15(S)-Hydroxyeicosatetraenoic Acid Induces Angiogenesis via Activation of PI3K-Akt-mTOR-S6K1 Signaling

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Abstract

To determine whether the lipoxygenase metabolites of arachidonic acid, 5(S)-, 12(S)-, and 15(S)-hydroxyeicosate-traenoic acids [5(S)-HETE, 12(S)-HETE, and 15(S)-HETE, respectively] are angiogenic, we have studied their effects on human dermal microvascular endothelial cell (HDMVEC) tube formation and migration. All three HETEs stimulated HDMVEC tube formation and migration. Because 15(S)-HETE was found to be more potent than 5(S)-HETE and 12(S)-HETE in HDMVEC tube formation, we next focused on elucidation of the signaling mechanisms underlying its angiogenic activity. 15(S)-HETE stimulated Akt and S6K1 phosphorylation in HDMVEC in a time-dependent manner. Wortmannin and LY294002, two specific inhibitors of phosphatidylinositol 3-kinase (PI3K), blocked both Akt and S6K1 phosphorylation, whereas rapamycin, a specific inhibitor of Akt downstream effector, mammalian target of rapamycin (mTOR), suppressed only S6K1 phosphorylation induced by 15(S)-HETE suggesting that this eicosanoid activates the PI3K-Akt-mTOR-S6K1 signaling in HDMVEC. Wortmannin, LY294002, and rapamycin also inhibited 15(S)-HETE-induced HDMVEC tube formation and migration. In addition, all three HETEs stimulated angiogenesis as measured by *in vivo* Matrigel plug assay with 15(S)-HETE being more potent. Pharmacologic inhibition of PI3K-Akt-mTOR-S6K1 signaling completely suppressed 15(S)-HETE-induced *in vivo* angiogenesis. Consistent with these observations, adenoviral-mediated expression of dominant-negative Akt also blocked 15(S)-HETE–induced HDMVEC tube formation and migration and *in vivo* angiogenesis. Together, these results show for the first time that 15(S)-HETE stimulates angiogenesis via activation of PI3K-Akt-mTOR-S6K1 signaling. (Cancer Res 2005; 65(16): 7283-91)

Introduction

Arachidonic acid is an important polyunsaturated fatty acid component of membrane phospholipids and is released acutely in response to a number of agonists, including growth factors, cytokines, and hormones in various cell types (1–4). Upon release, it is either metabolized via the cyclooxygenase (COX), lipoxygenase (LOX), or cytochrome *P*450 monooxygenase (CYP) pathways producing prostaglandins, hydroperoxyeicosatetraenoic acids, and epoxyeicosatrienoic acids (EET), respectively, or reincorporated into membrane phospholipids via esterification involving sequential actions of arachidonoyl-CoA synthetase and arachidonoyl-lysophospholipid transferase (1, 5). Arachidonic acid and its oxygenative metabolites, collectively known as eicosanoids, are involved in the regulation of important biological activities such as maintenance of vascular tone (5, 6). In addition, these lipid molecules have been reported to mediate various intracellular signaling events in response to different stimulants (7–12). Studies over the past several years also suggest that the COX, LOX, and CYP metabolites of arachidonic acid stimulate the proliferative capacity of various cell types and play a role in tumor progression (13–20). In addition, eicosanoids have been shown to be associated with the pathogenesis of vascular diseases such as atherosclerosis and restenosis (21, 22). Despite the involvement of eicosanoids in the development of various types of cancers and vascular diseases, their mechanisms of actions are less understood.

Formation of new capillaries, a process known as angiogenesis, plays an important role in embryonic development and wound healing (23, 24). In addition, angiogenesis is a crucial player in the progression of various tumors and vessel wall diseases (23–27). Migration and proliferation of microvascular endothelial cells are essential events of angiogenesis (25). Factors such as fibroblast growth factor and vascular endothelial growth factor that influence endothelial cell migration and proliferation are therefore likely involved in embryonic development and disease processes (23–27). The reports that eicosanoids such as 12(S)-HETE, 12(R)-HETE, 11,12-EET, and 14,15-EET induce angiogenesis (19, 28–30) provide clues for elucidation of the possible mechanisms by which these lipid mediators could be involved in tumor progression and vessel wall lesions. Phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR)-S6K1 signaling has been reported to play a major role in mediating cell survival and proliferation and angiogenesis in response to various stimulants (21–36). Previously, we have reported that 5(S)-HETE, the 5-LOX metabolite of arachidonic acid, stimulates DNA synthesis via activation of PI3K-Akt-mTOR-S6K1 in human dermal microvascular endothelial cell (HDMVEC; ref. 37). To understand better the role of eicosanoids in the development of various types of cancers as well as vessel wall diseases, we have undertaken a systematic study to identify eicosanoids with potent angiogenic activities and elucidate the underlying signaling mechanisms. Here, we report for the first time that 15(S)-HETE, the 15-LOX metabolite of arachidonic acid, stimulates HDMVEC tube formation and migration *in vitro* and angiogenesis *in vivo*. In addition, the present observations indicate that activation of PI3K-Akt-mTOR-S6K1 signaling is essential for 15(S)-HETE-induced HDMVEC tube formation and migration and Matrigel plug angiogenesis.

Materials and Methods

Reagents. Aprotinin, DTT, Drabkin's reagent, HEPES, leupeptin, phenyl-methylsulfonyl fluoride (PMSF), sodium deoxycholate, and sodium orthovanadate were purchased from Sigma Chemical Co. (St. Louis, MO). 5(S)-HETE, 12(S)-HETE, and 15(S)-HETE were bought from Cayman Chemicals (Ann Arbor, MI). Growth factor–reduced Matrigel was obtained from BD Biosciences (Bedford, MA). LY294002 was procured from Calbiochem

Chemicals (San Diego, CA). Rapamycin and wortmannin were bought from Biomol (Plymouth Meeting, PA). Phospho-specific anti-Akt, anti-S6K1, and normal anti-Akt and S6K1 antibodies were obtained from Cell Signaling Technology (Beverly, MA).

Cell culture. HDMVEC were bought from Clonetics (Walkersville, MD). Cells were grown in endothelial basal medium-2 (EBM-2; CC-3156) containing EGM-2 MV SingleQuots (CC-4147; Cambrex Bio Science Walkersville, Inc., Walkersville, MD). Cultures were maintained at 37°C in a humidified 95% air and 5% CO_2 atmosphere. Cells were quiesced by incubating in EBM-2 for 24 hours and used to perform the experiments unless otherwise indicated.

