Eicosanoid Regulation of Vascular Endothelial Growth Factor Expression and Angiogenesis in Microvessel Endothelial Cells*

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12(R)-Hydroxy-5,8,14-eicosatrienoic acid (HETrE) is a potent inflammatory and angiogenic eicosanoid in ocular and dermal tissues. Previous studies suggested that 12(R)-HETrE activates microvessel endothelial cells via a high affinity binding site; however, the cellular mechanisms underlying 12(R)-HETrE angiogenic activity are unexplored. Because the synthesis of 12(R)-HETrE is induced in response to hypoxic injury, we examined its interactions with vascular endothelial growth factor (VEGF) in rabbit limbal microvessel endothelial cells. Addition of 12(R)-HETrE (0.1 nM) to the cells increased VEGF mRNA levels with maximum 5-fold increase at 45 min. The increase in VEGF mRNA was followed by an increase in immunoreactive VEGF protein. 12(R)-HETrE (0.1 nM) rapidly activated the extracellular signal-regulated kinases (ERKs) ERK1 and ERK2. Moreover, preincubation of cells with PD98059, a selective inhibitor of MEK-1, inhibited 12(R)-HETrE-induced VEGF mRNA. Addition of VEGF antibody to cells grown in Matrigel-coated culture plates inhibited 12(R)-HETrE-induced capillary tube-like formation, suggesting that VEGF mediates, at least in part, the angiogenic response to 12(R)-HETrE. The results indicate that in microvessel endothelial cells, 12(R)-HETrE induces VEGF expression via activation of ERK1/2 and that VEGF mediates, at least in part, the angiogenic activity of 12(R)-HETrE. Given the fact that both VEGF and 12(R)-HETrE are produced in the cornea after hypoxic injury, their interaction may be an important determinant in the development of neovascularized tissues.

A wide variety of disorders of the cornea evoke a vasculogenic response; a considerable amount of information has accumulated about the circumstances under which newly formed blood vessels sprout and extend centripetally into the cornea. Thus, the cornea has become a standard model for the study of angiogenesis. Vascularized corneas are clinically significant because they diminish visual acuity.

Diverse mediators have been implicated in the process of corneal angiogenesis including prostaglandins, vasoactive amines, epithelial angiogenic factors, and components of leukocytic extracts (1). We have identified a corneal epithelial-derived eicosanoid, 12(R)-hydroxy-5,8,14-eicosatrienoic acid (HETrE)[1] and characterized it as a novel inflammatory and angiogenic mediator; it is produced by the corneal epithelium in response to injury, displays potent inflammatory properties, is a mitogen for microvascular endothelial cells, and is angiogenic *in vitro* and *in vivo*. Moreover, after hypoxic and chemical injury to the corneal epithelium, 12(R)-HETrE is produced at a rate of 60–100 pmol/h/mg, which is sufficient to elicit its effect on the adjacent capillary endothelial cells of the limbal vessels. The increased synthesis of this eicosanoid after injury is observed within 1 day of the injury, sustained for several days (up to 7–9 days), and correlates well ($r = 0.963$; $p < 0.04$) with the degree of inflammation (2–4). *In vitro*, 12(R)-HETrE synthesis is markedly increased in response to hypoxia (5). Moreover, 12(R)-HETrE is readily released into the incubation medium of cultured injured rabbit corneas, and its levels were found to be dramatically increased in tears from inflamed human eyes (6, 7) suggesting a paracrine role for 12(R)-HETrE. We therefore postulate that 12(R)-HETrE is a tissue-derived angiogenic factor whose synthesis is induced in response to injury, acting in a paracrine manner on microvessels to activate endothelial cells via a specific receptor/binding site resulting in an angiogenic phenotype.

It should be noted that the synthesis and activity of 12(R)-HETrE extend beyond the ocular surface; its synthesis has been documented in rat epidermal microsomes (8, 9) and porcine neutrophils (10). 12(R)-HETrE is a potent chemoattractant for human neutrophils (11), and in guinea pig skin, it enhances delayed-type hypersensitivity inflammatory reactions at doses as low as 1 fmol (12).

The cellular mechanisms underlying the angiogenic activity of 12(R)-HETrE are still unclear. We have identified several events involved in the signal transduction of endothelial cells stimulated by 12(R)-HETrE including the demonstration of a putative receptor in limbal microvessel endothelial cells (13), a protein kinase C-dependent nuclear factor κB activation (14), and increased c-*fos*, c-*jun* and c-*myc* oncogene expression (15). The mechanism by which 12(R)-HETrE promotes the angiogenic response of the endothelial cells may involve the induction of peptide angiogenic factors, primarily VEGF. The current study examines whether VEGF is a component of the angiogenic activity of 12(R)-HETrE and characterizes mechanisms underlying the effect of 12(R)-HETrE on VEGF expression.

