Eicosanoid regulation of angiogenesis: role of endothelial arachidonate 12-lipoxygenase
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Angiogenesis, the formation of new capillaries from preexisting blood vessels, is a multistep, highly orchestrated process involving vessel sprouting, endothelial cell migration, proliferation, tube differentiation, and survival. Eicosanoids, arachidonic acid (AA)-derived metabolites, have potent biologic activities on vascular endothelial cells. Endothelial cells can synthesize various eicosanoids, including the 12-lipoxygenase (LOX) product 12(S)-hydroxyeicosatetraenoic acid (HETE). Here we demonstrate that endogenous 12-LOX is involved in endothelial cell angiogenic responses. First, the 12-LOX inhibitor, N-benzyl-N-hydroxy-5-phenylpentanamidine (BHPP), reduced endothelial cell proliferation stimulated either by basic fibroblast growth factor (bFGF) or by vascular endothelial growth factor (VEGF). Second, 12-LOX inhibitors blocked VEGF-induced endothelial cell migration, and this blockage could be partially reversed by the addition of 12(S)-HETE. Third, pretreatment of an angiogenic endothelial cell line, RV-ECT, with BHPP significantly inhibited the formation of tubelike/cordlike structures within Matrigel. Fourth, overexpression of 12-LOX in the CD4 endothelial cell line significantly stimulated cell migration and tube differentiation. In agreement with the critical role of 12-LOX in endothelial cell angiogenic responses in vitro, the 12-LOX inhibitor BHPP significantly reduced bFGF-induced angiogenesis in vivo using a Matrigel implantation bioassay. These findings demonstrate that AA metabolism in endothelial cells, especially the 12-LOX pathway, plays a critical role in angiogenesis. (Blood. 2000;95:2304-2311)

Introduction

The formation of new capillaries from preexisting vessels, a process termed angiogenesis, is tightly regulated in physiologic processes such as embryonic development, wound repair, and hypertyperhemia of normal organs. In contrast, persistent unregulated angiogenesis underscores many pathologic conditions, such as tumor growth and metastasis, diabetic retinopathy, atherosclerosis, and chronic inflammation. Angiogenesis is a complex process involving an extensive interplay between cells, soluble factors, and extracellular matrix molecules that culminate in the proliferation, migration, and tube differentiation of endothelial cells. A plethora of angiogenesis regulators such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) can elicit various angiogenic responses from endothelial cells. An understanding of endothelial cell metabolism and the signaling that underlies angiogenesis is important because it provides potential therapeutic targets to inhibit or enhance angiogenesis.

12-lipoxygenases (12-LOX) are a family of isozymes that belong to the LOX superfamily. These enzymes catalyze the stereospecific oxygenation of arachidonic acid (AA) to form 12(S)-hydroperoxyeicosatetraenoic acid (HPETE) and 12(S)-hydroxyeicosatetraenoic acid (HETE). At least 3 types of 12-LOX have been well characterized: platelet-type, leukocyte-type, and epidermal 12-LOX. Platelet-type 12-LOX exclusively uses AA released from glycerophospholipid pools to synthesize 12(S)-HPETE and 12(S)-HETE, whereas leukocyte-type 12-LOX can also synthesize 15(S)-HETE and 12(S)-HETE. In addition to leukocytes and platelets, the expression of 12-LOX isozymes has been detected in various types of cells, such as smooth muscle cells, keratinocytes, endothelial cells, and tumor cells. Elevated 12-LOX activity has been implicated in hypertension, vaso-occlusion in sickle cell disease, inflammation, thrombosis, and mouse skin tumor development. In human prostate carcinoma, the level of 12-LOX expression has been correlated with tumor stage.