Cell migration assay. Cell migration was done using a modified Boyden chamber method as described by Nagata et al. (38). The cell culture inserts containing membranes with 10 mm in diameter and 8.0-μm pore size (Nalge Nunc International, Rochester, NY) were placed in a 24-well tissue culture plate (Costar, Corning Inc., Corning, NY). The lower surface of the porous membrane was coated with 0.5% gelatin at 4°C overnight and blocked with 0.1% heat-inactivated bovine serum albumin at 37°C for 1 hour. HDMVEC were quiesced for 24 hours in EBM-2, washed with HBSS, trypsinized, and neutralized with TNS (Cambrex Bio Science Walkersville). Cells were seeded into the upper chamber at 1×10^5 cells per well. Vehicle or eicosanoid of interest were added to the lower chamber at the indicated concentration. Both the upper and lower chambers contain EBM-2. After 8 hours of incubation at 37°C, nonmigrated cells were removed from the upper side of the membrane with cotton swabs and the cells on the lower surface of the membrane were fixed in methanol for 15 minutes. The membrane was then stained with Giemsa-Wright stain for 10 minutes (Sigma Chemical) and washed once each with 50% and 100% alcohol. Cells were counted in five randomly selected squares per well under light microscope (Model, Eclipse 50i, Nikon, Tokyo, Japan) and presented as number of migrated cells per field.

Cell survival assay. HDMVEC survival was measured using trypan blue dye exclusion assay. After various treatments, cells were trypsinized, neutralized, and pelleted by centrifugation at 2,000 rpm for 5 minutes at 4°C. Cell pellets were suspended in PBS and trypan blue dye was added to a final concentration of 0.2% and the stained and unstained cells were counted by hemacytometer. Cell viability was determined as ratio of unstained cells (living cells) to total number of counted cells (unstained + stained cells).

DNA synthesis. HDMVEC with and without appropriate treatments were labeled with 1 μCi/mL [^{3}H]-thymidine for the last 20 hours of the 24-hour incubation period. After labeling, cells were washed with cold PBS, trypsinized, and collected by centrifugation. The cell pellet was suspended in cold 10% (w/v) trichloroacetic acid (TCA) and vortexed vigorously to lyse cells. After standing on ice for 20 minutes, the cell lysate mixture was passed through a glass fiber filter (GF/C, Whatman, Clifton, NJ). The filter was washed once with cold 5% TCA and once with cold 70% (v/v) ethanol. The filter was dried, placed in a scintillation vial containing the scintillant fluid, and the radioactivity was measured in a Beckman liquid scintillation counter (LS 5000TA).

Matrigel plug angiogenesis assay. Matrigel plug angiogenesis assay was done essentially as described by Medhora et al. (30). All the animal protocols were done in accordance with the relevant guidelines and regulations approved by the Internal Animal Care and Use Committee of the University of Tennessee Health Science Center. C57BL/6 mice (8 weeks old) were lightly anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and were injected s.c. with 0.5 mL of growth factor–reduced Matrigel that was premixed with vehicle or 50 μmol/L of a specific eicosanoid along the dorsal midline. The injection was made rapidly with a B-D 26G1/2 needle to ensure the entire content was delivered in one plug. The mice were allowed to recover, and 7 days later, the animals were sacrificed by inhalation of CO_2 and the Matrigel plugs were harvested from underneath the skin. The plugs were homogenized in 1 mL of deionized H_2O on ice and cleared by centrifugation at 10,000 rpm for 6 minutes at 4°C. The supernatant was collected and used in duplicate to measure hemoglobin content with Drabkin's reagent along with hemoglobin standards

essentially as suggested by the manufacturer (Sigma Chemical). The absorbance was read at 540 nm in an ELISA plate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA). These experiments were repeated at least thrice with four mice for each group and the values are expressed as g/d of hemoglobin/mg plug.

Tube formation assay. Tube formation assay was done as described by Nagata et al. (38). Ninety-six-well culture plates (Costar, Corning) were coated with growth factor–reduced Matrigel (BD Biosciences) in a total volume of 20 μL and allowed to solidify for 30 minutes at 37°C. HDMVEC were trypsinized, neutralized with TNS, and resuspended at 5×10^4/mL and 200 μL of this cell suspension were added into each well. Vehicle or eicosanoid of interest at the indicated concentration were added to the appropriate well and the cells were incubated at 37°C for 6 hours. Tube formation was observed under an inverted microscope (Model, Eclipse TS100, Nikon). Images were captured with a CCD color camera (Model, KP-D20AU, Hitachi, Tokyo, Japan) attached to the microscope and tube length was measured using the NIH Image J 1.31v Program.

Western blot analysis. After appropriate treatments, HDMVEC were rinsed with cold PBS and frozen immediately in liquid nitrogen. Cells were lysed by thawing in 500 μL of lysis buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/mL PMSF, 100 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mmol/L sodium orthovanadate) and scraped into 1.5-mL Eppendorf tubes. After standing on ice for 20 minutes, the cell lysates were cleared by centrifugation at 12,000 rpm for 20 minutes at 4°C. Cell lysates containing equal amount of protein were resolved by electrophoresis on 0.1% SDS and 10% polyacrylamide gels. The proteins were transferred electrophoretically to a nitrocellulose membrane (Hybond, Amersham Pharmacia Biotech, Piscataway, NJ). After blocking in 10 mmol/L Tris-HCl buffer (pH 8.0) containing 150 mmol/L sodium chloride, 0.1% Tween 20, and 5% (w/v) nonfat dry milk, the membrane was treated with appropriate primary antibodies followed by incubation with horseradish peroxidase–conjugated secondary antibodies. The antigen-antibody complexes were detected using chemiluminescence reagent kit (Amersham Pharmacia Biotech).

Adenoviral vector construction. Adenoviral vectors expressing either dominant-negative Akt (Ad-dnAkt) or constitutively active Akt (Ad-myrAkt) were constructed by Fujio et al. (39) and was generously provided to us by Dr. Kenneth Walsh (Boston University School of Medicine, Boston, MA) for use in our experiments. To construct Ad-GFP vector, the GFP DNA fragment was excised from pEGFP-N3 (Clontech, Palo Alto, CA) by digestion of the plasmid with *Sal*I and *Not*I and subcloned into an entry vector, pENTR3C (Invitrogen, Carlsbad, CA), producing pENTR3C-GFP. The pENTR3C-GFP was transformed into *Escherichia coli* DH5α and the plasmid was amplified. The plasmid was recombined with pAd/CMV/V5-DEST as described by the manufacturer (Invitrogen) producing pAdGFP plasmid and verified by DNA sequencing. The pAdGFP was linearized with *Pac*I and transfected into HEK293A cells. The resulting adenovirus was further amplified by infection of HEK293A cells and purified by cesium chloride gradient ultracentrifugation (40). AdGFP, Ad-dnAkt, and Ad-myrAkt virus were titrated using standard plaque assay (40).

Statistics. All the experiments were repeated thrice with similar results. Data on HDMVEC tube formation and migration and Matrigel plug angiogenesis are presented as mean ± SD. The treatment effects were analyzed by Student's *t* test. *P*s < 0.05 were considered statistically significant. In the case of Western blot analysis, one representative set of data is shown.

