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The CYP4A/F-20-HETE System: A Regulator of Endothelial Precursor Cells Derived from Human Umbilical Cord Blood

Austin M. Guo, Branislava Janic, Ju Sheng, John R. Falck, Richard J. Roman, Paul A. Edwards, Ali S. Arbab and A. Guillermo Scicli

Henry Ford Hospital, Detroit, MI 48202 (A.M.G., B.J., J.S., P.A.E., A.S.A., A.G.S.); University of Texas Southwestern Medical Center, Dallas, TX 75390 (J.R.F.); University of Mississippi Medical Center, Jackson MS 39216 (R.J.R); Department of Pharmacology, New York Medical College, Valhalla, NY 10595 (A.M.G.); Department of Pharmacology, Wuhan University, Wuhan, PR China (A.M.G.).

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b) Send correspondence to:

Austin M. Guo, PhD

Department of Pharmacology

New York Medical College

15 Dana Road, BSB 546A

Valhalla, NY 10595

Tel # 914-594-4625

Fax # 914-347-4956

Email: Austin_guo@nymc.edu

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endothelial growth factor; SDF-1α: stroma-derived factor-1-alpha; CXCR-4: chemokine receptor type 4; HIF-1α: hypoxia-inducible factor-1-alpha; MSC: mesenchymal stem cells; DHE: dihydroethidium.

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Abstract

Endothelial precursor cells (EPC) contribute to physiological and pathological neovascularization. Previous data have suggested that the CYP4A/F-20-HETE system regulates neovascularization. Therefore, we studied whether the angiogenic effects of the CYP4A/F-20-HETE system involve regulation of EPC function. We extracted human umbilical cord blood and isolated EPC, which express AC133^{+} CD34+ and KDR surface markers and contain mRNA and protein for CYP4A11 and CYP4A22 enzymes, as opposed to mesenchymal stem cells which only express negligible amounts of CYP4A11/22. When EPC were incubated with arachidonic acid (AA), they produced 20-HETE which stimulated the cells to proliferate and migrate, as did vascular endothelial growth factor (VEGF). Incubation with 1 μM HET0016, a selective inhibitor of 20-HETE synthesis, reduced the proliferative and migratory effects of VEGF and also significantly abolished EPC migration mediated by stroma-derived factor-1-alpha (SDF-1α), as did (6,15) 20-hydroxyeicosadienoic acid (20-HEDE). Co-culturing EPC and endothelial cells (EC) on a Matrigel matrix led to tube formation which in turn was inhibited by both HET0016 and 20-HEDE. We concluded that the CYP4A/F-20-HETE system is expressed in EPC and can act as both an autocrine and paracrine regulatory factor.

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Introduction

Angiogenesis, the growth of new blood vessels from existing ones, and vasculogenesis, the *de novo* formation of blood vessels, are essential for organ development. They also play a critical role in tissue repair and regeneration and the response to ischemia and cardiovascular repair/regeneration (Carmeliet, 2005). Unchecked neovascularization is also associated with human diseases, including cancer and age-related macular degeneration. Thus the ability to regulate angiogenic responses is critical. Therapeutic manipulation of blood vessel formation (either excessive or impaired) requires a precise understanding of the numerous factors capable of regulating neovascularization (Pearson, 2010).

Circulating endothelial progenitor cells (EPC) contribute to postnatal vasculogenesis and angiogenesis, and a growing body of evidence suggests that they play an important role in neovascularization and hence repair of damaged tissue (Kirton and Xu, 2010;Janic and Arbab, 2010;Jarajapu and Grant, 2010). Neovascularization may involve sprouting from neighboring vessels in response to increases in locally formed vascular endothelial growth factor (VEGF) and may also be supported by mobilization and functional incorporation of bone marrow-derived EPC. Therefore, EPC derived from the bone marrow may provide a novel therapeutic target in patients with vascular disease. Neovascularization also plays a critical role in tumor formation, where it involves recruitment of circulating EPC from bone marrow as well as sprouting of vessels from pre-existing endothelial cells (EC) (Janic and Arbab, 2010;Kirton and Xu, 2010;Patenaude et al., 2010;Pearson, 2010). Although EPC are considered to be originated in bone marrow they are also present in other tissues including cord blood.

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VEGF plays a critical role in regulation of EPC function by increasing mobilization of EPC from the bone marrow and mediating their migration into the circulation (Li et al., 2006; Rosti et al., 2007). In turn EPC promote vascular growth and repair by increasing production of VEGF and other factors within ischemic tissue (Tepper et al., 2005). EPC express CXCR4, which allows migration to sites of neovascularization in response to its ligand, stroma-derived factor-1-alpha (SDF-1α), typically released by target tissues (Folkins et al., 2009;Weidt et al., 2007).

Cytochrome P450 4A (CYP4A) is a monooxygenase that metabolizes arachidonic acid (AA) to 20-hydroxyeicosatetraenoic acid (20-HETE). We have shown that 20-HETE stimulates production of both VEGF and hypoxia-inducible factor-1-alpha (HIF-1α), essential regulators of angiogenic responses in EC. In addition, 20-HETE increases EC proliferation and migration, both essential steps in the angiogenic cascade (Guo et al., 2009;Guo et al., 2007). We have also shown that 20-HETE induces neovascularization in the rat cornea (Chen et al., 2005). Thus 20-HETE activates both release of angiogenic factors and the growth responses of vascular cells *in vivo*. We examined the hypothesis that the CYP4A/F-20-HETE system regulates neovascularization responses by altering proliferation and migration of EPC. For this, we extracted human umbilical cord blood and isolated mononuclear cells positive for AC133 and CD34 (Janic et al., 2010;Janic and Arbab, 2010;Peichev et al., 2000). We have found that these cells express CYP4A11 and synthesize 20-HETE and are targeted by the proliferative and angiogenic effects of 20-HETE.

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Methods

Isolation and *In Vitro* Culture of AC133+ EPC. Progenitor cells positive for the CD133+ marker (AC133+) were isolated from umbilical cord blood obtained from subjects who volunteered for Institutional Review Board (IRB) approved protocols. Mononuclear cells were separated out by Ficoll gradient centrifugation and AC133+ cells isolated by immunomagnetic positive selection using a MidiMACS system (Miltenyi Biotec, Auburn, CA). Freshly isolated AC133+ cells were suspended in Stemline II medium (Sigma, St. Louis, MO) supplemented with 40 ng/ml stem cell factor (SCF), 40 ng/ml FLT3 and 10 ng/ml thrombopoietin (TPO) (all from CellGenix, IL). They were maintained at 37^{o}C in a humidified incubator containing 5% CO_2 as a suspension culture for 30 days, keeping cell concentration at 5×10^5–1×10^6 cells/ml. Throughout this expansion period, the cells were cultured in Falcon 6-well dishes (BD Biosciences) and monitored daily using an inverted phase contrast microscope. Following mitosis viable cells were counted by Trypan blue exclusion assay and a growth curve was generated. Then mitosis was induced by adding freshly prepared medium so as to adjust the concentration to 5×10^5 cells/ml**.**

Flow Cytometry of Endothelial Lineage Cell Surface Markers on AC133+ EPC. Cells expanded in suspension under either normal growth conditions or conditions that facilitate cell differentiation were harvested, washed in ice-cold 1x PBS and incubated for 30 min on ice in the dark with a fluorescence-labeled antibody. Flow cytometry was performed with an LSR II flow cytometer (Becton Dickinson) and 10,000 or more events were analyzed for each sample. Live cells were gated based on their forward and side angle light scatter and analyzed using Cell Quest Pro software (Becton

Dickinson). The antibodies used for flow cytometry were: mouse anti-human CD133 IgG1 (Miltenyi Biotec, Auburn, CA), mouse anti-human CD34 IgG1 (BioLegend), mouse anti-human CD117 IgG1 (BioLegend), mouse anti-human CD29 IgG1 (BioLegend), mouse anti-human CD31 IgG1 (BioLegend), mouse anti-human CD54 IgG2a (BioLegend), mouse anti-human KDR (VEGFR2) IgG1 (R&D Systems), mouse anti-human VE-cadherin IgG2b (R&D Systems), mouse anti-human CD62E IgG2a (BioLegend), mouse anti-human CD184 IgG2a (BioLegend), and mouse anti-human CD105 IgG2a (BioLegend).