Along this line, we recently demonstrated that the overexpression of platelet-type 12-LOX in human prostate cancer PC3 cells stimulated tumor growth by elaborating tumor angiogenesis. In endothelial cells, it has been shown that 12-LOX activity is required for serum- and bFGF-stimulated endothelial cell proliferation and for minimally modified low-density lipoprotein-induced monocyte binding to endothelial cells. It has been shown that 12(S)-HETE can directly stimulate endothelial cell mitogenesis, migration, and tube differentiation. Because endothelial cell proliferation and migration are involved in angiogenesis, these observations prompted us to investigate the functional role of endothelial 12-LOX in angiogenesis. Here we report that endothelial 12-LOX activity is required for endothelial cell proliferation, migration, and tube differentiation in vitro and angiogenesis in vivo. This study suggests the importance of arachidonic acid metabolism in endothelial cell signaling as it relates to angiogenesis.

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Materials and methods

Inhibitors

BHPP, a 12-LOX inhibitor as previously described,24 was a generous gift from Biomide (Grosse Pointe Farms, MI). The IC_{50} for BHPP to inhibit platelet-type 12-LOX activity in tumor cells is approximately 0.2 to 1 µmol/L, depending on cell type.24,25 BHPP does not appreciably inhibit 5(S)-HETE or 15(S)-HETE synthesis in highly metastatic B16a cells.25

Using recombinant enzymes, the IC_{50} of BHPP for the murine platelet-type 12-LOX is 0.8 µmol/L with arachidonic acid as a substrate. The rank of selectivity of BHPP for lipoxigenase inhibition is murine platelet-type 12-LOX > murine epidermis-type 12-LOX > human 5-LOX >> murine leukocyte 12-LOX >> rabbit 15-LOX-1 (Furstenberger G, personal communication). Because of its selectivity toward the platelet-type 12-LOX, BHPP was extensively used in the current study. Other inhibitors for AA metabolism—5-, 8-, 11-, and 14-eicosatetraynoic acid (ETYA), nordihydroguaiaretic acid (NDGA), 5-LOX-activating protein (FLAP) inhibitor MK886, and cyclooxygenase (COX) inhibitor indomethacin—were purchased from Calbiochem (San Diego, CA). Their sites of action are illustrated in Figure 1.

Cell culture

The cord-forming angiogenic endothelial cell line RV-ECT was isolated from the RV-EC cell line established from rat brain resistance vessels as previously described.26 The mouse capillary endothelial cell line CD4 was originally established from mouse lung capillary blood vessels.27 Both the RV-ECT and the CD4 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVEC) and human foreskin dermal microvascular endothelial cells (HMVEC) were purchased from Clonetics (San Diego, CA), multiplied in EGM-2, and used from passages 4 to 10.

Detection of 12-LOX expression by reverse transcription–polymerase chain reaction

Total RNA was isolated from semiconfluent (70% to 80%) endothelial cells using Tri-reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s recommendations. After total RNA was isolated, it was further washed with 2 mol/L LiCl/5 mmol/L EDTA to reduce possible contamination from genomic DNA. Total RNA was reverse transcribed with oligo dT using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD). To detect the expression of platelet-type 12-LOX in human endothelial cells, nested polymerase chain reaction (PCR) was conducted using primers located in different exons of the ERA kit (Rapid Amplification of cDNA Ends kit; #18,73-027; Life Technologies). Three different combinations of primers were used for nested PCR. The first combination yielded the final product of 62 bp using AGACAATAGCAGCAGACT (1 U) and CT-CAGGGTAAAACA (119 L) as the first-round primer set and TGACCTCCTCCAAACAT (26 U) and TCAGGCGTCCGTTGCGTAA (70 L) as the second PCR primer set. The expected product size of the third combination was 88 bp using CAGGAGACATGCTTGTGAA (LOS2) and GAA-CAACTCATCACTCCTGCC (LO-AS2) as the first PCR primer set and AGACAATAGCAGCAGACT (1 U) and TCAGGCGTCCGTTGCGTAA (70 L) as the second primer set. The final PCR products were analyzed using 2% agarose gel.

