYP2C9 inhibitor miconazole.

Downregulation of EphB4 Expression
EphB4 expression was downregulated in HUVECs using an antisense approach, as described. Briefly, endothelial cells (80% confluent) were treated with either EphB4 antisense oligonucleotides (5'-ATGGAGGCCTCGCTCAGAAA-3', 2 μmol/L) or control oligonucleotides (EphB4 scrambled: 5'-TACCTGAAGGTCAGGCAAC-3', 2 μmol/L), using Gene Trans II according to the manufacturer’s instructions (MobiTec). Thereafter, cells were left to recover in medium containing 2% FCS. Mouse lung endothelial cells were infected with either control or CYP2C9 adenoviruses and then immediately transfected with either a control siRNA (GFP) or 1 of 3 siRNAs directed against EphB4 (siRNA1: 5'-AAG-UAG-GUC-AAG-UUC-GUG-A-3', siRNA2: 5'-UGU-CUC-CUA-UGU-CAA-GAU-U-3', siRNA3: 5'-AU-CUU-GAC-AU-A-GGA-GAC-3'), using TransPass HUVEC Transfection Reagent (New England BioLabs). Cells were then cultured in medium containing 2.5% FCS for an additional 48 hours.

Immunoblotting
Cells were lysed in sample buffer and separated by SDS-PAGE as described. Proteins were detected using specific antibodies as described in the results section.

Reporter Gene Assay
Porcine endothelial cells were transiently cotransfected with the EphB4 promoter luciferase construct as described, together with either pcDNA 3.1 or CYP2C9. After 36 hours, the cells were lysed, and luciferase activity was measured according to the manufacturer’s protocol (Promega).

Endothelial Cell Spheroids
Spheroids containing 400 cells were generated as described. After 24 hours in a collagen gel, angiogenesis was quantified by measuring the cumulative length of all capillary like sprouts originating from the central plain of an individual spheroid using a computer assisted microscope (Axio-Observer Z1, Zeiss). At least 5 randomly selected spheroids per experimental group and experiment were analyzed.

In Vivo Matrigel Plug Assay
Female C57BL/6 mice (8 weeks old) were lightly anesthetized with chloralhydrate (200 μL of a 4% solution, s.c.) and then injected with...
0.5 mL of Matrigel impregnated with heparin (0.0025 U/mL, s.c.) and 11,12-EET (10 µmol/L) and containing either a control siRNA (GFP; 2 µmol/L) or siRNA directed against EphB4 (siRNA1; 2 µmol/L) along the dorsal midline on each side of the spine. After 7 days the mice were euthanized, the Matrigel plugs removed, embedded in tissue tech (Sakura Finetec) and frozen. Plugs were then either cryo-sectioned (10 µm) and processed for staining for PECAM or harvested and washed with phosphate-buffered saline solution. The latter samples were mixed with water (200 µL), homogenized, and centrifuged at 14,000 rpm for 15 minutes to remove particulate material and the concentration of the hemoglobin contained in the plugs was determined at 595 nm using Drabkin reagent (Sigma). In some experiments Matrigel implants were fixed in zinc fixative overnight, dehydrated using alcohol and isopropanol, and processed for paraffin sectioning. EphB4 expression in the Matrigel plugs was determined using a polyglonal goat antinouse EphB4 antibody in combination with an enhanced detection method (Envision, DAKO) 3, 3′-diaminobenzidine enhanced liquid substrate system solution A and B (DAB) (Sigma) and Mayers Hematoxylin Solution (Sigma).

Vessel formation was quantified by analyzing pixel/area of at least 5 sections per plug using a computer-assisted program (Nikon NIS-Elements). The data obtained were normalized with respect to values recorded in the respective control.