Western Blotting. Homogenates were prepared from EPC and mesenchymal stem cells (MSC) using RIPA buffer as described previously (Guo et al., 2005). Equal amounts of protein (20-40 μg) were separated on a 14% Tris-glycine gel, transferred to a PVDF membrane and incubated with a CYP4A11 primary antibody [RDI Division of Fitzgerald Industries; #RDI-CYP4A11abr (new catalog # 20-R-CR041)] at a dilution of 1:500. The membranes were incubated with an anti-rabbit HRP antibody and developed by enhanced chemiluminescence (ECL). We then treated the blots with protein stripping buffer, re-blocked with blocking agent, and then immunostained with an antibody against human β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) using the same development system to serve as loading controls.

RT-PCR. RNA was extracted from EPC or MSC using TRIZOL reagent, treated with DNAse and concentration measured by absorbance at 260 nm. Then 1 μg RNA was reverse-transcribed using a SuperScript first-strand synthesis system (Invitrogen). Primers used for PCR were CYP4A11 forward 5'-AATTTGCCATGAACGAACGAGCTGA-3' and reverse 5'-

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TGTTCCAAAGGCCACAAGG-3'; CYP4A22 forward 5'-AATTTGCCATGAACCAGCTGA-3' and reverse 5'-GGTCCTTGTCTTCACAAGGG-3'; CYP4F2 forward 5′-CCCGAAACGGAATTGGTTCT-3′ and reverse 5′-CCAGCAGCACATATCACCGA-3′; CYP4F3 forward 5'-CCCAAGACGGAACTGGTTTT-3' and reverse 5'-GACCTTAAAGCCCTGGGGGT-3'; CYP2J2 forward ATGCTCGCGGCGATGGGCTC and reverse TAGTGCTGTCAGAGTGAACC; CYP2C8 forward TCTTACACGAAGTTACATTA and reverse TAGATCAGCTACAGTGCCAA; CYP2C9 forward TGAAGAAGAGCAGATGGCCT and reverse GAATGTTCACTAGATCTTCA. Amplification was performed at 95˚C for 3 min, followed by 40 cycles of 95˚C for 1 min, 55˚C for 1 min, and 72˚C for 1 min. The PCR reaction was extended at 72˚C for 10 min and the products separated by electrophoresis on 1% agarose gels. Expected product size for CYP4A11 was 500bp, CYP4A22 172bp, Cyp4F2 110bp, CYP4F3 160bp, CYP2J2 435bp, CYP2C8 169bp, and CYP2C9 147bp.

Real-time PCR. Real-time PCR were performed using LightCycler3 (Roche). The 20 μl PCR mix include 2 μl reverse transcription product, 2 μl LightCycler FastStart DNA Master SYBG mix, 0.5 unit of LightCycler Uracil-DNA Glycosylase, and 10 pmol of various primers. The reactions were incubated at 95°C for 10 min, followed by 55 cycles of 95°C for 10s, 55°C for 5s, and 72°C for 15s. The Ct data were determined using default threshold settings. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The $2^{-\Delta\Delta C}$t method was used to calculate the relative changes in the gene expression. The products were collected and molecular weight of the products was confirmed by electrophoresis on 1% agarose gels.

Cell Proliferation Assay. Proliferation studies were performed as described previously (Guo, Roman, Falck, Edwards, and Scicli, 2005;Guo et al., 2006). EPC were incubated with VEGF (20 ng/ml) or 20-HETE (1 μM) for 48hr. In some experiments the cultures were exposed to VEGF in the presence of 10 μM HET0016, a selective inhibitor of 20-HETE synthesis. The effects of VEGF and 20-HETE on proliferation were assessed by cell counting, using EPC treated with solvent (EtOH, 0.01%) as a control. Cell counts were normalized to EtOH and expressed as a percentage of control.

Cell Migration Assay. 1 x 10^5 EPC in medium lacking both serum and growth factors were placed in the upper chamber of a cell migration transwell (QCM Chemotaxis 5μm 24-well cell migration assay, Millipore, Billerica, MA) and VEGF (20 ng/ml), SDF-1 (100 ng/ml) or 20-HETE (1 μM) added to the bottom well for 16 hr to study their effects on EPC migration. In separate experiments, EPC were incubated with either 100 ng/ml SDF1 or 20 ng/ml VEGF in the presence or absence of 10 μM HET0016 and/or 10 μM 20-HEDE. In addition, 10 μM 20-HETE was added to one group to see if it would reverse the inhibitory effects of HET0016 on SDF-1-mediated EPC migration. Changes in the numbers of cells both attached to the membrane and contained in the lower chamber were quantified using the fluorescence based migration assay kit following the manufacturer's recommended protocol.

EC Tube Formation Assay. The ability of EPC or EC to form tubes resembling capillaries was assessed by seeding the cells in wells containing 35 mm BioCoat Matrigel (BD Bioscience). 1 x 10^5 EC either alone or in the presence of 2 x 10^4 EPC washed with PBS were seeded in wells containing Matrigel without serum or growth factors and tube formation assessed after ~ 18 hr incubation. To establish whether the

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CYP4A/F-20-HETE system contributes to the effects of EPC-assisted EC tube formation, a 20-HETE synthesis inhibitor, either 10 μM HET0016, 10 μM DDMS or 20-HETE antagonist ,10 μM 20-HEDE were added. Images were taken from random fields of Matrigel wells and the tube lengths were tracked using Sigma-plot and normalized to pixel counts.

Assessment of 20-HETE Formation. For this, 1 x 10^6 EPC were either left untreated or treated with 10 μM arachidonic acid (AA) overnight before harvesting the cells. Both cells and media were analyzed for the presence of 20-HETE and other lipid metabolites as described previously (Guo et al. JPET, 2008). d^6-20-hydroxyeicosatetraenoic acid (2 ng) was added to each samples prior to lipid extraction with ethyl acetate served as the internal positive standard. The organic phase were collected and dried under nitrogen.

Samples were reconstituted with 50% methanol and water and the metabolites separated by high-performance liquid chromatography (HPLC) on a Betabasic C_{18} column (150 × 2.1 mm, 3 μm; Thermo Electron, Belletonte, PA) at a flow rate of 0.2 ml/min using an isocratic elution starting from a 51:9:40:0.01 mixture of acetonitrile/methanol/water/acetic acid for 30 min followed by a step change to 68:13:19:0.01 acetonitrile/methanol/water/acetic acid for 15 min. The effluent was ionized using a negative ion electrospray (450°C, 4,500 V) with the collision-activated dissociation gas set at 7 L/min. All transitions had a scan time of 0.2 s and a unit resolution in both Q1 and Q3 set at 0.7 ± 0.1 full width at half maximum. Peaks eluting with a mass/charge ratio (*m*/*z*) of 319 > 301 (HETEs and EETs), 337 > 319 (DiHETEs), 319 > 245 (20-HETE), 325 > 251 (d6-20-HETE), 351 > 271 (prostaglandin $_{D2}$ and $_{E2}$),

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353 > 309 ($_{PGF2}$), 369 > 245 ($_{PGF1\alpha}$), and 369 > 195 (thromboxane A2) were monitored as multiple reactions using a triple quadrupole mass spectrometer (Foster City, CA).