[1] The abbreviations used are: HETrE, hydroxy-5,8,14-eicosatrienoic acid; VEGF, vascular endothelial growth factor; RLME, rabbit limbal microvessel endothelial; MAP, mitogen-activated protein; MAPK, MAP kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; HPLC, high pressure liquid chromatography.

EXPERIMENTAL PROCEDURES

Materials—12(*R*)-HETrE and 12(*S*)-HETrE were synthesized and purified as described previously (16). The identity and purity of each compound were confirmed by chiral HPLC and gas chromatography/mass spectrometry. Stock solutions were prepared in ethanol and stored at −80 °C. The ethanol stocks were dried under nitrogen, and dilutions for each experiment were prepared in 0.01% Me$_2$SO. Actinomycin D, cycloheximide, and PD98059 were purchased from Sigma. Anti-ERK1/2 and anti-phospho-specific ERK antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Cell Culture—Primary cultures of rabbit limbal microvascular endothelial (RLME) cells were obtained via an *in vitro* angiogenesis assay as described previously (17). Cells were grown, and their endothelial identity was examined at random by measuring factor VIII antigen immunofluorescence. Cells were cultured in Dulbecco's modified Eagle's medium (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (Cellgro), 1% antibiotic-antimycotic (Cellgro), and endothelial cell growth supplements (ECGS; Sigma). Before each experiment, cells were washed twice in phosphate-buffered saline, pH 7.6, and quiesced in antibiotic- and growth factor-free Dulbecco's modified Eagle's medium containing 0.5% fetal bovine serum for 24–36 h.

Measurements of VEGF mRNA Expression—Monolayer-grown cells were promptly homogenized in Trireagent (Sigma). Cell homogenates were quick-frozen and stored at −80 °C until use. Total RNA was extracted (18) and denatured in the presence of a 1× RNA sample loading buffer (Sigma). For Northern analysis, denatured RNA was electrophoresed on 1.2% agarose formaldehyde gels and transferred to Hybond-N+ membranes (Amersham Biosciences). For slot blot hybridization, denatured RNA was blotted to Hybond-N+ membranes using a slot blot filtration manifold (Schleicher & Schuell). Membranes were cross-linked (1200 mkJ/cm^2), dried at 80 °C for 2 h, and incubated overnight in hybridization buffer containing the ^{32}P-end-labeled mouse VEGF cDNA probe at 65 °C. Hybridization signals were visualized by radiography and quantitated using Scion Image software (National Institutes of Health, Bethesda, MD) and a Kodak gray color scale as a standard. Each membrane was stripped and rehybridized with a ^{32}P-end-labeled β-tubulin cDNA probe. VEGF mRNA levels were normalized to β-tubulin to correct loading variation.

Measurements of VEGF Protein Levels—VEGF protein levels in the culture medium were measured by enzyme-linked immunosorbent assay using a Mouse VEGF Quantikine M Immunoassay Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Quantitation of samples was determined from the least squares regression analysis of a linear standard curve obtained with recombinant mouse VEGF as control. Protein concentration was determined by the Bradford method (Sigma).

Western Blot—Cells were harvested, resuspended in lysis buffer (Cell Signaling Technology), and sonicated (4 °C, 5 × 1 min). Cell-free homogenates were prepared by centrifugation at 6000 × *g* for 5 min. Aliquots of cell-free homogenates (150 μg) were denatured in Laemmli loading buffer (2 min at 95 °C), resolved on 11% or 14% SDS-polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were hybridized with the antibodies indicated in the text, and immunoreactive bands were detected using chemiluminescence substrates according to the manufacturer's instructions and visualized after exposure to Hyperfilm™ ECL (Amersham Biosciences). The autoradiographed films were scanned, and densitometry analysis was performed with Scion Image software using a Kodak gray color scale as a standard.

ERK Kinase Assay—Kinase assays were performed using a p44/p42 (ERK1/2) MAPK kinase assay kit (Cell Signaling Technology) according to the manufacturer's protocol. Briefly, activated ERK was precipitated from cell lysates using immobilized phospho-ERK1/2 MAP kinase (Tyr^{202}/Tyr^{204}) antibodies. The precipitates were incubated with recombinant Elk-1, a specific ERK substrate, and ATP. The reaction was terminated by adding boiling gel-loading buffer. ERK activity was detected by immunoblotting the products of the kinase reaction with anti-phospho-Elk-specific antibody.