Results

Hydroxyeicosatetraenoic acids stimulate human dermal microvascular endothelial cell tube formation and migration. A body of data accumulated in the past several years indicates that the COX, LOX, and CYP metabolites of arachidonic acid are involved in the maintenance of survival and proliferative capacities of various types of normal and cancer cells and play

a role in tumor progression and vessel wall diseases (2, 4, 5, 12–20). Despite these evidences linking arachidonic acid metabolites to the development of various cancers including colon and prostate cancers and vessel wall diseases, very little is known with regard to the mechanisms by which eicosanoids influence these lesions. A few studies in recent years have shown that eicosanoids, such as 12(S)-HETE, 12(R)-HETE, 11,12-EET, and 14,15-EET, are involved in the regulation of angiogenesis (19, 28–30). Because angiogenesis plays a crucial role in tumor growth and vessel wall diseases, we have undertaken a systematic study to identify the eicosanoids with potent angiogenic capacities and elucidate the underlying signaling mechanisms. Microvascular endothelial cell proliferation, migration, and tube formation are important events in angiogenesis. Towards understanding the capacity of eicosanoids in the regulation of angiogenesis, we have previously reported that 5(S)-HETE, the 5-LOX metabolite of arachidonic acid, enhances the ability of HDMVEC to proliferate via activating the PI3K-Akt-mTOR-S6K1 signaling (37). In this report, we focused on studying the effects of various LOX metabolites of arachidonic acid [i.e., 5(S)-HETE, 12(S)-HETE, and 15(S)-HETE] in stimulating angiogenesis using *in vitro* and *in vivo* model systems. Quiescent HDMVEC were seeded onto 96-well plate coated with growth factor–reduced Matrigel and treated with vehicle or 0.1 μmol/L of 5(S)-HETE, 12(S)-HETE, or 15(S)-HETE for 6 hours at 37°C and tube formation was measured. As shown in Fig. 1A, all three HETEs tested were found to stimulate HDMVEC tube formation by about 1.5- to 2-fold compared with control. We next determined the effect of these eicosanoids on HDMVEC migration using a modified Boyden chamber method. Quiescent HDMVEC were added to the upper chamber of the culture inserts that were placed in a 24-well plate and the vehicle or 0.1 μmol/L of the indicated HETE were added to the lower chamber. After incubation at 37°C for the indicated time, the migrated cells in the lower surface of the membrane were fixed, stained with Giemsa-Wright stain, and counted under a light microscope. All three HETEs induced HDMVEC migration by about 1.6- to 2-fold compared with control (Fig. 1B). Although all the three HETEs were found to possess similar capacity to stimulate HDMVEC migration, 15(S)-HETE was observed to be more effective on stimulating HDMVEC tube formation than the other two lipid molecules. Because microvascular endothelial cells normally do not exist in quiescence state in tumors, we also tested the ability of 15(S)-HETE on inducing tube formation and migration of proliferating HDMVEC. Consistent with its effect on quiescent cells, 15(S)-HETE also stimulated both tube formation and migration of proliferating HDMVEC, although to a lesser degree (Fig. 2A and B). Given the more effectiveness of 15(S)-HETE in stimulating HDMVEC tube formation than 5(S)-HETE or 12(S)-HETE, we next focused on investigating the mechanisms of its angiogenic actions.

15(S)-hydroxyeicosatetraenoic acid stimulates Akt and S6K1 phosphorylation in human dermal microvascular endothelial cell in a time-dependent manner. Akt, a serine/threonine kinase, has been shown to play an essential role in the mediation of angiogenesis in response to various stimulants including hypoxia and nitric oxide (34–36, 38). Previously, we have reported that 5(S)-HETE, the 5-LOX metabolite of arachidonic acid, stimulates HDMVEC DNA synthesis via activation of PI3K-Akt-mTOR-S6K1 signaling (37). To find whether the PI3K-Akt-mTOR-S6K1 signaling also plays a role in angiogenesis induced by 15(S)-HETE, we have studied first the time course effect of this
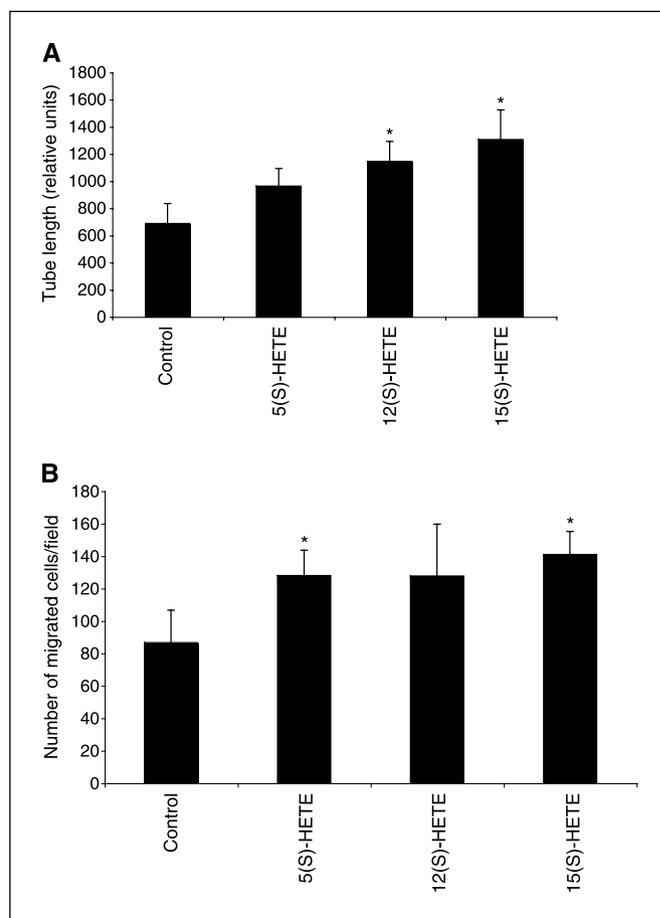
Figure 1. HETEs induce HDMVEC tube formation and migration. *A,* quiescent HDMVEC were seeded onto 96-well plate coated with growth factor–reduced Matrigel. Cells were then treated with vehicle or 0.1 μmol/L of the indicated HETE for 6 hours at 37°C and tube formation was observed under an inverted microscope. The images were captured with CCD color camera and the tube length was measured using the NIH Image J 1.31v program. *B,* quiescent HDMVEC were added to the upper chamber of the cell culture inserts that were placed in a 24-well plate. Vehicle or 0.1 μmol/L of the indicated HETE was added to the lower chamber. After 8 hours of incubation at 37°C, the inserts were lifted out from the 24-well plate and the nonmigrated cells were removed from the upper side of the membrane with cotton swab. The migrated cells on the lower side of the membrane were fixed in methanol, stained with Giemsa-Wright, and counted under light microscope. Representative of three independent experiments. *Columns,* means; *bars,* ±SD. *, *P* < 0.05 versus control.