To study the expression of leukocyte-type 12-LOX in RV-ECT, the following pairs of primers were designed based on the leukocyte-type 12-LOX sequence characterized from rat brain and used for nested PCR: first-round PCR primers, GCGCAAGGCCAAGCAT (lower primer) and CATCCTGTAGGGACACAT (upper primer). The expected size of the second-round PCR product was 342 bp using GCGGAGAAGCAGGAGTAA (lower primer) and ACCTATTGCTCATTTGTGCC (upper primer) as second-round PCR primers.

Immunoblot analysis of 12-LOX expression

Semiconfluent confluent (70% to 80%) HUVEC, HMVEC, RV-EC, RV-ECT, and CD4 endothelial cells were rinsed with ice-cold PBS, scraped into lysis buffer containing 20 mmol/L Tris-HCl, pH 7.5, 2 mmol/L EDTA, 0.5 mmol/L EGTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L leupeptin, 0.15 mmol/L pepstatin A, 1 mmol/L dithiothreitol, and 1% NP-40. Protein concentration was measured using BCA protein assay kit (Pierce, Rockford, IL). Human platelet lysates (10-40 ng) or human epidermoid carcinoma A431 cell lysates (30 µg) were used for positive control. Cell lysates (80 µg) from each sample were loaded onto a minigel for electrophoresis separation. The proteins in the gel were then transferred onto a polyvinylidene difluoride membrane and processed for immunodetection using a rabbit polyclonal antibody to human platelet-type 12-LOX obtained from Oxford Biomedical Research (Oxford, MI). This antibody reacts strongly with platelet-type 12-LOX from various species, with slight cross-reactivity with 5-LOX and 15-LOX at higher concentrations. Horse-radish peroxidase-conjugated goat antirabbit IgG antibodies and enhanced chemiluminescent reagent was purchased from Amersham (Arlington Heights, IL).

Figure 1. Scheme of AA metabolism. The AA released from phospholipids by PLA2 activity is metabolized by the COX pathways to various prostaglandins and thromboxanes (left), by the LOX pathways to various HETEs, leukotrienes, and lipoxins (middle), and by P-450 epoxygenase to epoxyeicosatrienoic acids (right). The proposed site of action for the inhibitors used in this study is shown in parentheses.
Measurement of 12(S)-HETE levels by enzyme immunoassay

To measure 12(S)-HETE synthesis in cell culture, RV-ECT cells (4 × 10^6 cells) were plated and grown to 80% to 90% confluence in DMEM supplemented with 10% FBS and then serum starved overnight in serum-free DMEM. Fresh serum-free DMEM with 1 µmol/L arachidonic acid was added 1 hour before VEGF treatment. BHPP was added to a final concentration of 10 µmol/L 30 minutes before VEGF treatment. Recombinant human VEGF was added in a final concentration of 10 ng/mL. After 30 minutes of treatment, cells were washed in PBS once and harvested using cell scrapers. After centrifugation, the cell pellets were resuspended in cell lysis buffer and treated with recombinant human VEGF and bFGF (R&D Systems, Minneapolis, MN) plus various amounts of BHPP. The concentration of recombinant human VEGF and bFGF was 10 ng/mL. After 2 days, the plates were processed to quantitate the absorbance at 490 nm (A490) using an MTS cell proliferation assay kit. The A490 reading 2 to 3 hours after plating was used as a baseline. The number of cells was expressed as the percentage of increase from the A490 baseline.

Endothelial cell tube/cord formation assay

Cord-forming RV-ECT cells or CD4 transfectants (1 × 10^3 cells) were plated on a 24-well plate precoated with a thin layer of Matrigel (Becton Dickinson). After overnight incubation, BHPP was added. After 24 hours of treatment, the media were removed and the confluent monolayer was overlaid with 0.5 mL diluted Matrigel (Becton Dickinson; final concentration, 5 mg/mL). After solidifying at 37°C, 0.5 mL DMEM – 10% FBS medium was carefully added without disturbing the gel. The formation of a tubelike structure was monitored microscopically every 6 hours and recorded.