In Vitro Capillary Tube-like Formation—Assessment of *in vitro* capillary tube-like formation utilized growth factor reduced basement membrane Matrigel matrix (BD Biosciences-Discovery Labware). The Matrigel was thawed overnight at 4 °C and mixed to homogeneity using cooled pipette tips. Aliquots of Matrigel (250 μl) were distributed as a thin layer on the bottom of 12-well cell culture plates and left for polymerization at 37 °C for 30 min. Quiesced RLME cells were resuspended in Dulbecco's modified Eagle's medium containing 0.5% fetal bovine serum to give a final cell concentration of $1 × 10^5$ cells/ml, plated

onto the Matrigel-coated surface, and incubated for 1 h in a 37 °C humidified incubator. The medium was aspirated to remove nonattached cells and substituted by fresh medium containing vehicle or the test compounds. Tube-like structure formation was examined at 8, 16, and 24 h after treatment. Cultures were photographed, and the length of the tube-like was structures was quantified using Image Pro-Express Software (Cyber Media).

RESULTS

12(R)-HETrE Induces VEGF Expression—Incubation of RLME cells with 12(*R*)-HETrE at concentration of 0.1 nM resulted in a rapid induction of VEGF expression. As seen in Fig. 1*A,* 12(*R*)-HETrE increased VEGF mRNA levels in a time-dependent manner. A 5-fold increase over the control levels was observed 45 min after the addition of 12(*R*)-HETrE, and these levels gradually declined to the control levels by 48 h. Incubation of cells with cycloheximide did not affect 12(*R*)-HETrE-induced VEGF mRNA, suggesting that the induction does not require *de novo* protein synthesis. On the other hand, addition of actinomycin D abolished 12(*R*)-HETrE-induced VEGF expression, indicating that this effect requires *de novo* RNA synthesis (Fig. 1*B*). To determine whether the effect of 12(*R*)-HETrE is due in part to an increase in the stability of VEGF mRNA, we performed a standard mRNA decay assay using actinomycin D. As seen in Fig. 1*C,* the half-life of VEGF mRNA was 9.15 ± 1.26 h in the absence of 12(*R*)-HETrE and 13.46 ± 1.46 h in the presence of 12(*R*)-HETrE (*n* = 3; *p* = 0.018), suggesting that both transcriptional activation and mRNA stabilization accounted for the increase in VEGF mRNA induced by 12(*R*)-HETrE. 12(*R*)-HETrE increased VEGF mRNA in a concentration-dependent manner, with 0.1 nM 12(*R*)-HETrE having the maximal effect (Fig. 2). Moreover, this effect was stereospecific because the S enantiomer did not significantly affect VEGF expression at concentrations of up to 10 nM (Fig. 2); VEGF mRNA levels were 115 ± 26%, 65 ± 31%, and 99 ± 22% of control levels at 0.1, 1, and 10 nM 12(*S*)-HETrE (mean + S.E.; *n* = 3). Northern blot analysis indicated the presence of two VEGF transcripts with estimated sizes of 2.4 and 4.2 kb. 12(*R*)-HETrE induced the level of expression of both transcripts in a concentration-dependent manner (Fig. 3).

The increase in VEGF mRNA in response to 12(*R*)-HETrE was followed by an increase in its protein levels. As seen in Fig. 4*A,* VEGF protein levels in the culture media of cells incubated with 0.1 nM 12(*R*)-HETrE increased in a time-dependent manner with a significant increase at 3 h and a 4-fold increase at 24 h after the addition of 12(*R*)-HETrE. The 12(*R*)-HETrE-stimulated increase in VEGF protein was also concentration-dependent (Fig. 4*B*).