eicosanoid on activation of Akt and S6K1 in HDMVEC. Quiescent HDMVEC were treated with vehicle or 0.1 μmol/L 15(S)-HETE for various times and cell extracts were prepared. An equal amount of protein from control and each treatment was analyzed for Akt and S6K1 phosphorylation using their phospho-specific antibodies. 15(S)-HETE stimulated the serine phosphorylation of Akt (Ser^{473}) and S6K1 (Thr^{421}/Ser^{424}) in a time-dependent manner in HDMVEC with a maximum effect at 30 minutes of treatment and these increases were sustained for at least 2 hours (Fig. 3A). To understand the upstream mediators of 15(S)-HETE-stimulated Akt and S6K1 phosphorylation, we now determined the effects of wortmannin and LY294002, two specific inhibitors of PI3K (41, 42). Quiescent HDMVEC were treated with vehicle or 0.1 μmol/L 15(S)-HETE in the presence and absence of wortmannin (1 μmol/L) or LY294002 (10 μmol/L) for 30 minutes and cell extracts were prepared and analyzed for Akt and S6K1 phosphorylation as described above. Wortmannin and LY294002
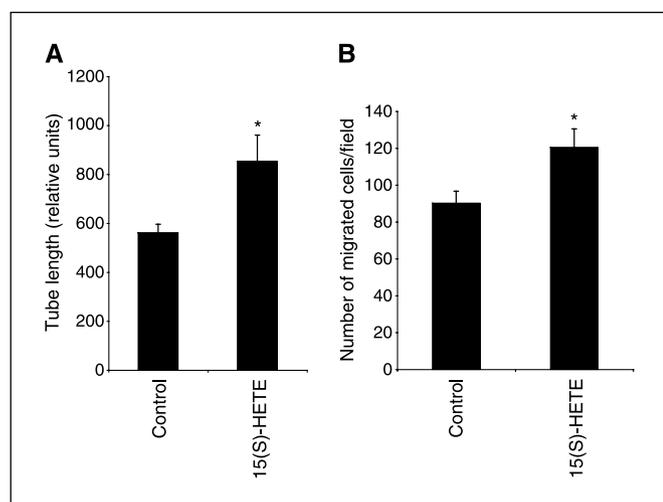
Figure 2. 15(S)-HETE induces tube formation and migration of proliferating HDMVEC. *A,* proliferating HDMVEC were seeded onto 96-well plate coated with growth factor–reduced Matrigel. Cells were then treated with vehicle or 0.1 μmol/L of 15(S)-HETE for 6 hours at 37°C and tube formation was observed under an inverted microscope. The images were captured with CCD color camera and the tube length was measured using the NIH Image J 1.31v program. *B,* proliferating HDMVEC were added to the upper chamber of the cell culture inserts that were placed in a 24-well plate. Vehicle or 0.1 μmol/L of 15(S)-HETE was added to the lower chamber. After 8 hours of incubation at 37°C, the inserts were lifted out from the 24-well plate and the nonmigrated cells were removed from the upper side of the membrane with cotton swab. The migrated cells on the lower side of the membrane were fixed in methanol, stained with Giemsa-Wright, and counted under light microscope. Representative of three independent experiments. *Columns,* means; *bars,* ±SD. *, $P < 0.05$ versus control.

completely inhibited 15(S)-HETE-stimulated Akt and S6K1 phosphorylation (Fig. 3B). These results suggest that Akt and S6K1 phosphorylation stimulated by 15(S)-HETE were dependent on activation of PI3K. To understand the sequential activation of the PI3K-Akt-mTOR-S6K1 signaling pathway by 15(S)-HETE, we also tested the effect of rapamycin, a specific inhibitor of mTOR, which is an immediate downstream effector of Akt and upstream regulator of S6K1 (43, 44). As shown in Fig. 3B, rapamycin (50 ng/mL) while having no effect on Akt phosphorylation inhibited S6K1 phosphorylation induced by 15(S)-HETE. Rapamycin alone to some extent stimulated Akt phosphorylation. These results indicate that 15(S)-HETE activates PI3K-Akt-mTOR-S6K1 signaling in this sequential manner in HDMVEC.

15(S)-hydroxyeicosatetraenoic acid–induced human dermal microvascular endothelial cell tube formation and migration require activation of PI3K-Akt-mTOR-S6K1 signaling. To find the role of PI3K-Akt-mTOR-S6K1 signaling in 15(S)-HETE-induced angiogenic events, we next studied the effect of wortmannin, LY294002, and rapamycin on HDMVEC tube formation. Quiescent HDMVEC were seeded onto 96-well plate coated with growth factor–reduced Matrigel and treated with vehicle or 0.1 μmol/L of 15(S)-HETE in the presence and absence of wortmannin (1 μmol/L), LY294002 (10 μmol/L), or rapamycin (50 ng/mL) for 6 hours and tube formation was measured. All three pharmacologic inhibitors significantly suppressed both basal and 15(S)-HETE-induced HDMVEC tube formation (Fig. 4A). To determine the involvement of PI3K-Akt-mTOR-S6K1 signaling in 15(S)-HETE-induced HDMVEC migration, quiescent cells were added to the upper chamber of the culture inserts that were placed in a 24-well plate and the vehicle or 0.1 μmol/L 15(S)-HETE in combination with and without wortmannin (1 μmol/L), LY294002