Matrigel implantation assay for angiogenesis

The Matrigel (Becton Dickinson) implantation assay was performed as described by Ito et al.29 with the following modifications. Matrigel 0.4 mL premixed with bFGF (5 µg/ml) with and without BHPP (0.9 mg/ml) was injected subcutaneously into nude mice (4 mice/group). Mice were killed 5 days after injection and dissected to expose the implants for recording using an SP SZ-4060 stereomicroscope (Olympus America, Melville, NY). The amount of blood retained in the Matrigel was further assessed by measuring the hemoglobin levels using Drabkin’s reagent (Sigma Diagnostics, St. Louis, MO).

Stable transfection of CD4 endothelial cells and characterization

Semiconfluent CD4 endothelial cells were transfected with a pcDNA 3.1 expression construct containing human platelet-type 12-lipoxygenase cDNA, which was a gift from Dr Colin Funk (University of Pennsylvania). Empty vector was used as a control. Transfection was performed using lipofectin reagent (Life Technologies). Transfectants were selected using 300 µg/mL geneticin (G418) in DMEM with 10% FBS. The expression of 12-LOX in CD4 transfectants was characterized by reverse transcription (RT)-PCR and Northern blot analysis for mRNA expression and by immunoblot for 12-LOX protein expression.

Endothelial cell proliferation assay

HUVEC cells were used to study the effects of 12-LOX inhibitors on endothelial cell proliferation. Cells were harvested by trypsinization, resuspended in EGM-2, and plated in a 96-well plate at 2000 cells per well. After overnight incubation, the media were changed to fresh EBM-2 with 1% FBS and treated with recombinant human VEGF-A or bFGF (R&D Systems, Minneapolis, MN) plus various amounts of BHPP. The concentrations of BHPP used were 0, 1, 10, and 50 µmol/L unless otherwise indicated. The final concentration of recombinant human VEGF and bFGF was 10 µmol/L. After 2 days, the plates were processed to quantitate the number of cells using an MTS cell proliferation assay kit (Promega, Madison, WI). The absorbance at 490 nm (A_490) indicated the relative proliferation. The final concentration of recombinant human VEGF and bFGF was 10 µmol/L 30 minutes before VEGF treatment. Recombinant human VEGF was added in a final concentration of 10 ng/mL. After 30 minutes of treatment, cells were washed in PBS once and harvested using cell scrapers. After centrifugation, the cell pellets were resuspended in cell lysis buffer and treated with recombinant human VEGF and bFGF (R&D Systems, Minneapolis, MN) plus various amounts of BHPP. The concentrations of BHPP used were 0, 1, 10, and 50 µmol/L unless otherwise indicated. The final concentration of recombinant human VEGF and bFGF was 10 µmol/L. After 2 days, the plates were processed to quantitate the number of cells using an MTS cell proliferation assay kit (Promega, Madison, WI). The absorbance at 490 nm (A_490) indicated the relative cell proliferation. As shown in Figure 2C, primary cultures of HUVEC and HMVEC had the highest levels of 12-LOX expression, whereas CD4, an endothelial cell line derived from mouse pulmonary microvasculature,27 had the lowest 12-LOX expression.

Immunoblot analysis using a rabbit polyclonal antibody against human platelet-type 12-LOX revealed that 12-LOX is also expressed in endothelial cells at the protein level. As shown in Figure 2C, primary cultures of HUVEC and HMVEC had the highest levels of 12-LOX expression, whereas CD4, an endothelial cell line derived from mouse pulmonary microvasculature,27 had the lowest 12-LOX expression.

When RV-ECT cells were treated with VEGF for 30 minutes, a 5-fold increase in 12(S)-HETE biosynthesis was observed (Figure 2D). The increased 12-LOX activity was inhibited by pretreating RV-ECT cells with BHPP (10 µmol/L). Because BHPP is 20-fold more selective toward platelet-type 12-LOX than leukocyte-type 12-LOX, the results suggest that the increased 12(S)-HETE synthesis on VEGF treatment was probably caused by the increased activity of platelet-type 12-LOX.