**Results**

**Effect of CYP2C9-Derived EETs on Endothelial EphB4 Expression In Vitro and In Vivo**

Although CYP2C protein is expressed in native endothelial cells, expression levels decrease rapidly after cell isolation. Therefore, to determine whether or not a link exists between CYP2C9 and EphB4 expression, human umbilical vein endothelial cells were infected with either a control (CYP2C9 antisense) adenovirus or CYP2C9 overexpression viruses. Although low levels of EphB4 were detected in the endothelial cells used, CYP2C9 overexpression resulted in a 4-fold increase in EphB4 expression within 24 hours compared to control virus-treated cells (Figure 1A). The increase in EphB4 expression could be attributed to elevated CYP epoxygenase activity as the CYP2C9-induced (48 hours) increase in its expression was inhibited in the presence of the epoxygenase inhibitor, MSPPPOH (10 µmol/L, Figure 1B). Moreover, exogenously applied 11,12-EET (1 µmol/L) was also able to elicit a time-dependent increase in endothelial EphB4 expression (Figure 1C). Although ephrinB2, the main ligand for EphB4 was expressed by the endothelial cells used, there was no detectable change in the expression of the protein after the overexpression of CYP2C9 (data not shown).

To ensure that similar changes in EphB4 expression can be observed in endothelial cells in situ, isolated mesenteric arteries and vein were incubated with either solvent or 11,12-EET (1 µmol/L) and EphB4 was assessed by immunofluorescence staining after 18 hours. Quantification of fluorescence staining confirmed the EET-induced increase in EphB4 levels in mesenteric arteries (Figure 2A). Although EphB4 is reportedly to be preferentially expressed in veins, a much weaker response was detected in mesenteric vein endothelial cells treated with 11,12-EET (supplemental Figure I, available online at http://atvb.ahajournals.org).

To determine whether a link exists between 11,12-EET and the in vivo expression of EphB4, Matrigel plugs were impregnated with 11,12-EET and implanted in mice for a total of 14 days. Thereafter, the expression of EphB4 in the Matrigel plug vessels was assessed by immunohistochemis-
try. As in the in vitro assays, we were able to detect EphB4 positive vessels in the Matrigel plugs (Figure 2B).

Effect of CYP2C9-Overexpression and Inhibition With LY 294002 on EphB4 Expression

As EETs, in particular 11,12- and 14,15-EET, have been reported to activate phosphatidylinositol 3-kinase (PI 3-K)/Akt signaling pathway in endothelial cells,9,12 we assessed the effects of CYP2C9 overexpression on the activity of the EphB4 promoter and the consequences of PI 3-K inhibition on this response.

The overexpression of CYP2C9 significantly increased EphB4 promoter activity compared to that detected in cells transfected with pcDNA (Figure 3A). This increase in luciferase activity was prevented in cells treated with the PI 3-K inhibitor LY 294002 (1.5 μmol/L). Similar effects on the expression of EphB4 protein were observed (Figure 3B).

The overexpression of CYP2C9 resulted in the phosphorylation of Akt (Figure 3C), an observation that confirms our previous report.12 In the same cells, the tyrosine phosphorylation of EphB4 was enhanced (supplemental Figure II). We next determined whether or not a dominant negative Akt mutant could inhibit the CYP2C9-induced increase in EphB4 expression. As before, EphB4 expression increased in cells treated with CYP2C9 adenoviruses and transfected with pcDNA (Figure 3D). However, no increase in EphB4 was observed in cells overexpressing CYP2C9 and transfected with the dominant negative Akt mutant.

Effect of EphB4 Antisense Oligonucleotides on CYP2C9-Induced Endothelial Cell Sprouting In Vitro

To assess the impact of EphB4 on CYP2C9-mediated angiogenesis in vitro, we performed a collagen-based in vitro spheroid assay using cells treated with scrambled or antisense oligonucleotides directed against EphB4.

Whereas control (scrambled) oligonucleotides were without effect, the EphB4 antisense oligonucleotides decreased basal EphB4 protein levels in human endothelial cells and completely prevented the CYP2C9-induced increase in protein expression (Figure 4A). We next incubated CYP2C9-overexpressing endothelial cells with EphB4 antisense oligonucleotides before generating spheroids and assessing sprouting. We observed a significantly attenuated CYP2C9-induced (24 hours) sprout formation in the presence of the EphB4 antisense oligonucleotides (Figure 4B).

Inhibition of 11,12-EET-Induced Angiogenesis In Vivo by EphB4 siRNA

To confirm the importance of EphB4 in CYP2C9-induced angiogenesis in vivo, we assessed the effect of including siRNA directed against murine EphB4 in solvent or 11,12-EET-impregnated Matrigel plugs.