12(R)-HETrE Induces VEGF Expression via a MAPK-dependent Pathway—The mechanism by which 12(*R*)-HETrE induces VEGF expression is unknown. The p44/p42 (ERK1/2) MAP kinase signaling pathway has been implicated in a wide range of cellular functions including the control of VEGF expression in endothelial cells (19). We examined whether the 12(*R*)-HETrE-induced increase in VEGF expression in RLME cells involves the activation of the ERK1/2 MAP kinases. After the addition of 0.1 nM 12(*R*)-HETrE to the cells, both ERK1 and ERK2 were transiently activated as determined by Western blot analyses with antibody against the phosphorylated forms of ERK1/2 (Fig. 5). Kinase activation peaked at 5 min and gradually decreased to control levels by 60 min. Additional *in vitro* kinase assays demonstrated an increase in phosphorylation of Elk, the MAPK-specific substrate, which paralleled the increase in ERK1/2 activity (Fig. 6, *A* and *B*). The increase in kinase activity was inhibited by the MEK inhibitor PD98059 (Fig. 6*C*), suggesting that MEK, the immediate upstream dual specificity kinase to ERK, is involved in 12(*R*)-HETrE-stimulated ERK1/2 activity. The observation that activation of
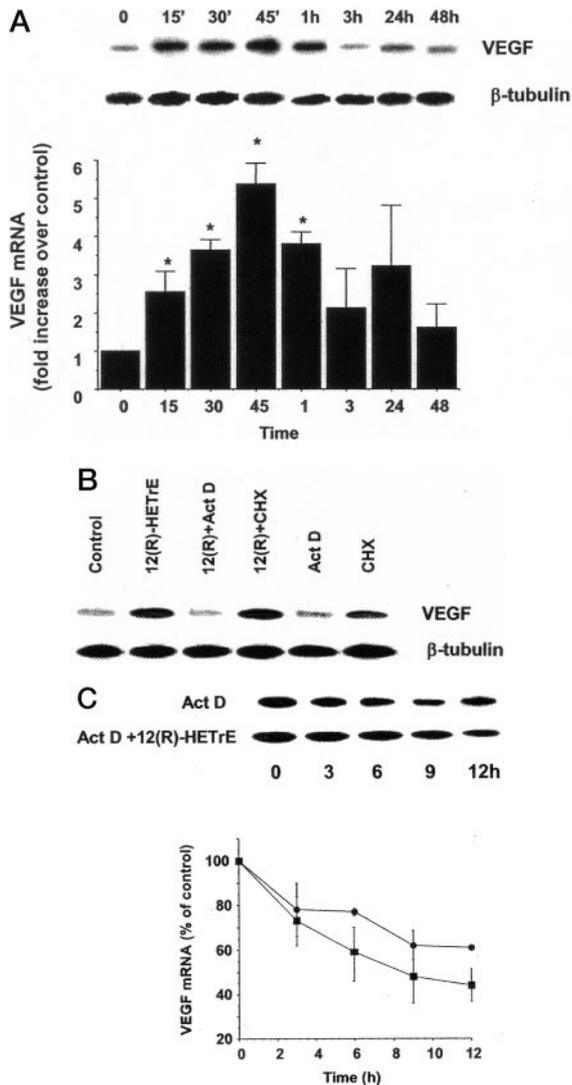
FIG. 1. **12(*R*)-HETrE induction of VEGF mRNA in RLME cells.** *A*, time-dependent induction of VEGF mRNA. Subconfluent RLME cells (50–70%) were quiesced for 36 h in serum-deprived medium and further exposed to 0.1 nM 12(*R*)-HETrE. At the indicated time points, cells were lysed, and total RNA was isolated and analyzed by slot blot hybridization with a mouse cDNA probe as described under "Experimental Procedures." Membranes were stripped and reprobed with β-tubulin as a housekeeping gene. VEGF mRNA levels were normalized to β-tubulin to correct loading variations. Results of densitometry analysis represent fold increases from control of the ratio of VEGF mRNA to β-tubulin mRNA in nontreated cells (t = 0 min). The results are the mean ± S.E.; n = 3; *, $p < 0.05$ from the control. *B*, effect of actinomycin D (*Act D*) and cycloheximide (*CHX*) on 12(*R*)-HETrE-induced VEGF mRNA. Quiesced subconfluent RLME cells were treated with 12(*R*)-HETrE (0.1 nM) with and without actinomycin D (10 μM) or cycloheximide (10 μM) for 45 min. Concentrations of both actinomycin D and cycloheximide were 10 μM. VEGF mRNA was measured as described above. The blot is representative of three separate experiments. *C*, effect of 12(*R*)-HETrE on VEGF mRNA half-life. Quiesced subconfluent RLME cells were incubated with either 12(R)-HETrE (0.1 nM) (●) or vehicle (■) for 45 min before the addition of actinomycin D (10 μM). Total RNA was extracted from cells at the indicated times after the addition of actinomycin D. VEGF mRNA level was analyzed by slot blot hybridization as described above. VEGF mRNA levels were normalized to β-tubulin, and the decay rates were plotted as a percentage of the 0 h value against time. Half-life of VEGF mRNA was 13.46 ± 1.46 h in the absence of 12(*R*)-HETrE and 9.15 ± 1.26 h in the presence of 12(*R*)-HETrE (mean ± S.E.; n = 3; p = 0.018 between groups).