12-Lipoxygenase expression and activity in endothelial cells

Several studies have provided evidence that endothelial cells synthesize various lipoxygenase products such as 5(S)-HETE, 12(S)-HETE, and 15(S)-HETE.30 The expression of platelet-type 12-LOX in HUVEC cells was previously detected by RT-PCR.6 Using primers selective for platelet-type 12-LOX, the expression of 12-LOX mRNA was confirmed in HUVEC cells and also detected in microvascular endothelial cells, HMVEC (Figure 2A). In RV-ECT, an endothelial cell line derived from rat brain resistance microvessel, a faint band of PCR product was also present (Figure 2A). To further confirm the expression of platelet-type 12-LOX in RV-ECT cells, we designed 7 primers on the basis of the partial sequence obtained from rat Walker 256 cells.31 The expression of platelet-type 12-LOX was detected by nested PCR using 3 different combinations of these 7 primers (Figure 2B). Interestingly, in addition to platelet-type 12-LOX, RV-ECT cells also expressed another isoform of 12-LOX that presumably was leukocyte-type as detected by using primers designed on the basis of the rat leukocyte-type 12-LOX sequence29 (data not shown), suggesting that RV-ECT cells expressed both platelet- and leukocyte-type 12-LOX.
shown in Figure 2E, inhibition of 12-LOX activity by BHPP significantly inhibited bFGF- or VEGF-stimulated HUVEC proliferation, suggesting that 12-LOX activity is required for the endothelial cell proliferative responses to bFGF or VEGF.

Involvement of endogenous 12-lipoxygenase in endothelial cell migration

Endothelial cell migration is a requisite step in angiogenesis. It has been shown that the activation of phospholipase A₂ is required for endothelial cell migration in response to bFGF. To study whether AA released by phospholipase A₂ modulates endothelial cell migration, we selected the RV-ECT cell line, which can grow in DMEM–10% FBS without bFGF or VEGF supplementation, for cell migration assay. First we examined the migratory response of RV-ECT cells toward exogenous AA, bFGF, and VEGF. As shown in Figure 3A, AA increased endothelial cell migration by 70% to 80%, a level comparable to that of bFGF but less than VEGF. This observation is consistent with the report that VEGF is a stronger chemotactic factor than bFGF. Because mobilization of AA has been observed in endothelial cells on stimulation with angiogenic factors such as VEGF, bFGF, and angiogenin, we studied the effect of various inhibitors of arachidonic acid metabolism on endothelial cell migration stimulated by VEGF. As shown in Figure 3B, ETYA, a promiscuous inhibitor for AA metabolism, inhibited VEGF-stimulated RV-ECT migration. NDGA, a general LOX inhibitor, also significantly reduced VEGF-stimulated RV-ECT migration. In contrast, indomethacin, a general COX inhibitor, had no effect. The results suggest that the LOX pathway, but not the COX pathway, of AA metabolism is involved in RV-ECT migration. In the LOX pathways, AA can be used by 5-LOX to synthesize 5(S)-HETE and leukotrienes, by 12-LOX to synthesize mainly 12(S)-HETE, and by 15-LOX to synthesize 15(S)-HETE. To delineate which pathway(s) is involved in endothelial cell migration, we first studied the effect of various types of HETEs on RV-ECT cell migration. As shown in Figure 3C, among the various types of HETEs tested, only 12(S)-HETE, but not 5(S)-HETE, 15(S)-HETE, or 12(R)-HETE, could stimulate endothelial cell migration. The stimulation of endothelial cell migration by 12(S)-HETE is consistent with previous observations. To study whether the endogenous synthesis of 12(S)-HETE is involved in endothelial cell migration in response to VEGF, we examined the effect of BHPP on VEGF-stimulated endothelial cell migration. As shown in