(10 μmol/L), or rapamycin (50 ng/mL) were added to the lower chamber. After incubation at 37°C for 8 hours, the migrated cells in the lower surface of the membrane were fixed, stained with Giemsa-Wright stain, and counted under a light microscope. Consistent with their effects on tube formation, wortmannin, LY294002, and rapamycin also significantly blocked both basal and 15(S)-HETE-induced HDMVEC migration (Fig. 4B). Because the PI3K-Akt-mTOR-S6K1 pathway plays a vital role in cell survival, it is quite possible that inhibition of this signaling cascade may trigger cell death and thereby account for the observed decreases in tube formation and migration of HDMVEC. To find whether this is the case, we first determined the effect of 15(S)-HETE on HDMVEC DNA synthesis. Quiescent cells were treated with and without 0.1 μmol/L of 15(S)-HETE for 24 hours and DNA synthesis was measured by labeling cells with 1 μCi/mL of [^{3}H]-thymidine for the last 20 hours of the 24-hour incubation period. As shown in Fig. 5A, 15(S)-HETE had no effect on HDMVEC DNA synthesis. We next studied the effect of inhibition of the PI3K-Akt-mTOR-S6K1 signaling on HDMVEC survival rate using trypan blue dye exclusion assay. About 80% of HDMVEC were found to be viable after 24 hours of maintenance in quiescent medium (EBM-2) and pharmacologic inhibition of PI3K or mTOR for 8 hours did not cause any additional cell death suggesting that the observed decreases in tube formation and migration of these cells by wortmannin, LY294002, and rapamycin were not due to increased apoptosis (Fig. 5B). Instead, these results suggest that PI3K-Akt-mTOR-S6K1 signaling plays a vital role both in basal and 15(S)-HETE-induced HDMVEC tube formation and migration. To strengthen this view, we also
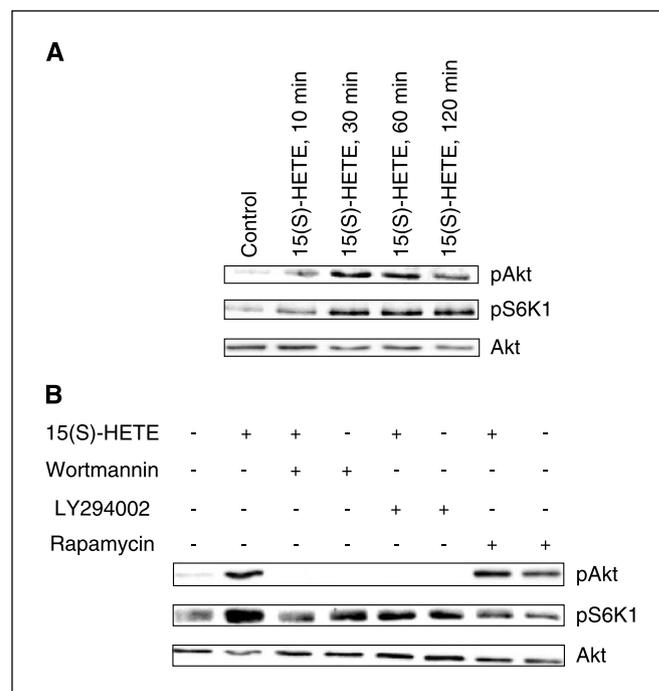
Figure 3. 15(S)-HETE stimulates phosphorylation of Akt and S6K1 in HDMVEC in a time- and PI3K-dependent manner. Quiescent HDMVEC were treated with vehicle or 0.1 μmol/L 15(S)-HETE for the indicated times (*A*) or in the presence and absence of wortmannin (1 μmol/L), LY294002 (10 μmol/L), or rapamycin (50 ng/mL) for 30 minutes (*B*), and cell extracts were prepared. Equal amounts of protein (30 μg) from control and each treatment were analyzed by Western blotting for pAkt and pS6K1 using their phospho-specific antibodies. As a loading control, the same blot was reprobed with anti-Akt antibodies.
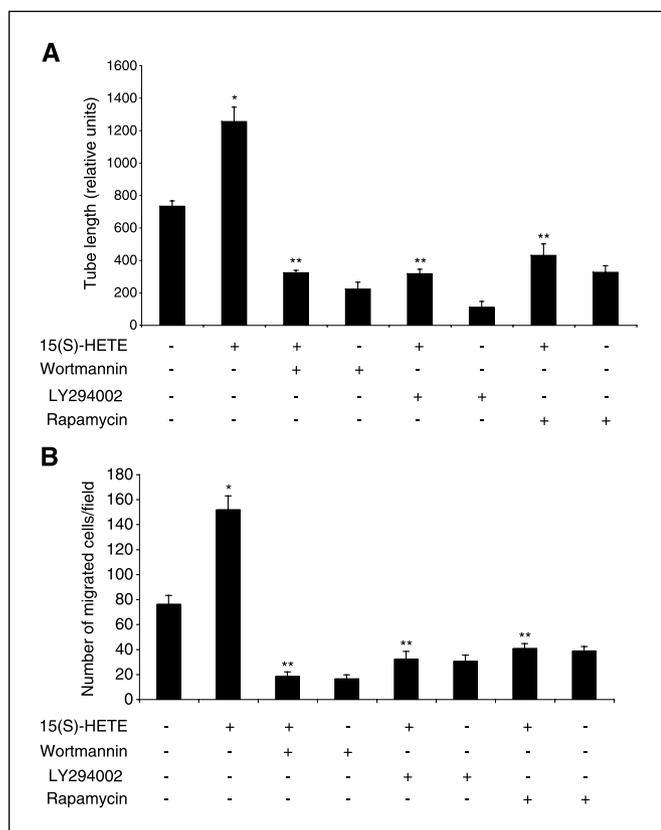
Figure 4. Pharmacologic blockade of PI3K and mTOR suppresses 15(S)-HETE-induced HDMVEC tube formation and migration. *A,* quiescent HDMVEC were seeded onto 96-well plate coated with growth factor–reduced Matrigel. Cells were then treated with vehicle or 0.1 μmol/L 15(S)-HETE in the presence and absence of wortmannin (1 μmol/L), LY294002 (10 μmol/L), or rapamycin (50 ng/mL) for 6 hours at 37°C and tube formation was observed under an inverted microscope. The images were captured with CCD color camera and the tube length was measured using the NIH Image J 1.31v program. *B,* quiescent HDMVEC were added to the upper chamber of the cell culture inserts that were placed in a 24-well plate. Vehicle or 0.1 μmol/L 15(S)-HETE was added to the lower chamber in the presence and absence of wortmannin (1 μmol/L), LY294002 (10 μmol/L), or rapamycin (50 ng/mL). After 8 hours of incubation at 37°C, the inserts were lifted out from the 24-well plate and the nonmigrated cells were removed from the upper side of the membrane with cotton swab. The migrated cells on the lower side of the membrane were fixed in methanol, stained with Giemsa-Wright, and counted under light microscope. Representative of three independent experiments. *Columns,* means; *bars,* ±SD. *, $P < 0.01$ versus control; **, $P < 0.01$ versus 15(S)-HETE treatment alone.

studied the effect of overexpression of constitutively active Akt (myrAkt) on S6K1 phosphorylation, tube formation and migration. Similar to the effects of 15(S)-HETE, adenoviral-mediated expression of constitutively active Akt alone was sufficient to cause a robust increase in S6K1 phosphorylation in HDMVEC and their capacity of tube formation and migration (Fig. 5C-F).

15(S)-hydroxyeicosatetraenoic acid–induced Matrigel plug angiogenesis is dependent on activation of PI3K-Akt-mTOR-S6K1 signaling. To relate the effects of 15(S)-HETE on tube formation and migration to angiogenesis *in vivo*, we next determined the effects of all three HETEs on angiogenesis using a Matrigel plug assay. Mice were injected with growth factor-reduced Matrigel containing either vehicle or 50 μmol/L of the indicated HETE underneath the skin, and 7 days later, the plugs were retrieved from the sacrificed animals and analyzed for hemoglobin content using Drabkin's reagent. As shown in Fig. 6A, all three HETEs induced angiogenesis by about 2-fold.

Consistent with its effect on HDMVEC tube formation and migration, 15(S)-HETE was found to be slightly more potent than 5(S)-HETE and 12(S)-HETE in inducing Matrigel plug angiogenesis. To understand the mechanisms, particularly the role of PI3K-Akt-mTOR-S6K1 signaling in 15(S)-HETE-induced angiogenesis *in vivo*, we tested the effects of LY294002 and rapamycin. LY294002 (250 μmol/L) or rapamycin (1 μg/mL) in combination with and without 50 μmol/L 15(S)-HETE were added to the Matrigel and injected into mice underneath the skin. Seven days later, the Matrigel plugs were retrieved and analyzed for hemoglobin. Both LY294002 and rapamycin significantly inhibited 15(S)-HETE-induced Matrigel plug angiogenesis (Fig. 6B).