Statistical Analysis. Data were analyzed using ANOVA followed by Tukey's test or Student's *t*-test when only two groups were studied, taking $p < 0.05$ as significant.

Results

Isolation and Characterization of EPC. Mononuclear cells were isolated from human umbilical cord blood. AC133+ and CD34+ cells were separated out by immunomagnetic positive selection and expression of AC133 and CD34 cell surface markers confirmed *in vitro* by flow cytometry (**Fig. 1A**). When these cells were plated and cultured on a fibronectin-coated surface in the presence of 2% FBS and 2 ng/ml VEGF for 10 days, they became differentiated, exhibiting a cobble-stone pattern and expression of mature EC-specific markers. Comparison of CD133, CD34 and CD117 (progenitor markers) and CD31, CD29, CD184, CD62, KDR, VE-cadherin and CD105 (endothelial lineage cell surface markers) on EPC before and after differentiation confirmed that the AC133+ and CD34+ EPC we isolated matured into EC (**Fig. 1B**).

CYP4A/F-20-HETE System in EPC. EPC expressed mRNA for CYP4A11, CYP4A22 and CYP4F2 -- all of which are known to produce 20-HETE - along with the epoxide-synthesizing enzymes CYP2C8 and CYP2C9 (**Fig. 2A**). CYP4F3 was not detectable in EPC by RT-PCR (data not shown). To determine the relative mRNA expressions of these genes, real-time PCR were also performed in EPC. We found that CYP4A11 and CYP4A22 have relatively high expression in EPC, while CYP4F2, CYP2C8 and CYP2C9 are minimally expressed (**Fig. 2B**).

Western blots confirmed that EPC also express CYPA11/22 protein (**Fig. 3**). Due to the relatively high expression of CYP4A11 in EPC, we examined whether this 20-HETE synthase is differentially expressed in another different type of stem cells, mesenchymal stem cells. Interestingly, RT-PCR of cDNA from MSC isolated from

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umbilical cord blood showed negligible expression of CYP4A11 (**Fig 3B**). Similar observation was also recorded in the CYP4A11 protein expression.

When these cells were incubated with medium containing AA (10 μM) for 60 min and 20-HETE analyzed by LC-MS/MS, they were shown to produce 20-HETE along with 5-,12- and 15-HETE and several EETs (**Fig. 4**). The majority of 20-HETE in EPC was detected in the culture media, not in the cell pellets.

Effects of 20-HETE on EPC Proliferation and Migration. Additional studies were performed to determine the effects of 20-HETE on EPC proliferation and migration, with VEGF and SDF-1α serving as corresponding positive control. While both 20-HETE and VEGF increased EPC proliferation and migration, on a molar basis VEGF was more potent than 20-HETE. Blocking 20-HETE synthesis with HET0016 inhibited both proliferation and migration responses to VEGF and SDF-1α (**Figs. 5A and 5B**). In addition, both HET0016 and 20-HEDE prevented SDF-1α from accelerating EPC migration. We also found that addition of 20-HETE partially reversed the inhibitory effect of HET0016 on SDF-1α mediated EPC migration.

Effects of 20-HETE Synthesis Inhibitors on EPC-induced EC Tube Formation. Co-culture of EPC with EC markedly increased tube formation as seen on Matrigel assay, and this effect was significantly inhibited by HET0016 (**Fig. 6**). DDMS, another inhibitor of 20-HETE synthesis, had a similar effect on EPC-mediated EC tube formation even though it is chemically dissimilar to HET0016, and so did 20-HEDE (WIT002) (**Fig. 6**).

Effects of 20-HETE on Expression of the Angiogenic Regulators VEGF and HIF-1, eNOS Phosphorylation and Dihydroethidium (DHE) Staining. 20-HETE

increased expression of HIF-1α and VEGF as well as phosphorylation of serine 1177 in eNOS (**Fig. 7A**). It also increased DHE staining in EPC, most likely by stimulating superoxide formation (**Fig. 7B)**.

Discussion

Over the past decade there has been considerable interest in the role of endothelial progenitor cells (EPC) in angiogenesis. EPC are considered to be bone marrow-derived cells that have the capacity to proliferate and migrate to sites of neovascularization where they differentiate into mature EC. However, EPC are also present in other tissues. Many groups have used peripheral and placental umbilical cord blood as a source of EPC (Harris and Rogers, 2007). We isolated cells expressing CD34, AC133 and VEGFR2 from placental cord blood. These cells are thought to represent an immature EPC population that when plated on a matrix can give rise to AC133-/CD34+/VEGFR2+, which are more mature and have limited proliferative capacity (Janic, Guo, Iskander, Varma, Scicli, and Arbab, 2010;Janic and Arbab, 2010). Such cells behave like EPC, migrating to sites of neovascularization and becoming integrated into vascular networks (Janic, Guo, Iskander, Varma, Scicli, and Arbab, 2010;Janic and Arbab, 2010). Cord blood AC133+ cells represent a pluripotent adult progenitor cell population that could be valuable in vascular repair; moreover, cord blood generates more EPC than any other source of EPC (Harris and Rogers, 2007).

We and others (Buysschaert et al., 2007;Jiang et al., 2004;Chen, Guo, Wygle, Edwards, Falck, Roman, and Scicli, 2005;Guo, Arbab, Falck, Chen, Edwards, Roman, and Scicli, 2007) have shown that the CYP4A-20-HETE system plays an important role in angiogenesis. 20-HETE is angiogenic both *in vitro* and *in vivo* (Jiang, Mezentsev, Kemp, Byun, Falck, Miano, Nasjletti, Abraham, and Laniado-Schwartzman, 2004;Guo, Arbab, Falck, Chen, Edwards, Roman, and Scicli, 2007;Guo, Scicli, Sheng, Falck, Edwards, and Scicli, 2009) and activates VEGF and HIF-1α in EC (Guo, Scicli, Sheng,

Falck, Edwards, and Scicli, 2009;Guo, Arbab, Falck, Chen, Edwards, Roman, and Scicli, 2007). In the vasculature the main source of 20-HETE is the smooth muscle layer (Gebremedhin et al., 2000), although in the lung it is also produced by EC (Zhu et al., 2002). We have tested a total of 6 batches of EPC randomly isolated from more than 20 donors. CYP4A11 and CYP4A22 mRNA (RT-PCR) and protein (Western blot) were expressed in each of the six batches. Three additional batches from different donors were also subjected for real time PCR analysis and showed mRNA expression of both isozymes. The ratio of one isoform to another tended to vary. This may simply reflect the different donors, since cord blood was obtained from volunteers without regard for age, race or health status. Although we failed to detect CYP4F3 RNA in EPC by RT-PCR, we did not specifically analyze whether CYP4F3a or CYP4F3b RNA was expressed. Since these P450s may have distinct patterns of cell distribution, our studies still cannot completely rule out the participation of a CYP4F3 P450 in AA ω-hydroxylation by EPC.