Figure 2. Expression of platelet-type 12-LOX in endothelial cells and its role in cell proliferation. (A) RT-PCR detection of 12-LOX expression in endothelial cells. Total RNA was isolated and processed for double-round RT-PCR using primers designed on the basis of human platelet-type 12-LOX sequence as described in "Materials and Methods." Control, no RNA present in PCR or RT reaction mixtures as controls for the quality of PCR; RT(−), no reverse transcriptase present in RT reaction mixtures as controls for the possible contamination of DNA in RNA samples; RT(+), reverse transcription present. (B) RT-PCR detection of platelet-type 12-LOX expression in RV-ECT cells. The primer combinations for 1st, 2nd, and 3rd are described in "Materials and methods." The target sizes of the final PCR product from 1st, 2nd, and 3rd primer combinations are 111 bp, 62 bp, and 88 bp, respectively. (C) Immunoblot analysis of 12-LOX expression in endothelial cells. The blot was probed with a rabbit polyclonal antibody to human platelet-type 12-LOX. (D) Inhibition of VEGF-stimulated 12-LOX activity by BHPP. Cell treatment and measurement of 12(S)-HETE are detailed in "Materials and methods." Columns, average levels of 12(S)-HETE per 1 × 10⁶ cells (n = 3); bars, SE. a, P < .01 when compared with the unstimulated control; b, P < .05 when compared to the VEGF-stimulated cells. (E) Involvement of endothelial 12-LOX in bFGF- or VEGF-stimulated cell proliferation. HUVEC cells were plated in 96-well culture plates. Cell proliferation was stimulated with 10 ng/ml bFGF (open triangle) or 10 ng/ml VEGF (filled circle) in EBM-2 with 2% FBS. Cells with no bFGF or VEGF stimulation were used as controls (open circle). 48 hours after treatment with graded levels of BHPP, cell numbers were measured by an MTS method as described in "Materials and methods." Data point, mean from quadruplicate determination; bars, SE from quadruplicate of treatment.

Figure 3. Arachidonate metabolites in endothelial cell migration. The migration assays were performed as described in "Materials and methods." (A) Stimulation of RV-ECT migration by arachidonic acid (1 µmol/L), bFGF (10 ng/ml), and VEGF (10 ng/ml). (B) VEGF-stimulated RV-ECT migration involves lipoxygenase-dependent arachidonic acid metabolism. Treatment: VEGF, 10 ng/ml; ETYA, 5 µmol/L; NDGA, 50 µmol/L; indomethacin, 50 µmol/L. (−), absence of treatment (vehicle only); (+), presence of treatment. (C) Differential effects on RV-ECT cell migration by various HETEs. (D) Modulation of RV-ECT cell migration by 12-LOX inhibitor BHPP and 12(S)-HETE. Columns, percentage of the average number of cells migrated when compared with the controls; bars, SE. (A, P < .05; b, P < .01; Student’s t-test). All migration assays were repeated at least 4 times.
activities of VEGF, we used bFGF premixed with Matrigel to investigate the role of angiogenesis in vivo by bFGF requires the angiogenic activity can compromise angiogenesis in vivo. Because the induction of angiogenesis in vivo by bFGF requires the angiogenic activities of VEGF, we used bFGF premixed with Matrigel to induce angiogenesis. As shown in Figure 6A, bFGF induced massive angiogenesis around and within the implant (upper panel, left). When dissected out, the implanted Matrigel retained a large volume of blood within the gel (upper panel, right). Matrigel implants without bFGF had little or no angiogenic activities in vivo (bottom panel). Inclusion of the 12-LOX inhibitor BHPP in the implants significantly reduced the ability of bFGF to induce angiogenesis (Figure 6A, middle panel), suggesting that 12-LOX is involved in angiogenesis in vivo. Figure 6B shows the hemoglobin levels in the dissected Matrigel. As shown in the Figure, BHPP significantly reduced the hemoglobin levels in Matrigel (P < .05), suggesting a reduction of angiogenesis. The reduction of angiogenesis was closely correlated with the levels of 12(S)-HETE in the Matrigel implants as shown in Figure 6C. Taken together, the data suggest a critical role of 12-LOX activity in angiogenesis in vivo.