Adenoviral-mediated overexpression of dominant-negative Akt suppresses 15(S)-hydroxyeicosatetraenoic acid–induced human dermal microvascular endothelial cell tube formation and migration *in vitro* and Matrigel plug angiogenesis *in vivo*. To confirm the role of PI3K-Akt-mTOR-S6K1 signaling in 15(S)-HETE-induced angiogenesis, we have also used a dominant-negative mutant approach. HDMVEC were infected with adenovirus expressing either GFP or dominant-negative Akt at a multiplication of infection of 80, quiesced for 24 hours, treated with vehicle or 0.1 μmol/L 15(S)-HETE for the desired length of time and tested for S6K1 phosphorylation levels, tube formation, and migration as described above. Adenoviral-mediated expression of dominant-negative Akt but not GFP completely blocked 15(S)-HETE-induced S6K1 phosphorylation (Fig. 7A). Similarly, adenoviral-mediated expression of dominant-negative Akt but not GFP also blocked 15(S)-HETE-induced HDMVEC tube formation and migration (Fig. 7B and C). To test the effect of dominant-negative Akt on 15(S)-HETE-induced angiogenesis *in vivo*, adenovirus expressing GFP or dominant-negative Akt (1 × 10^8 plaque forming units) in combination with and without 50 μmol/L 15(S)-HETE were mixed with growth factor–reduced Matrigel and injected into mice underneath the skin. Two weeks later, the Matrigel plugs were retrieved and assayed for hemoglobin content as described above. Adenoviral-mediated expression of dominant-negative Akt but not GFP completely blocked the angiogenesis induced by 15(S)-HETE (Fig. 8).

Discussion

The major findings of the present study are as follows. (a) Among the three LOX products of arachidonic acid tested, 15(S)-HETE was found to be more potent in inducing angiogenesis as measured by HDMVEC tube formation and *in vivo* Matrigel plug assay. (b) 15(S)-HETE stimulated phosphorylation of Akt and S6K1 in HDMVEC in a time-dependent manner. (c) Wortmannin and LY294002, two specific inhibitors of PI3K, suppressed both Akt and S6K1 phosphorylation whereas rapamycin, a specific inhibitor of mTOR, blocked only S6K1 phosphorylation induced by 15(S)-HETE suggesting that this eicosanoid possess the ability to activate the PI3K-Akt-mTOR-S6K1 signaling in HDMVEC. (d) Pharmacologic blockade of activation of PI3K-Akt-mTOR-S6K1 signaling inhibited 15(S)-HETE-induced tube formation and migration of HDMVEC *in vitro* and Matrigel plug angiogenesis *in vivo*. (e) Adenoviral-mediated expression of wild-type Akt enhanced tube formation and migration of HDMVEC. (f) Adenoviral-mediated expression of dominant-negative Akt suppressed 15(S)-HETE-induced tube formation and migration of HDMVEC *in vitro* and Matrigel plug angiogenesis *in vivo*.
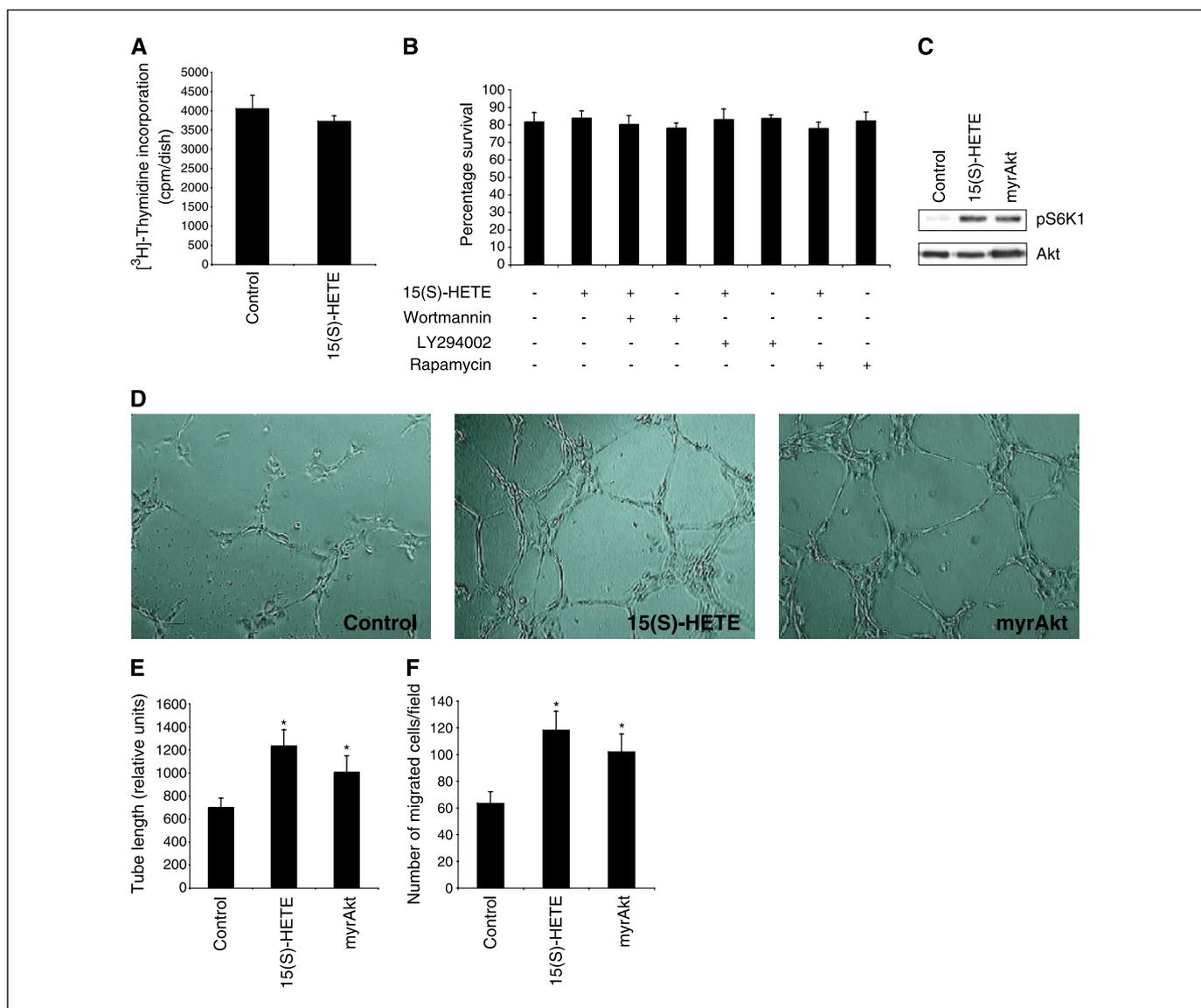
Figure 5. Short-term inhibition of PI3K-Akt-mTOR-S6K1 signaling does not affect HDMVEC survival rate and adenoviral-mediated overexpression of constitutively active Akt enhances S6K1 phosphorylation in these cells and their ability of tube formation and migration. *A,* quiescent HDMVEC were treated with vehicle or 0.1 μmol/L 15(*S*)-HETE for 24 hours and DNA synthesis was measured by labeling cells with 1 μCi/mL of [^{3}H]-thymidine for the last 20 hours of the 24-hour incubation period. *B,* quiescent HDMVEC were treated with vehicle or 0.1 μmol/L 15(*S*)-HETE in the presence and absence of wortmannin (1 μmol/L), LY294002 (10 μmol/L), or rapamycin (50 ng/mL) for 8 hours at 37°C and their survival rate was measured by trypan blue dye exclusion assay. *C,* HDMVEC that were infected with Ad-GFP or Ad-myrAkt at a multiplication of infection of 80, were quiesced, treated with vehicle or 0.1 μmol/L of 15(*S*)-HETE for 30 minutes, cell extracts prepared and analyzed for S6K1 phosphorylation using its phospho-specific antibodies. The same blot was reprobed with normal anti-Akt antibodies. *D* and *E,* HDMVEC that were infected with Ad-GFP or Ad-myrAkt at a multiplication of infection of 80 were quiesced and seeded onto 96-well plate coated with growth factor–reduced Matrigel. Cells were then treated with vehicle or 0.1 μmol/L of 15(*S*)-HETE for 6 hours at 37°C and tube formation was observed under an inverted microscope. The images were captured with CCD color camera and the tube length was measured using the NIH Image J 1.31v program. *F,* HDMVEC that were infected with Ad-GFP or Ad-myrAkt at a multiplication of infection of 80 were quiesced and added to the upper chamber of the cell culture inserts that were placed in a 24-well plate. Vehicle or 0.1 μmol/L of 15(*S*)-HETE was added to the lower chamber. After 8 hours of incubation at 37°C, the inserts were lifted out from the 24-well plate and the nonmigrated cells were removed from the upper side of the membrane with cotton swab. The migrated cells on the lower side of the membrane were fixed in methanol, stained with Giemsa-Wright, and counted under light microscope. Representative of three independent experiments. *Columns,* means; *bars,* $\pm$SD. *, $P < 0.05$ versus control.