As observed before in human umbilical vein endothelial cells, the overexpression of CYP2C9 in murine lung endothelial cells increase the expression of EphB4 (Figure 5). We then determined the ability of a control siRNA (GFP) and 3 different EphB4 siRNAs to downregulate murine endothelial EphB4 expression. Two of the siRNA tested (siRNA1 and siRNA2) prevented at least the CYP2C9-induced increase in EphB4 expression but only 1 of them (siRNA1) also decreased basal levels of the protein.

Matrigel plugs impregnated with either solvent or 11,12-EET together with either a control siRNA (GFP) or the siRNA1 characterized above were then implanted in mice for a total of 7 days. Whereas PECAM-1–positive endothelial cells could be detected in plugs treated with solvent, PECAM and β-actin positive vessels were detected in plugs impreg-
nated with 11,12-EET (Figure 6A). These plugs were also blood perfused as they contained measurable levels of hemoglobin (Figure 6B). There was no evidence of blood perfusion of the plugs containing either the CTL nor the EphB4 siRNA in the absence of 11,12-EET (supplemental Figure III). However, the inclusion of EphB4 siRNA1 in the Matrigel plugs completely prevented 11,12-EET-stimulated formation of PECAM-1– and β(H9252)-actin–expressing vessels and the accumulation of hemoglobin. As expected, the siRNA directed against EphB4 also attenuated the number of PECAM-1–positive cells detected in Matrigel impregnated with bFGF (supplemental Figure III).

Discussion
The results of the present investigation indicate that the overexpression of CYP2C9 as well as the direct application of 11,12-EET increases EphB4 mRNA and protein expression in human and murine endothelial cells. Moreover, the increase in EphB4 seems to be essential for the initiation of CYP epoxygenase/EET-induced angiogenesis as endothelial cell sprouting and the formation of vessels in Matrigel in vivo were prevented by the downregulation (antisense oligonucleotides and siRNA) of EphB4.

Our findings suggest that cytochrome P450-derived EETs enzymes play an important role regulating the expression of key molecules involved in angiogenesis and vascular development. However, the role of CYP epoxygenase-derived EETs may have gone largely unnoticed because of the lack of readily available selective tools to activate and inhibit the different enzymes. There are in addition other more basic considerations such as the fragility of CYP expression in some cultured endothelial cells as although CYP epoxygenases are expressed in native endothelial cells, their expression can decrease rapidly after cell isolation.25 This means that the majority of the in vitro assays of endothelial cell sprouting and angiogenesis have been determined in cells that express little or no CYP epoxygenase. Rather intriguingly, EET-induced angiogenesis has been observed when CYP levels are elevated in endothelial cells (either as the result of overexpression of a CYP epoxygenase,7,26 its induction by stimuli such as cyclic strain and hypoxia19,27), or when the EETs are derived from an extraendothelial source (such as EET-generating astrocytes,28,29 an EET-impregnated methylcellulose disk7 or CYP overexpressing tumor cells26,30). This implies that both intra- and extracellular sources of EETs are involved in angiogenesis. The molecular mechanisms by which EETs can stimulate such cellular responses are unclear, and although EET-specific binding sites have been reported on rat aortic smooth muscle cells31 and guinea pig monocytes,32 a specific EET receptor has yet to be identified. EETs can, however, affect several angiogenic signaling cascades.5 One EET-activated pathway clearly linked to angiogenesis is the transactivation of the EGF receptor6,7 which leads in turn to the activation of the serine threonine kinase Akt and an enhanced expression of cyclin D1.7,33,34 The activation of Akt by EETs also induces the phosphorylation, and therefore inhibition of the forkhead factors FoxO1 and FoxO3a and
subsequently a decrease in the expression of the cyclin-dependent kinase inhibitor p27kip1. The results of the present study indicate that CYP2C9 overexpression and the addition of exogenous 11,12-EET increase the expression of EphB4 in cultured human and murine endothelial cells and that the signaling cascade activated involves PI 3-K and Akt.