Discussion

In this study we demonstrated that endothelial cells from different species (rat and human) and different organs (brain, umbilical cord, and foreskin) express platelet-type 12-LOX and elucidated its important role in endothelial cell responses to angiogenic stimuli.
Inhibition of 12-LOX activity by BHPP, a platelet-type selective inhibitor, attenuated the endothelial cell mitogenic and migratory responses to the angiogenic factors bFGF and VEGF and the tubelike differentiation on Matrigel. Forced expression of 12-LOX in the CD4 endothelial cells stimulated cell migration and promoted tube differentiation. Inhibition of 12-LOX activity by BHPP significantly reduced angiogenesis in vivo. Our findings suggest that eicosanoids from the arachidonic acid metabolism through the 12-LOX pathway, ie 12(S)-HETE, are involved in modulating angiogenesis.

There are seemingly conflicting reports regarding the isozymes of 12-LOX expressed in endothelial cells as both leukocyte type 12-LOX and platelet-type 12-LOX are reported. In the current study, we demonstrated that platelet-type 12-LOX was expressed in HUVEC and HMVEC as well as in RV-ECT, an endothelial cell line originally isolated from rat brain resistance blood vessels. RV-ECT cells also express another isoform of 12-LOX originally isolated from rat brain and close to leukocyte-type.29 When RV-ECT cells were treated with VEGF, there was a 5-fold increase in 12(S)-HETE biosynthetic activity inhibitable by BHPP pretreatment. Because BHPP is much more selective toward platelet-type 12-LOX than leukocyte-type (Furstenberger G, personal communication), the results suggest that it is the platelet-type 12-LOX, not the leukocyte-type 12-LOX, that is activated in endothelial cells during angiogenic responses.

Arachidonic metabolites have been implicated in angiogenesis since the inhibition of arachidonic acid metabolism by α-guaiaconic acid (GR-12) attenuated endothelial cell migration, tube formation, and angiogenesis in vivo.36 Cellular mobilization of AA is usually achieved by PLA2 cleavage of phospholipids. The activation of PLA2 and the subsequent mobilization of AA have been observed in endothelial cells in response to various extracellular cues such as angiogenin, bFGF, zinc, and phorbol ester.35,34,37,38 As a potent angiogenic factor, bFGF stimulates the migration and proliferation of vascular endothelial cells. Abrogation of the release of AA in endothelial cells by the inhibition of PLA2 activity inhibited bFGF-stimulated cell proliferation19 and migration.30 VEGF also can rapidly increase phosphorylation and activity of cytosolic PLA2 and stimulate the release of AA in HUVEC.32 In a recent study, it was shown that the inhibition of PLA2 activity in granuloma by SB 203 347 significantly reduced angiogenesis,39 suggesting that the activation of PLA2 and the subsequent mobilization of AA and lyso phospholipid are intrinsic steps during angiogenesis.
The downstream events for released AA include the synthesis of various prostaglandins and thromboxanes through the COX pathway, various HETEs and lipoxins through the LOX pathways, and various epoxyeicosatrienonic acids through cytochrome P-450 epoxygenase. In this study, we found that ETYA, a general inhibitor for arachidonic acid-derived metabolism, and NDGA, an agent that can inhibit LOX activity, inhibited endothelial cell migration stimulated by VEGF. On the other hand a general COX inhibitor, indomethacin, had no appreciable effect. In contrast, BHPP, which is a selective platelet-type 12-LOX inhibitor, blocked VEGF-stimulated endothelial cell migration. The involvement of 12-LOX and its AA metabolite in endothelial cell migration was further strengthened by the observations that exogenously added 12(S)-HETE directly stimulated RV-ECT migration and partially reversed the inhibitory effect of BHPP on cell migration. Finally, the overexpression of 12-LOX in CD4 endothelial cells significantly stimulated cell migration in a 12-LOX activity-dependent manner. The findings collectively suggest the role of endothelial 12-LOX and its AA metabolite, 12(S)-HETE, in endothelial cell migration.