Together, these observations show that 15(*S*)-HETE induces angiogenesis via activation of PI3K-Akt-mTOR-S6K1 signaling. Many studies over the past several years have indicated that the COX, LOX, and CYP metabolites of arachidonic acid stimulate growth in several cell types and play a role in tumor progression and vessel wall diseases (2, 4, 5, 12–20). Despite the indications that these lipid molecules are involved in the development of various cancers and vessel wall diseases, very little is known with

regard to possible mechanisms of their actions. Both the Janus-activated kinase (Jak) and Src family of protein tyrosine kinases play a role in receptor tyrosine kinase (RTK) and GPCR agonist-induced cell proliferation and tumor progression (45, 46). The work from our laboratory as well as others showed that like RTK and GPCR agonists, 5(*S*)-HETE and 14,15-EET, the 5-LOX, and CYP2C9 metabolites of arachidonic acid, activate Jak-2 and Src protein tyrosine kinases in mediating their mitogenic effects in
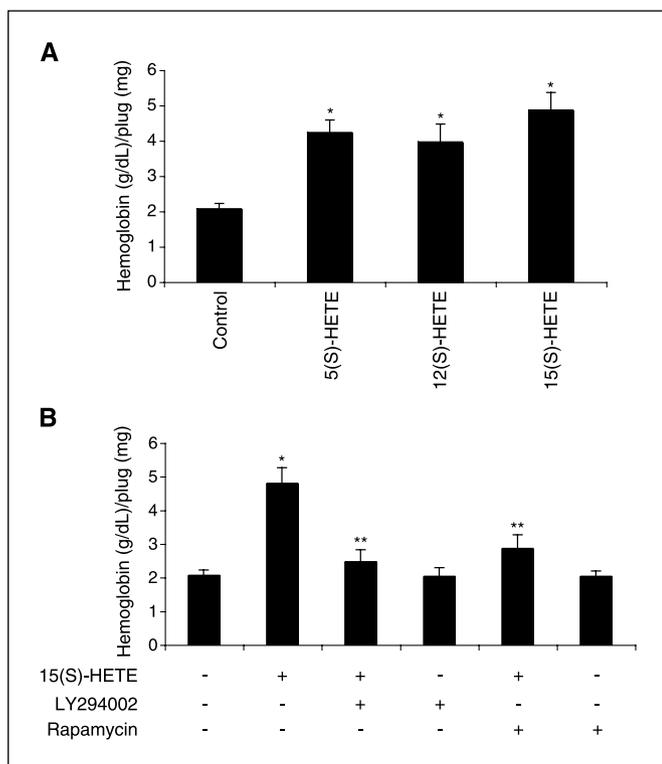
Figure 6. *A,* HETEs induce angiogenesis. C57BL/6 mice were injected s.c. with 0.5 mL of growth factor–reduced Matrigel premixed with vehicle or 50 μmol/L of the indicated eicosanoid. Seven days later, the animals were sacrificed and the Matrigel plugs were harvested from underneath the skin and analyzed for hemoglobin with Drabkin's reagent. *B,* blockade of PI3K and mTOR suppresses 15(*S*)-HETE–induced angiogenesis. C57BL/6 mice were injected s.c. with 0.5 mL of growth factor–reduced Matrigel premixed with vehicle or 50 μmol/L 15(*S*)-HETE along with and without LY294002 (250 μmol/L) or rapamycin (1 μg/mL). Seven days later, the animals were sacrificed and the Matrigel plugs were harvested from underneath the skin and analyzed for hemoglobin with Drabkin's reagent. Representative of three independent experiments. *Columns,* mean; *bars,* ±SD. *, $P < 0.01$ versus control; **, $P < 0.01$ versus 15(*S*)-HETE treatment alone.

HDMVEC and renal epithelial cells, respectively (16, 37). Similarly, arachidonic acid and its 5-LOX metabolites have been reported to stimulate small GTPase proteins and their downstream effectors, mitogen-activated protein kinases, in various cell types including fibroblasts mediating proliferation and/or cytoskeletal remodeling (8–11). It was also shown that 12(*S*)-HETE and 11, 12-EET, the 12-LOX, and CYP2C9 products of arachidonic acid, activate Akt towards stimulating growth in prostate cancer cells and endothelial cells, respectively (15, 47). Akt activation was also shown to be involved in 14,15-EET-mediated renal epithelial cell survival (48). Whereas these observations clearly provide evidence for the efficacy of some of the eicosanoids in stimulating cell growth and activating the potential underlying signaling events, the mechanisms by which these lipid molecules influence tumor and/or atherosclerotic plaque progression are less clear.