Although EPC cell pellets produced very little 20-HETE under basal conditions, they released substantial amounts into the medium (~200 pg/million cells) when incubated with AA, showing that they contained enzymatically active 20-HETE synthases. EPC responded to exogenous 20-HETE by proliferating and migrating, showing that 20-HETE stimulates basic functions of EPC. It is worth noting that 20-HETE is highly unstable in cell culturing system based on our previous experiences working with EC. Thus, it is most likely that the increased EPC proliferation observed at 48hr after 20-HETE addition is resulted from its immediate mitogenic effects. 20-HETE may reach EPC *via* either a paracrine or autocrine pathway (or both), since ω-

hydroxylases are microsomal enzymes (Roman, 2002). EPC supernatants also contained small amounts of various epoxides, a result consistent with low levels of epoxide producing enzymes being present.

We previously reported that 20-HETE synthesis inhibitors blocked the proliferative and migratory responses of EC to VEGF; indeed the selective AA ω-hydroxylase inhibitor HET0016 not only blunted EC growth and migration responses to VEGF *in vitro* but also angiogenic responses to VEGF and other growth factors *in vivo* (Chen, Guo, Wygle, Edwards, Falck, Roman, and Scicli, 2005;Guo, Arbab, Falck, Chen, Edwards, Roman, and Scicli, 2007). To see if blocking the CYP4A/F-20-HETE system would have a similar action on the proliferative and migratory responses of EPC to VEGF, we co-incubated VEGF with HET0016 and found that both responses were entirely eliminated. Although we do not know exactly how ω-hydroxylase inhibitors such as HET0016 suppress responses to VEGF, it seems unlikely that HET0016 acts on sites near EPC since blocking 20-HETE synthesis with a structurally and mechanistically different inhibitor, DDMS, had the same effect. HET0016 lessened the migratory effect of SDF-1α on EPC, suggesting that some metabolite produced by ω-hydroxylase, either from AA or some other lipid substrate, is necessary in order for VEGF or SDF-1α to activate its receptor signaling cascade. Adding 20-HETE partially reversed the inhibitory effects of HET0016. The fact that HET0016 inhibited two separate factors (VEGF and SDF-1α) that act *via* different receptors suggests that 20-HETE synthesis is an essential component of their common signaling pathway. Further studies are required to elucidate the exact mechanism involved. When we used 20-

HEDE, a competitive antagonist of the vasoconstrictor actions of 20-HETE, we found that it markedly reduced EPC migratory responses to VEGF and SDF-1α.

EC plated on Matrigel formed a network of tube-like structures resembling capillaries, indirectly demonstrating that differentiation of EC is necessarily the initial step in the angiogenic cascade (Arnaoutova et al., 2009). When HUVEC or EPC were plated on Matrigel in the absence of growth factors, they did not form tubes; however, when EPC and EC were plated together, a network of EC tubes was formed, suggesting that EPC secrete some factor(s) that induce EC to differentiate and form tubes which contributes to angiogenesis (Koga et al., 2009). The fact that the tube formation induced by EPC was inhibited by HET0016, DDMS and 20-HEDE is consistent with 20-HETE being an important mediator of EPC-induced EC differentiation.

In human microvascular EC, 20-HETE increases phosphorylation of VEGF and NOS and also enhances superoxide formation, which in turn increases HIF-1α (Guo, Scicli, Sheng, Falck, Edwards, and Scicli, 2009;Guo, Arbab, Falck, Chen, Edwards, Roman, and Scicli, 2007). We found that 20-HETE increased both superoxide and HIF-1α in EPC. Production of both SDF-1α and VEGF is regulated by HIF-1α (Hoenig et al., 2008) and both were increased by 20-HETE.

Phosphorylation of serine 1177 has been associated with increased synthesis of nitric oxide (NO) (Butt et al., 2000), and we found that it was markedly increased in EPC treated with 20-HETE just as others reported in EC (Cheng et al., 2008;Bodiga et al., 2010), pointing up the close similarities between these progenitor cells and mature EC. Both the proliferative and migratory effects of 20-HETE could be directly due to

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stimulation of some unknown receptor (Miyata and Roman, 2005); or they might be secondary to increases in NO and VEGF (Sautina et al., 2010).

The increases in SDF-1α induced by 20-HETE are worth noting. It might be that 20-HETE caused EPC to attract CXCR4+ cells such as stem cells and leukocytes. CXCL12 (SDF-1)/CXCR4 is believed to be involved in angiogenesis (Teicher, 2010) and has been identified as a key factor in recruitment of stem cells to injured tissue. Increased expression of SDF-1 secondary to 20-HETE stimulation could have multiple effects, including recruitment of bone marrow-derived stem cells and local inhibition of apoptosis of cells expressing CXCR4 (Penn, 2010).

Our research raises the question of whether EPC derived from umbilical cords behave like EPC derived from bone marrow. Functionally both cell lines are very similar. Vessels derived from the umbilical cord exhibit normal blood flow, permselectivity to macromolecules, and induction of leukocyte-EC interactions in response to cytokine activation just like normal vessels (Au et al., 2008). CD133+ cells derived from cord blood and EPC derived from mouse bone marrow showed no differences in migration and accumulation (Arbab et al., 2008;Arbab et al., 2006;Anderson et al., 2005). Patients at high risk of cardiovascular disease have fewer EPC, and those they do have exhibit greater senescence *in vitro*. Treatment with EPC derived from human umbilical cord blood could help restore stem cell function and enhance neovascularization (Senegaglia et al., 2010), particularly in the sick and elderly. Future studies should examine whether increasing 20-HETE stimulation of EPC by overexpression of 20-HETE synthase would heighten production of SDF-1, VEGF

and NO in these patients, which could increase angiogenic transduction pathways and help in tissue repair.

In summary, AC133+ and CD34+ cells isolated from human umbilical cord blood express mRNA and protein for CYP4A11 and CYP4A22 and produce 20-HETE in the presence of AA. EPC respond to 20-HETE with increased migration and proliferation and increases in genes known to regulate angiogenesis. Selective inhibitors of 20-HETE synthesis attenuate VEGF-induced migration and proliferation of EPC and hence EPC-induced EC tube formation. These data suggest that the CYP4A/F-20-HETE system plays an important role in regulation of the EPC functions associated with angiogenic responses.

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Authorship contributions

1. Participated in research design: Guo, Janic, Edwards, Arbab, Falck, Roman, and Scicli.
2. Conducted experiments: Guo, Sheng, Arbab, and Roman.
3. Contributed new reagents or analytic tools: Janic, Arbab, and Falck.
4. Performed data analysis: Guo, Arbab, and Roman.
5. Wrote or contributed to the writing of the manuscript: Guo, Janic, Edwards, Arbab, Roman, and Scicli.
6. Other: None.

Footnotes

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Figure Legends

Figure 1. Flow Cytometry of Cell Surface Markers on EPC. ***Panel A.*** Expression of AC133 and CD34 progenitor markers on EPC was analyzed by flow cytometry. ***Panel B.*** Expression of CD133, CD34 and CD117 (progenitor markers) and CD31, CD29, CD184, CD62, KDR, VE-cadherin and CD105 (endothelial lineage cell surface markers) on EPC. Before differentiation: 4-day-old EPC cultures; After differentiation: EPC were induced to differentiate into mature EC for 2 weeks. We used AC133+ EPC (before differentiation) for all studies.

Figure 2. mRNA Analysis of CYP Enzymes in EPC. ***Panel A.*** RNA was extracted from cord blood EPC using TRIzol and reverse-transcribed. cDNAs were amplified by RT-PCR using CYP4A11, CYP4A22, CYP4F2, CYP2C8, and CYP2C9 primers. GAPDH served as a loading control. Representative gels from three separate experiments are shown. ***Panel B.*** Real-time PCR analysis of CYP4A11, CYP4A22, CYP4F2, CYP2C8, and CYP2C9 relative mRNA expression were also performed in EPC. GAPDH was again used as control. Data represent at least three individual experiments in triplicate.