In addition to its role in endothelial cell migration, we found that 12-LOX is involved in endothelial cell tube formation. Pretreatment of the RV-ECT cell line with BHPP significantly inhibited the formation of vessel-like structures within Matrigel. The second line of evidence is the observation that the overexpression of 12-LOX in CD4 endothelial cells promoted the formation of tubelike structures on Matrigel. Interestingly, it has been extensively documented that exogenous 12(S)-HETE can induce a reversible retraction of endothelial cell monolayers cultured on collagen by regulating PKC and αvβ3 integrin.\(^{40,41}\) It remains to be determined, however, whether endothelial cell retraction is an early event of tube differentiation on matrix proteins such as collagens or Matrigel. Studies are under way to determine this potential relationship.

It has been reported that the 12-LOX pathway of AA metabolism is required in bFGF-stimulated endothelial cell proliferation.\(^{14}\) We demonstrated that the inhibition of 12-LOX activity also compromised VEGF-stimulated endothelial cell proliferation. The role of 12-LOX in endothelial cell proliferation, together with the finding that 12-LOX is involved in endothelial cell migration and tube differentiation, implicate the mobilization of arachidonic acid and the generation of 12(S)-HETE through the 12-LOX pathway in endothelial cells as an early event in the intracellular cascade of angiogenic responses. Currently we are exploring the mechanism by which 12-LOX and 12(S)-HETE participate in the signaling events elicited by VEGF or bFGF in endothelial cells.

The involvement of 12-LOX in endothelial cell angiogenic responses in vitro is further corroborated by the observation that the 12-LOX inhibitor BHPP significantly reduced bFGF-stimulated angiogenesis in vivo. Because bFGF induces angiogenesis by modulating endothelial cell expression of VEGF, which in turn contributes to angiogenesis in an autocrine mechanism,\(^{39}\) BHPP may have inhibited angiogenesis by attenuating endothelial cell angiogenic responses to bFGF and VEGF.

It should be noted that although platelet-type 12-LOX uses arachidonic acid to synthesize 12(S)-HETE almost exclusively, platelet-type 12-LOX has been shown to use leukotriene A\(_4\) to synthesize lipoxin\(^{12-24}\) and also 5(S)-HETE and 15(S)-HETE to form 5(S), 12(S)-DiHETE and 14(R), 15(S)-DiHETE, respectively.\(^{43}\) It awaits further studies regarding whether other 12-LOX products, additional to 12(S)-HETE, is angiogenic. In vivo, 12(S)-HETE is a prominent product of arachidonic acid metabolism through the LOX pathway in platelets. The pro-angiogenic function of 12(S)-HETE as delineated in the current study and in our previous reports\(^{12,13}\) implicates the possible involvement of platelets in angiogenesis during tumor growth and metastasis. Indeed, platelets are intimately involved in tumor angiogenesis,\(^{46,47}\) and platelet aggregation stimulates the release of VEGF.\(^{48}\) Clinically, 30% to 60% of patients with advanced cancer have platelet abnormalities such as thrombocytosis and many other thromboembolic disorders. In addition, activated platelets have been frequently associated with many malignant tumors.\(^{49}\) Obviously, the involvement of platelets adds to the complexity of tumor angiogenesis regulation.

In summary, our data suggest the important role of 12(S)-HETE generated by the 12-LOX pathway in angiogenesis and suggest the possibility of using 12-LOX inhibitors to treat angiogenic diseases such as tumor growth and arthritis. Studies are under way to evaluate the efficacy of 12-LOX inhibitors against solid tumor growth in vivo.

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References


17. Tang DG, Grossi IM, Chen YQ, Diglio CA, Honn KV. 12(S)-HETE promotes tumor-cell adhesion by increasing surface expression of \( \alpha_v \beta_3 \) integrins on ECs. Int J Cancer. 1993;54:102.


40. Tang DG, Diglio CA, Honn KV. 12(S)-HETE-induced microvascular endothelial cell retraction results from PKC-dependent rearrangement of cytoskeletal elements and \( \alpha_b \beta_3 \) integrins. Prosta. glandins. 1993;45:249.


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