Signaling by the EphB4 receptor and its ligand ephrinB2 plays an important role in vascular development during embryogenesis. Although the exact consequences of Eph-ephrin interactions and forward and reverse signaling on endothelial cell function in adult animals are not clear, it has been proposed that the Eph-ephrin signals are likely to play a role in the spatial organization of developing/remodeling vasculature. The results of our investigation, however, indicate that EphB4 is essential for 11,12-EET-induced sprouting angiogenesis. Indeed, preventing the CYP2C9-induced upregulation of EphB4 markedly attenuated endothelial cell sprouting in a modified spheroid assay. Although a role for EphB4 in endothelial migration and tube formation has been proposed by others, these findings are somewhat controversial as EphB4 is classed as a negative regulator of blood vessel branching and vascular network formation, and is generally accepted to switch the vascularization program from sprouting angiogenesis to circumferential vessel growth. This implies that large vessels with few branches would be expected to form in vessels expressing EphB4.

Classically, ephrinB2 was thought to be expressed in arterial endothelial cells whereas EphB4 was restricted to venous endothelial cells. However, this no longer appears to be strictly the case, and although the arterial expression of ephrinB2 has been confirmed in many different models, EphB4 expression has also been detected in arterial endothelial cells. Moreover, EphB4 expression has been detected in remodeling rat mesenteric microvessels and particularly in capillary sprouts. In the present study, we also found atypical EphB4 expression and observed the strongest EET-induced expression of EphB4 in isolated murine mesenteric arteries, even though there was no detectable expression of the protein in the endothelium of freshly isolated vessels. In contrast, EphB4 was abundantly expressed in mesenteric venous endothelial cells but was not upregulated by EET treatment. We also observed that the vessels that formed within Matrigel plugs impregnated with 11,12-EET expressed EphB4, and that endothelial cell infiltration into Matrigel was prevented by its downregulation. Thus, EphB4 may also be implicated in capillary sprouting in specific situations, such as angiogenesis associated with inflammation eg, in the mouse ear (T. Korff, unpublished data, 2007).

Although we have previously reported that CYP epoxygenase-derived EETs can initiate angiogenesis, the results of the present investigation indicate for that 11,12-EET also stimulates vessel maturation as the vessels detected in EET-impregnated Matrigel were coated with 

**Figure 6. Effect of downregulating EphB4 on angiogenesis in vivo.** A, PECAM-1 (red) and α-actin (green) labeled cells in Matrigel plugs containing solvent (Sol) or 11,12-EET together with either a control siRNA or EphB4 siRNA. B, General appearance and hemoglobin (Hb) content of 11,12-EET-impregnated Matrigel plugs also containing either the CTL or EphB4 siRNA. The bar graphs summarize data from 3 to 4 animals per group; *P<0.05, **P<0.01 vs CTL siRNA-containing plugs.

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**Disclosures**

None.
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Figure I. Effect of 11,12-EET on EphB4 expression in mesenteric veins. Mesenteric veins were freshly prepared from adult mice and maintained for 18 hours in the presence of either solvent (Sol; 0.1% DMSO) or 11,12-EET (EET; 1 µmol/L). EphB4 (red) and PECAM-1 (green) expression were then assessed in paraffin embedded segments. **A&B** Show the results at two different magnifications while the bar graph (C) summarizes data obtained using vessels from 3 different animals.
Figure II. Effect of CYP2C9 overexpression on the tyrosine phosphorylation of EphB4 in human endothelial cells. Cells were treated with either control (CTL) or CYP2C9 adenoviruses 48 hours before harvesting and the immunoprecipitation (IP) of tyrosine phosphorylated (p-Tyr) proteins. The tyrosine phosphorylation of EphB4 was then assessed by western blotting using an antibody directed against EphB4.

The phosphotyrosine antibody used was from Santa Cruz Biotechnology (Heidelberg, Germany) and the polyclonal anti-mouse EphB4 antibody was from R&D Systems (Wiesbaden, Germany).
Figure III. (A) Effect of downregulating EphB4 on the general appearance of solvent and 11,12-EET-impregnated Matrigel plugs also containing either a CTL siRNA or siRNA1 directed against murine EphB4. (B) Effect of downregulating EphB4 on endothelial cell levels (PECAM-staining) in Matrigel plugs impregnated with bFGF (150 ng/ml) together with either a control siRNA or siRNA1 directed against murine EphB4 and recovered 7 days after implantation. The bar graphs summarize data from 3 animals per group; **P<0.01 versus CTL siRNA-containing plugs.