Figure 3. Comparison of CYP4A11 protein and mRNA Expression in EPC and MSC. ***Panel A*****.** Total EPC and MSC protein lysates were assayed for human CYP4A11 by Western blot. ***Panel B*****.** mRNA was extracted using TRIzol and reverse-transcribed. cDNAs were amplified by RT-PCR using a CYP4A11 primer.

Figure 4. LC-MS/MS Profile of AA Metabolites Produced by EPC. Pooled EPC (1×10^6) were either left untreated or treated with 10 μM AA overnight before harvesting the cells. Both the EPC growth media and EPC pellet were extracted using

ethyl acetate and lipids were separated by HPLC on a C_{18} column. LC-MS/MS was performed to assess 20-HETE production as we have previously described. 2 ng of d^6-20-HETE was added to each sample prior to ethyl acetate extraction served as the internal positive standard.

Figure 5. **Effects of 20-HETE and CYP4A/F Inhibitors on EPC Proliferation and Migration.** ***Panel A.*** 6-well culture plates containing EPC were treated with either VEGF (20 ng/ml), 20-HETE (1 μM) or VEGF plus HET0016 (10 μM) for 48 hr. Changes in cell number are measured, normalized, and expressed as a percentage of control. Mean ± SD of three separate experiments (each in duplicates are shown. ** $p < 0.05$ *vs.* control; ^ $p < 0.05$ *vs.* VEGF alone. ***Panel B.*** EPC were incubated for 16 hr, a) in the presence of either 100 ng/ml SDF1-α or 20 ng/ml VEGF or 1μM 20-HETE and b) with either 100 ng/ml SDF1 or 20 ng/ml VEGF in the presence or absence of 10 μM HET0016 and/or 10 μM 20-HEDE in separate experiments. In addition, 10 μM 20-HETE was also added to one group to see if it would reverse the inhibitory effect of HET0016 on SDF-1α-induced EPC migration. Changes in cell numbers both attached to the membrane and contained in the lower chamber were quantified using the fluorescence based migration assay kit following the manufacturer's recommended protocol. Data represent three separate experiments (each performed in duplicates. ** $p < 0.05$ *vs.* control; # $p < 0.05$ *vs.* SDF-1α; ^ $p < 0.05$ *vs.* SDF1-α and VEGF.

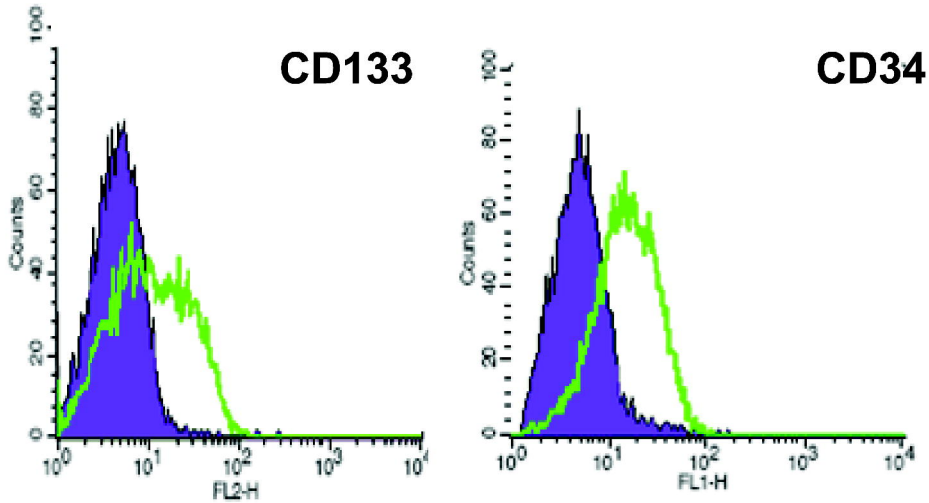
Figure 6. **Effects of EPC and CYP4A/F-20-HETE Inhibitors on Tube Formation by EC.** 1 x 10^5 EC either alone or in the presence of 2 x 10^4 EPC washed with PBS were seeded in wells containing Matrigel without serum or growth factors. In some wells, the ability of EPC to help EC form tubes was also tested in the presence of

a 20-HETE synthase inhibitor, either HET0016 (10 μM) or DDMS (10 μM), as well as 20-HETE antagonist 20-HEDE (10 μM). EC tube formation was assessed after ~ 18 hr incubation.

Figure 7. Effects of 20-HETE on HIF-1α, VEGF and SDF-1α Expression, eNOS Phosphorylation, and Superoxide Formation in EPC. ***Panel A.*** EPC were treated with 1 μM 20-HETE and protein lysates subjected to Western blot using anti-HIF-1α, anti-VEGF, anti-SDF-1α, and anti-phosphorylated eNOS serine 1177 antibodies with actin as a loading control. The blot represents three separate experiments. ***Panel B.*** EPC were treated with DHE for 30 min and fluorescence microscopy was used to assess formation of superoxide by 20-HETE. Representative images from three separate experiments are shown.

Fig. 1

A.



B.

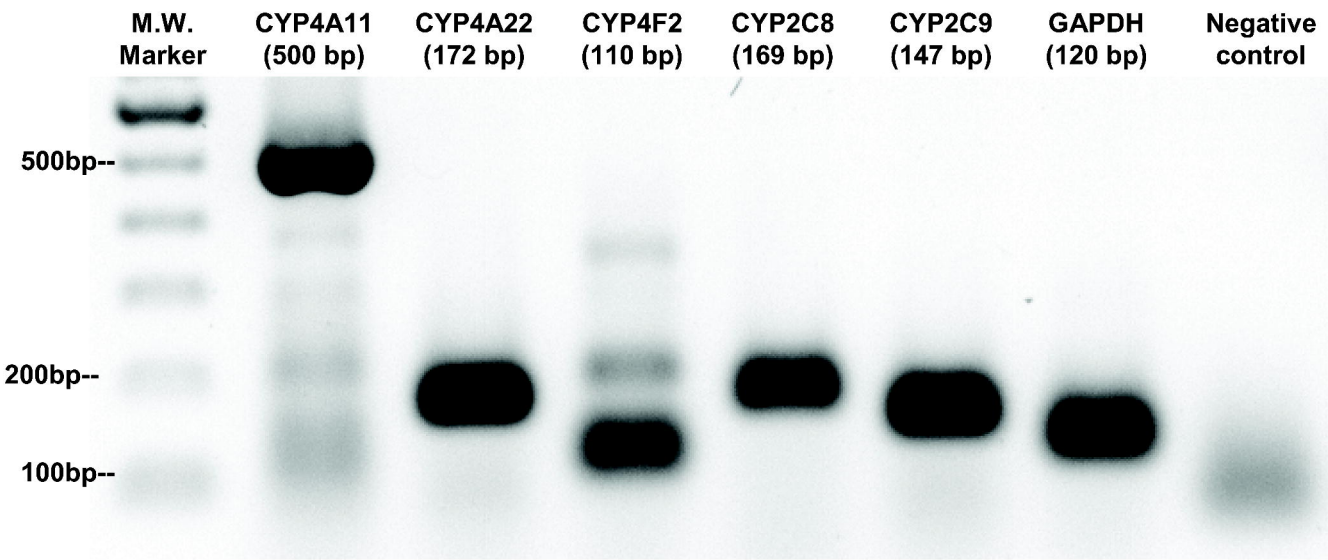
Markers / EPC	CD133	CD34	CD31	KDR	VE-cadherin	CD117	CD29	CD184	C62	CD105
Before differentiation	+	+	+	-	-	+	+	-	+	+
After differentiation	-	+	+	+	+	+	+	+	+	+

-: no expression;

+: with expression

Fig. 2

A.