Endothelial cell migration and proliferation are critical events for angiogenesis (25). In addition, angiogenesis is a major player in survival and progression of both tumors and atherosclerotic plaques (23–27). Towards understanding the role of eicosanoids in tumor growth, some recent studies from other laboratories have reported that 12(*S*)-HETE, 12(*R*)-HETE, 11,12, EET and 14,15-EET, the 12-LOX, and CYP4B1 and CYP2C9 metabolites of arachidonic

acid, respectively, induce angiogenesis (19, 28–30). In this aspect, the present study identifies 15(*S*)-HETE, the 15-LOX metabolite of arachidonic acid, as another potent inducer of angiogenesis. In fact, 15(*S*)-HETE was found to be more potent than 5(*S*)-HETE or 12(*S*)-HETE in stimulating angiogenesis as measured by HDMVEC tube formation and Matrigel plug assay. In addition, increased levels of 12/15-LOX and 15-LOX-2, the enzymes that convert arachidonic acid to 15(*S*)-HETE, were reported in various lung and prostate carcinomas (18, 49). Furthermore, increased expression of 15-LOX was reported in pulmonary arteries in response to hypoxia, a condition that triggers angiogenesis (50). Increased levels of 15-HETE were also reported in atherosclerotic plaques compared with normal arteries (51, 52). In view of these observations and the present findings, it is clear that 15(*S*)-HETE could be a potent arachidonic acid–derived lipid mediator involved in the development of various carcinomas as well as in the progression of atherosclerotic plaques. In contrast to 5(*S*)-HETE, 15(*S*)-HETE had no noticeable effect on HDMVEC DNA synthesis suggesting that this eicosanoid influences angiogenesis
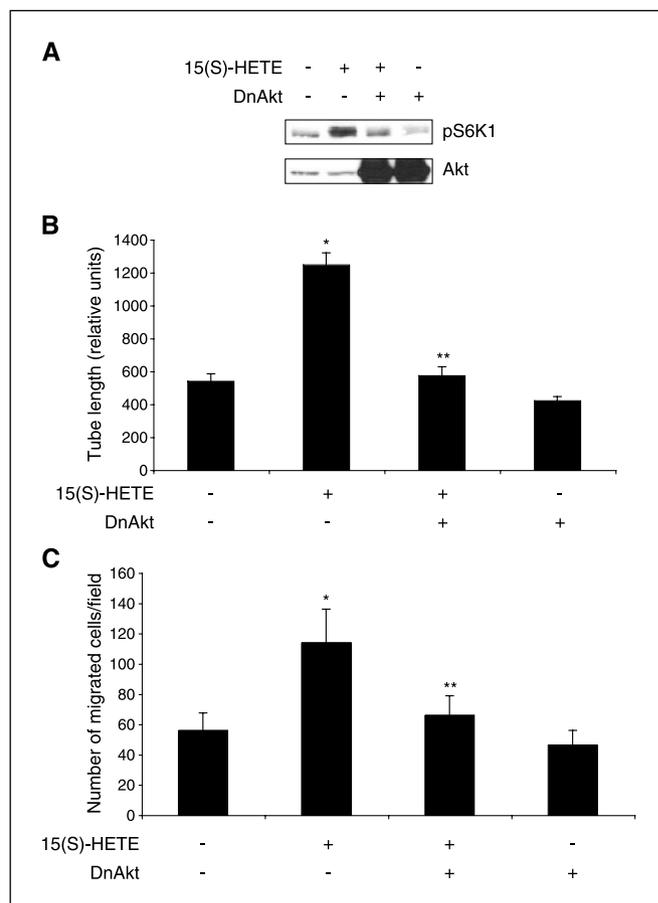
Figure 7. Adenoviral-mediated expression of dominant-negative Akt but not GFP suppresses 15(*S*)-HETE–induced S6K1 phosphorylation, tube formation, and migration. HDMVEC were infected with adenovirus expressing either GFP or dominant-negative Akt at a multiplication of infection of 80, growth-arrested, treated with and without 1 μmol/L 15(*S*)-HETE for 30 minutes, 6, and 8 hours at 37°C to measure S6K1 phosphorylation, tube formation, and migration, respectively. *A,* phosphorylation levels of S6K1 were measured by Western blotting using its phospho-specific antibodies as described in Fig. 3 legend. *B* and *C,* tube formation and migration of HDMVEC were determined as described in Fig. 1 legend. Representative of three independent experiments. *Columns,* means; *bars,* ±SD. *, $P < 0.01$ versus control; **, $P < 0.01$ versus 15(*S*)-HETE treatment alone.
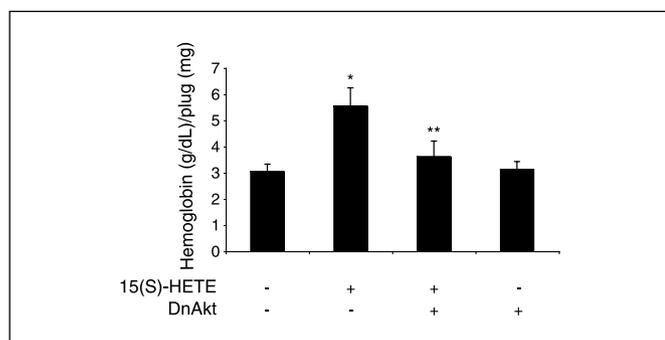
Figure 8. Adenoviral-mediated expression of dominant-negative Akt but not GFP suppresses 15(*S*)-HETE-induced angiogenesis. C57BL/6 mice were injected s.c. with 0.5 mL of growth factor–reduced Matrigel premixed with vehicle or 50 μmol/L 15(*S*)-HETE along with and without adenovirus expressing either GFP or dominant-negative Akt (1 × 10^8 plaque forming units). Two weeks later, the animals were sacrificed and the Matrigel plugs were harvested from underneath the skin and analyzed for hemoglobin with Drabkin's reagent. Representative of three independent experiments. *Columns,* mean; *bars,* ±SD. *, $P < 0.01$ versus control; **, $P < 0.01$ versus 15(*S*)-HETE treatment alone.

via enhancing the ability of these cells to migrate and form tubular structures. In this context, it is important to point out that molecules such as 15(*S*)-HETE that possess the capacity to enhance the ability of microvascular endothelial cells to migrate and form tubular structures in concert with mitogens may play a significant role in the progression of tumors and/or atherosclerotic plaques. A large number of studies have shown a role for Akt in mediating angiogenesis in response to several stimulants including hypoxia and nitric oxide (34, 35, 38). In the present study, we show that activation of PI3K-Akt-mTOR-S6K1 signaling is essential for 15(*S*)-HETE-induced angiogenesis. The finding that adenoviral-mediated expression of constitutively active Akt alone is sufficient to enhance HDMVEC migration and tube formation further lends support for the role of this signaling in angiogenesis. Additional work is needed to test whether activation of this signaling is a unifying mechanism of angiogenesis induced by various eicosanoids.

In summary, the present observations show that 15(*S*)-HETE induces angiogenesis and this phenomenon requires activation of PI3K-Akt-mTOR-S6K1 signaling.

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15(*S*)-Hydroxyeicosatetraenoic Acid Induces Angiogenesis via Activation of PI3K-Akt-mTOR-S6K1 Signaling

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