ERK1/2 is involved in 12(*R*)-HETrE-induced VEGF expression is further documented in Fig. 7. Preincubation of cells with PD98059 (5 μM) before the addition of 12(*R*)-HETrE (0.1 nM) inhibited the increase in VEGF mRNA by 50%.
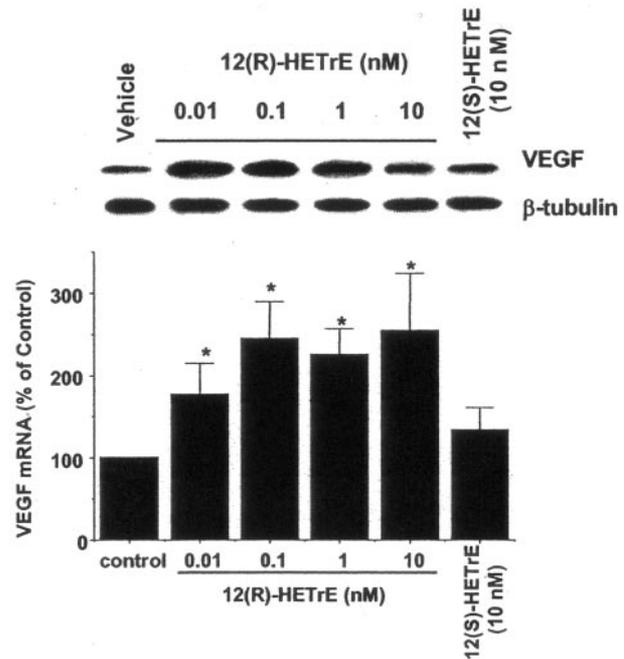

FIG. 2. **Concentration-dependent induction of VEGF mRNA synthesis by 12(*R*)-HETrE in RLME cells.** Subconfluent (50–70%) RLME cells were quiesced for 36 h in a serum-deprived medium and further incubated with 12(*R*)-HETrE (0.01–10 nM) or 12(*S*)-HETrE (10 nM) for 45 min. VEGF mRNA levels were measured by slot blot hybridization and densitometry analysis normalized to β-tubulin levels as described under "Experimental Procedures." Results are the mean ± S.E.; n = 4; *, $p < 0.05$ from the vehicle control.
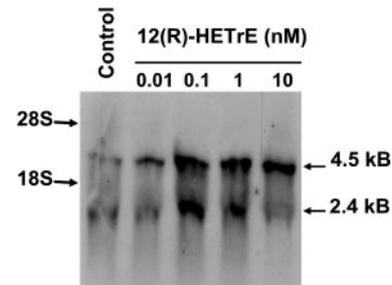

FIG. 3. **VEGF Northern blot.** Subconfluent (50–70%) RLME cells were quiesced for 36 h in serum-deprived medium and further incubated with 12(*R*)-HETrE (0.01–10 nM) for 45 min. VEGF mRNA levels were measured by Northern blot hybridization using the mouse VEGF cDNA probe as described under "Experimental Procedures."

Inhibition of VEGF Attenuates Angiogenic Activity of 12(R)-HETrE—We used the *in vitro* capillary tube-like formation in cells embedded in a coating of basement membrane Matrigel to assess the angiogenic activity of 12(*R*)-HETrE and examined whether it involves VEGF production. As seen in Fig. 8*A,* 12(*R*)-HETrE at a concentration as low as 1 pM stimulated capillary tube-like formation within 24 h. This effect was concentration-dependent, with the maximal stimulation achieved at 0.1 nM (Fig. 8*A*). Moreover, this effect was stereospecific because the *S* enantiomer, 12(*S*)-HETrE, at a concentration of up to 10 nM had little effect on tube-like capillary formation (Fig. 8*B*). Importantly, the addition of VEGF antibodies to the incubation medium greatly attenuated 12(*R*)-HETrE-stimulated capillary tube-like formation (Fig. 7*C*), indicating that the 12(*R*)-HETrE stimulatory effect is mediated, at least in part, by VEGF.