M.W. Marker
CYP4A11 (500 bp)
CYP4A22 (172 bp)
CYP4F2 (110 bp)
CYP2C8 (169 bp)
CYP2C9 (147 bp)
GAPDH (120 bp)
Negative control
500bp--
200bp--
100bp--

B.

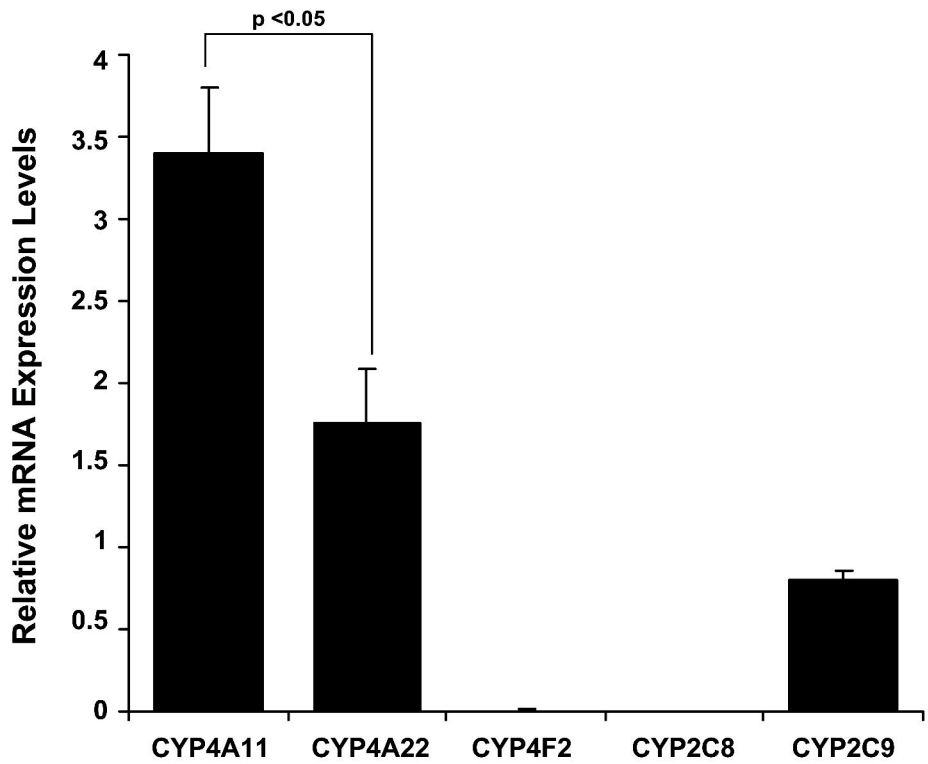

p <0.05
Relative mRNA Expression Levels
4
3.5
3
2.5
2
1.5
1
0.5
0
CYP4A11
CYP4A22
CYP4F2
CYP2C8
CYP2C9

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Fig. 3

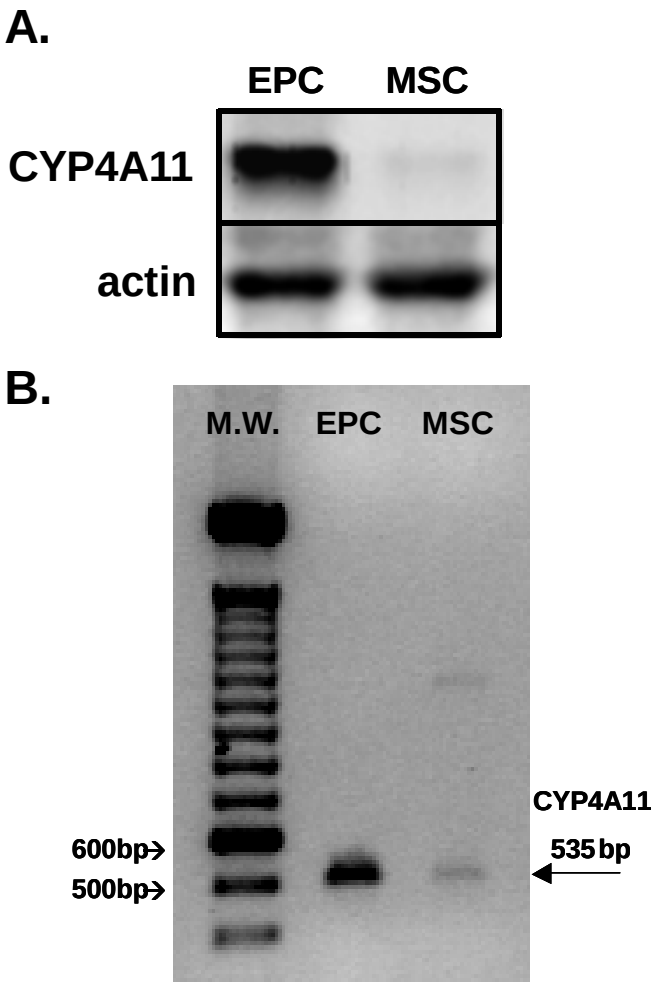


Fig. 4

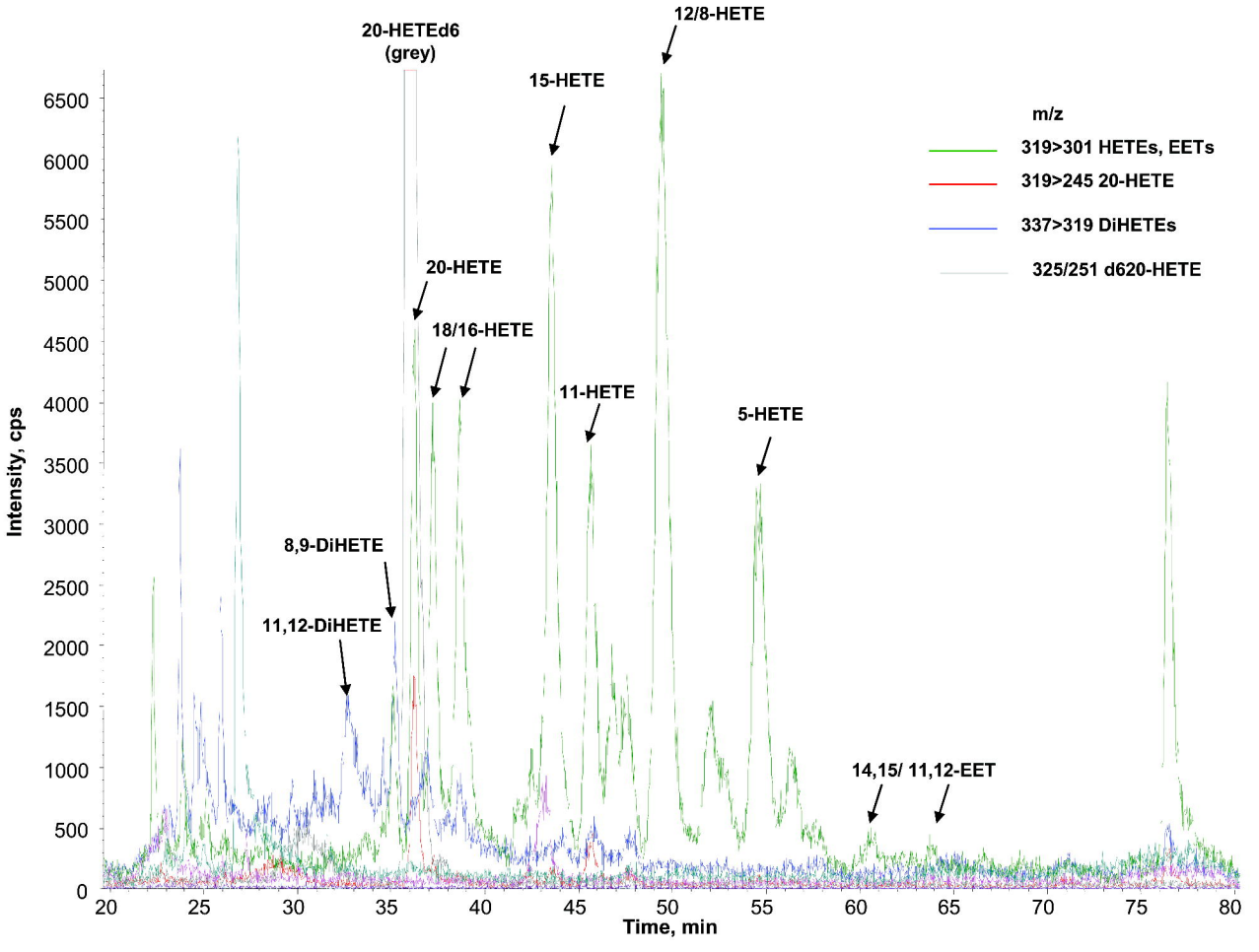
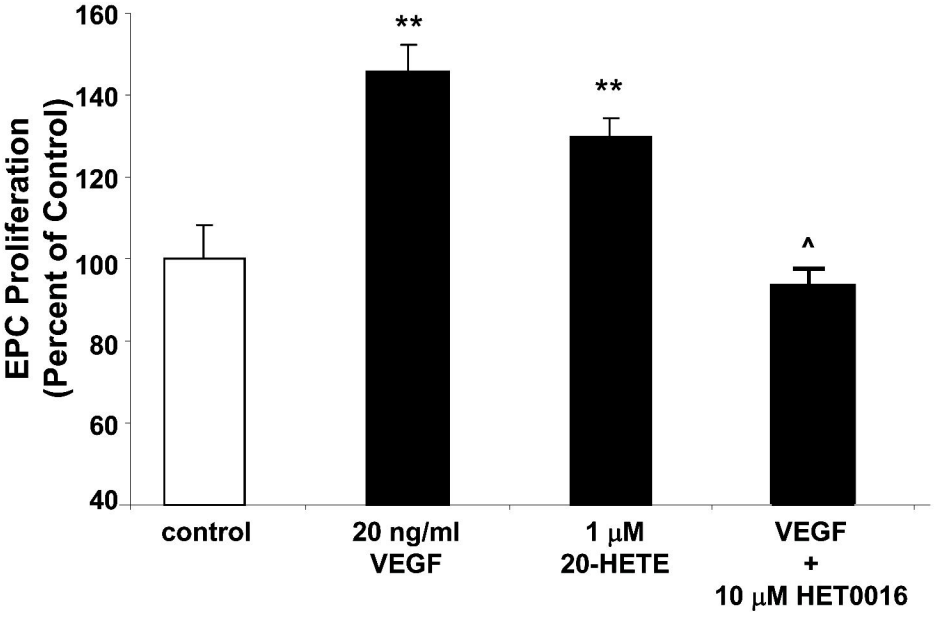

20-HETEd6
(grey)
12/8-HETE
15-HETE
20-HETE
18/16-HETE
11-HETE
5-HETE
8,9-DiHETE
11,12-DiHETE
14,15/ 11,12-EET
m/z
319>301 HETEs, EETs
319>245 20-HETE
337>319 DiHETEs
325/251 d620-HETE
Intensity, cps
Time, min
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6000
6500
20
25
30
35
40
45
50
55
60
65
70
75
80

Fig. 5

A.


EPC Proliferation
(Percent of Control)
160
140
120
100
80
60
40
**
**
^
control
20 ng/ml
VEGF
1 μM
20-HETE
VEGF
+
10 μM HET0016

B.

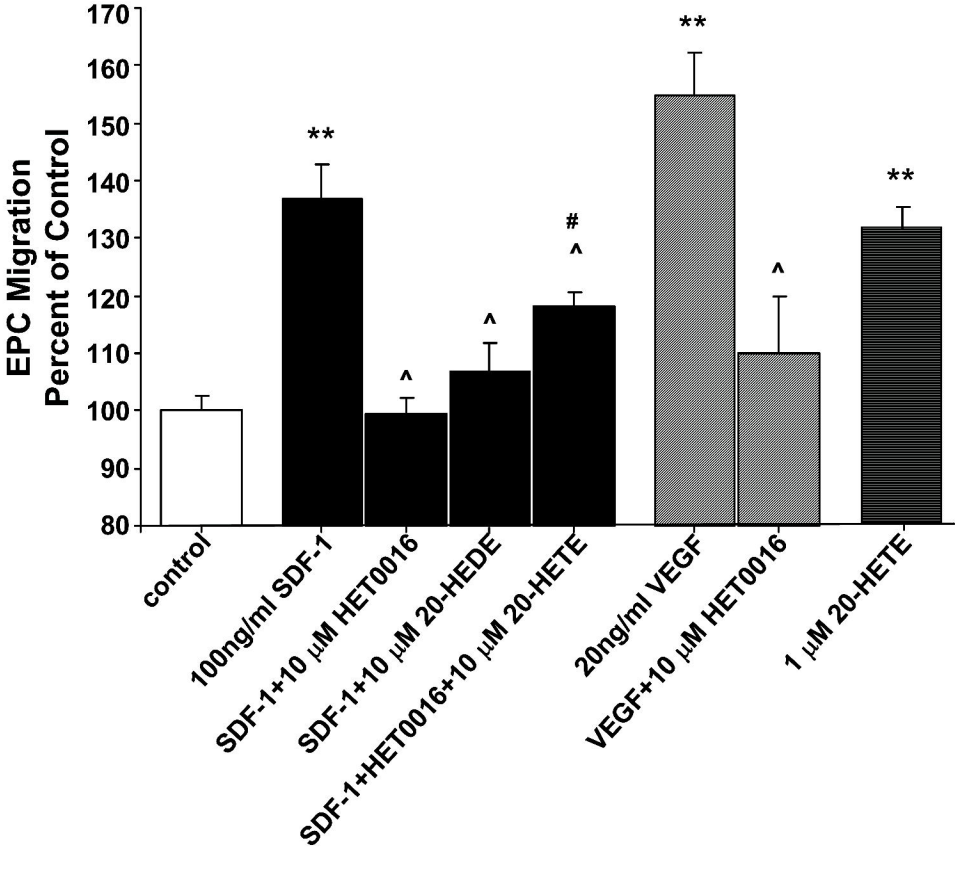

EPC Migration
Percent of Control
170
160
150
140
130
120
110
100
90
80
**
^
^
#
^
**
^
**
control
100ng/ml SDF-1
SDF-1+10 μM HET0016
SDF-1+10 μM 20-HEDE
SDF-1+HET0016+10 μM 20-HETE
20ng/ml VEGF
VEGF+10 μM HET0016
1 μM 20-HETE