DISCUSSION

12(*R*)-HETrE possesses biological activities *in vitro* and *in vivo* that are characteristic of a pro-inflammatory factor: vaso-
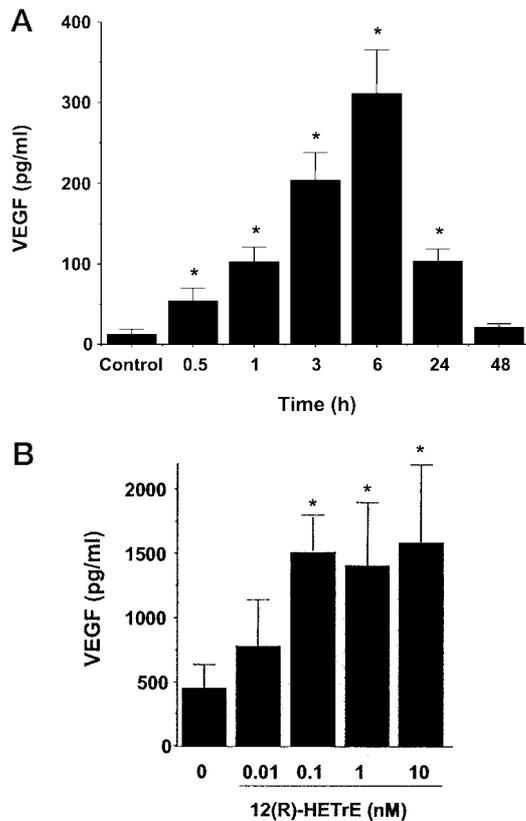
FIG. 4. **12(*R*)-HETrE stimulation of VEGF protein in culture medium of RLME cells.** *A*, time-dependent. Quiesced subconfluent RLME cells were treated with 12(*R*)-HETrE for 1–48 h. At the indicated time points, aliquots of cell culture medium (500 μl) were collected and analyzed for VEGF protein by enzyme-linked immunosorbent assay as described under "Experimental Procedures." Results are the mean ± S.E.; *n* = 5; *, *p* < 0.05 from the vehicle control. *B*, concentration-dependent. Quiesced subconfluent RLME cells were treated with various concentrations of 12(*R*)-HETrE (0.01–10 nM) for 24 h. Aliquots of cell culture medium (500 μl) were collected and analyzed for VEGF protein by enzyme-linked immunosorbent assay as described under "Experimental Procedures." Results are the mean ± S.E.; *n* = 5; *, *p* < 0.05 from the vehicle control.

dilation increased capillary permeability, neutrophil chemotaxis, and angiogenesis. The most fascinating aspect of these responses is that they are elicited by picogram to nanogram quantities, which readily can be found in injured tissues. The synthesis of 12(*R*)-HETrE in the corneal epithelium has been extensively studied. It is formed via a cytochrome P450-dependent pathway from either arachidonic acid or 12-hydroxyeicosatetraenoic acid (20). The production of this eicosanoid by the corneal epithelium in response to hypoxic or chemical injury correlated with the inflammatory response, which included neovascularization of the cornea. Furthermore, inhibition of its formation by depleting cytochrome P450 in the corneal epithelium attenuated the inflammatory response after hypoxic injury (3). Additional studies identified CYP4B1 as the hypoxia-induced cytochrome P450 enzyme involved in the synthesis of 12(*R*)-HETrE (21). Hypoxic injury to the corneal surface increases the expression of both CYP4B1 and VEGF (22), suggesting a potential interaction between these pathways in the implementation of the inflammatory and angiogenic response after such an injury.

The primary target cells of 12(*R*)-HETrE are the microvessel endothelial cells. Binding studies indicated the presence of a high affinity, low capacity receptor/binding site in these cells. Additional studies identified several events that occurred in microvessel endothelial cells in response to 12(*R*)-HETrE in-
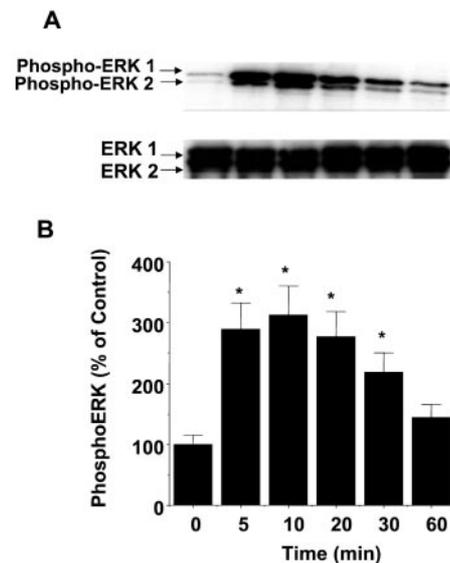

FIG. 5. **12(*R*)-HETrE activation of ERK1/2 in RLME cells.** Quiesced subconfluent RLME cells were treated with 12(*R*)-HETrE (0.1 nM) for the indicated times. Western blot analyses of whole cell lysates were performed with antibodies specific to the phosphorylated and unphosphorylated forms of ERK1/2 as described under "Experimental Procedures." Blots were stripped and reprobed with nonphosphorylated specific ERK antibody for loading control. *A*, a representative Western blot of whole cell lysates for activated (phosphorylated) ERK1/2 and nonphosphorylated ERK1/2. *B*, results of densitometry analysis of the ratio of phosphorylated to nonphosphorylated ERK1/2 are given as percentage of control (*t* = 0) and are the mean ± S.E., *n* = 3; *, *p* < 0.05 from control.
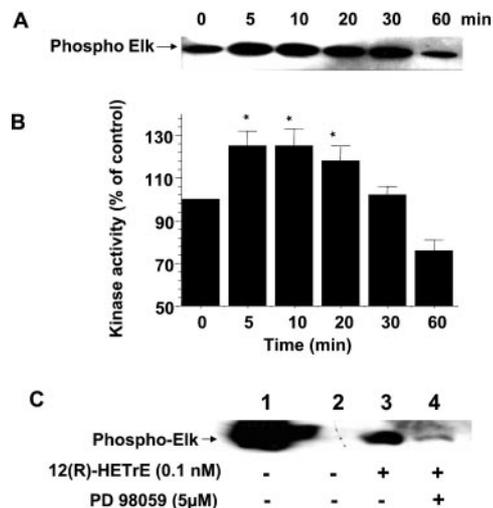

FIG. 6. **12(*R*)-HETrE stimulation of ERK1/2 activity in RLME cells.** Quiesced subconfluent RLME cells were incubated with 12(*R*)-HETrE (0.1 nM) for 5–60 min. ERK activity was determined using the specific substrate Elk as described under "Experimental Procedures." *A* and *B*, time-dependent stimulation of ERK1/2 activity. Results are given as a percentage of control (*t* = 0) and are the mean ± S.E.; *n* = 3; *, *p* < 0.05 from control. *C*, 12(*R*)-HETrE-activated ERK1/2 is inhibited by the MEK-specific inhibitor PD98059. Quiesced subconfluent RLME cells were preincubated with PD98059 (5 μM) for 15 min before the addition of 12(*R*)-HETrE (0.1 nM). ERK activity was determined using the specific substrate Elk as described under "Experimental Procedures." Western blot of whole cell lysates with anti-phospho-Elk antibody is representative of three blots from three separate experiments.

cluding a protein kinase C-dependent nuclear factor κB activation (14), increased c-*fos*, c-*jun*, and c-*myc* oncogene expression (15), and a Phospholipase C-inositol 1,4,5-trisphosphate-mediated increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).[2]

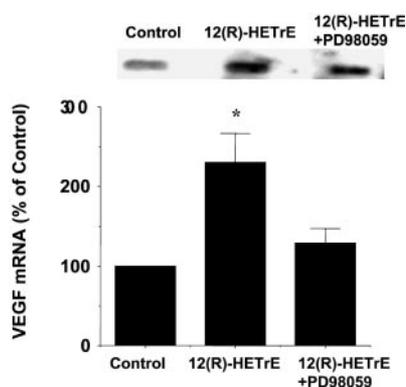[2] P. A. Mieyal and M. Laniado-Schwartzman, unpublished data.

FIG. 7. **Effect of MEK inhibition on 12(*R*)-HETrE-induced VEGF mRNA.** Quiesced subconfluent RLME cells (50–70%) were incubated with 12(*R*)-HETrE with either 12(*R*)-HETrE (0.1 nM) or 12(*R*)-HETrE (0.1 nM) plus PD98059 (5 μM) for 1 h. VEGF mRNA levels were measured by slot blot hybridization with the mouse VEGF cDNA and determined by densitometry analysis using β-tubulin mRNA for normalization. Results are given as a percentage of control untreated cells and are the mean ± S.E.; $n = 3$; *, $p < 0.05$ from control; #, $p < 0.05$ from 12(*R*)-HETrE-treated cells.

Each of these events may contribute to the angiogenic activity of 12(*R*)-HETrE because the transformation of endothelial cells from the resting state to an angiogenic phenotype is a complex process requiring multiple steps to occur before new capillaries are formed. We postulated that 12(*R*)-HETrE released by the injured tissue acts on neighboring microvessel endothelial cells and triggers key steps to initiate the angiogenic transformation. With regard to these key steps in angiogenic transformation, the current study links 12(*R*)-HETrE to the induction of VEGF, one of the most potent peptide angiogenic factors of endothelial cell origin.