Fig. 6

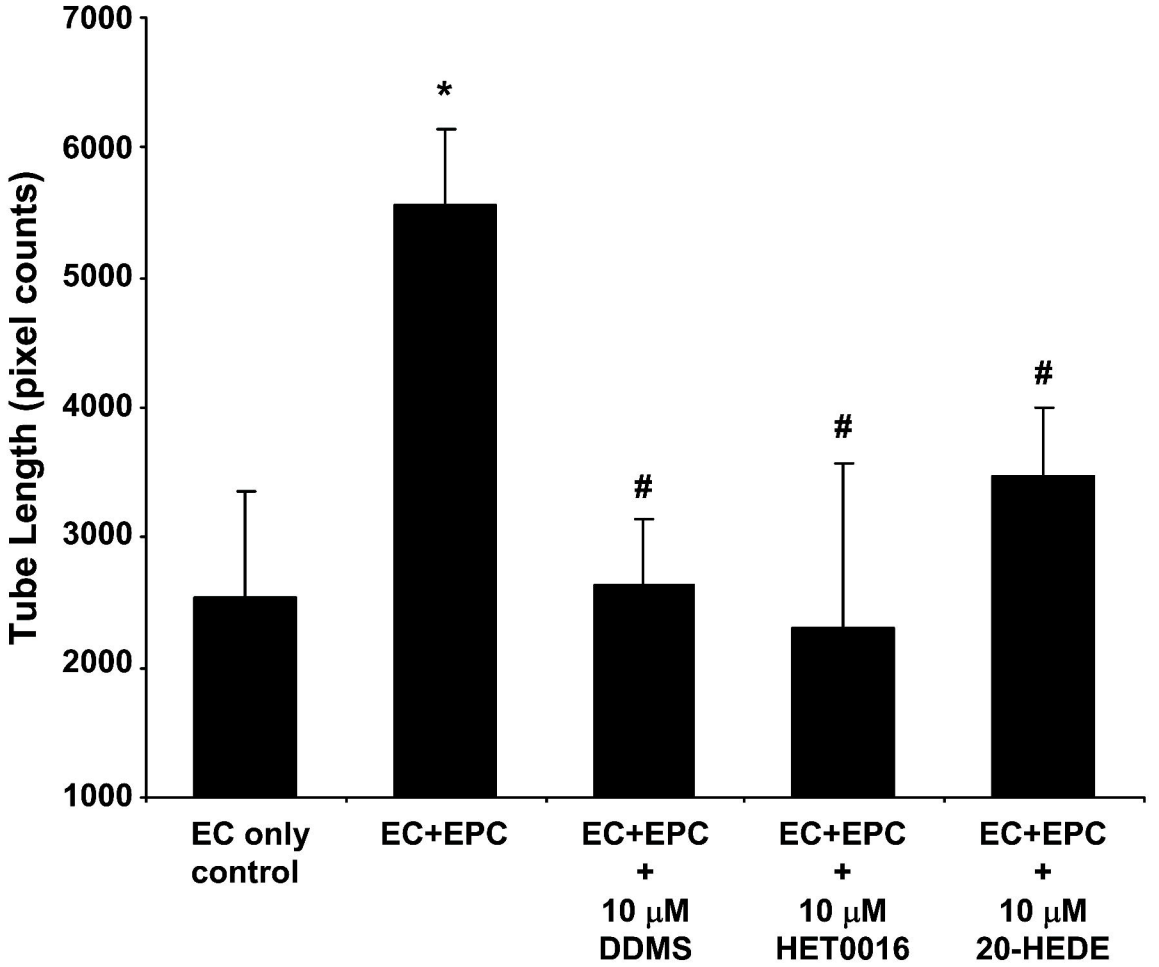
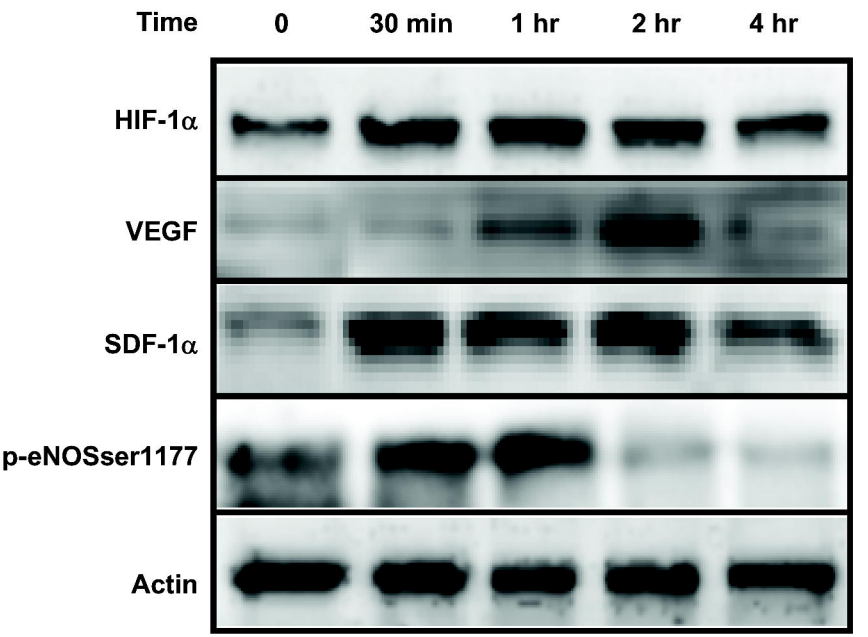

Tube Length (pixel counts)
7000
6000
5000
4000
3000
2000
1000
*
#
#
#
EC only control
EC+EPC
EC+EPC + 10 μM DDMS
EC+EPC + 10 μM HET0016
EC+EPC + 10 μM 20-HEDE

Fig. 7

A.


Time
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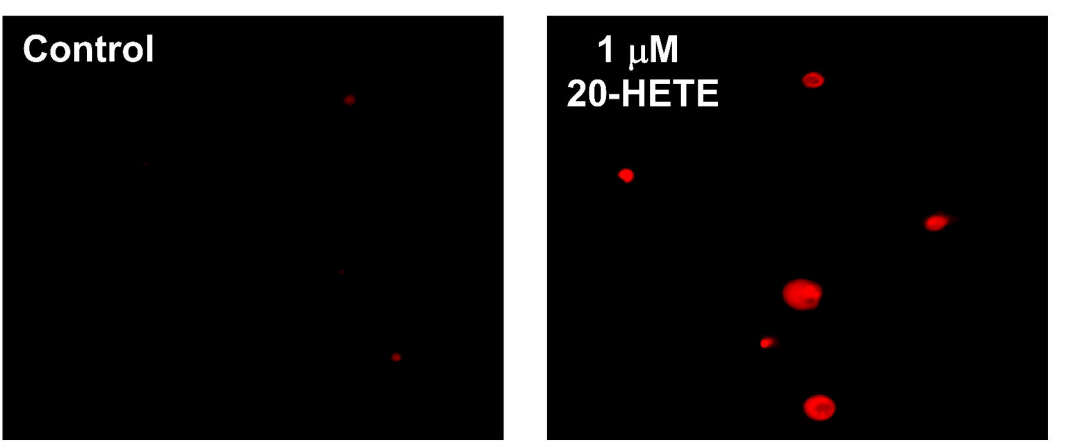
30 min
1 hr
2 hr
4 hr
HIF-1α
VEGF
SDF-1α
p-eNOSser1177
Actin

B.


Control
1 μM
20-HETE