The current study describes a novel mechanism for 12(*R*)-HETrE-induced angiogenesis. The results indicate that it is a potent inducer of VEGF expression in microvessel endothelial cells and that its angiogenic activity, measured as the ability to form capillary tube-like structures in culture, is mediated in part by VEGF. The results also suggest that 12(*R*)-HETrE induces VEGF expression via activation of the ERK1/2 MAPK signaling pathway, a pathway that has long been implicated in the regulation of VEGF expression in endothelial cells, especially under hypoxic conditions (23).

12(*R*)-HETrE is not the only angiogenic eicosanoid; however, as documented previously and seen in this study, it is one of the most potent. It stimulates capillary tube-like formation at concentrations as low as 1 pM. Its maximal stimulatory effect in inducing VEGF expression and eliciting an angiogenic response is obtained at 0.1 nM, a concentration that is approximately two times the estimated binding affinity of 12(*R*)-HETrE in these cells (13). Among other eicosanoids, prostaglandins have long been thought to promote angiogenesis, although they do not directly stimulate endothelial cell growth (24, 25). Their angiogenic effect is believed to be mediated by paracrine actions on angiogenic factors such as basic fibroblast growth factor and VEGF (26, 27). Other cyclooxygenase metabolites have been implicated in the process of angiogenesis. For example a thromboxane A2 agonist has been shown to increase endothelial cell proliferation and migration (28). Studies by Honn and colleagues (29) demonstrated that endothelial 12-lipoxygenase produces 12(*S*)-HETE, which in turn is capable of stimulating cell proliferation, migration, and capillary tube-like formation when added at nanomolar to micromolar concentrations. These investigators suggested that endogenous 12-lipoxygenase is involved in the endothelial cell angiogenic response by showing that enzymatic inhibitors re-

duced growth factor-stimulated cell proliferation, migration, and capillary tube-like formation. The cytochrome P450-derived epoxyeicosatrienoic acids have been shown to act as mitogens in various cell types including vascular endothelial cells (30). A potential role for epoxyeicosatrienoic acids in the regulation of angiogenesis has been implicated from studies showing that astrocytes are capable of inducing capillary angiogenesis that appears to be mediated in part by epoxyeicosatrienoic acids (31).

Our study shows that 12(*R*)-HETrE increased VEGF mRNA by a mechanism that includes both activation of transcription and stabilization of mRNA as evidenced by sensitivity to actinomycin D and an increased half-life of VEGF mRNA. The increase in VEGF mRNA was very rapid; a significant increase was seen 15 min after the addition of 12(*R*)-HETrE. Other eicosanoids such as prostaglandin E$_2$ have been shown to increase VEGF expression primarily by transcriptional activation and mostly within hours of cell exposure to these prostanoids (26, 27, 32–34). Our study also shows that 12(*R*)-HETrE increases VEGF mRNA via activation of a MAPK signaling pathway, namely, ERK1/2. 12(*R*)-HETrE at concentration of 0.1 nM increased ERK1/2 phosphorylation within 5 min, and this activation resulted in increased kinase activity. Moreover, the ability of PD98059 to inhibit 12(*R*)-HETrE-induced VEGF mRNA indicates that a MEK-1-dependent activation of ERK1/2 is part of the cellular mechanism that underlies the effect of 12(*R*)-HETrE on VEGF gene expression. Numerous studies have documented a key role for the ERK1/2 MAP kinase pathway in angiogenesis; these studies include evidence that ERK1/2 MAP kinase activity is critical for the control of endothelial cell proliferation and growth arrest and that these kinases promote VEGF expression via transcriptional activation (Ref. 19 and the references therein). More importantly, ERK1/2 MAP kinases have been shown to directly phosphorylate (HIF)-1α, a key step in the activation of HIF-1 transcription factor (23). The latter is essential for VEGF increased gene expression in response to hypoxia. The fact that 12(*R*)-HETrE is produced in response to hypoxia raises the possibility that this eicosanoid serves as amplifier of the hypoxic induction of VEGF through activation of the ERK1/2.

The relevance of 12(*R*)-HETrE is further enhanced by the fact that its formation is also seen in other tissues and that its topical application elicits biological activities typical of inflammatory factors. The presence of an eicosanoid with an HPLC retention time similar to that of 12-HETrE was documented in human psoriatic lesions (35). Rat epidermal microsomes have been shown to produce 12-HETrE enantiomers by an NADPH-dependent mechanism (8, 9). Production of 12-HETrE from 12-hydroxyeicosatetraenoic acid was also documented in porcine neutrophils (10). In guinea pig skin, 12(*R*)-HETrE increases delayed-type hypersensitivity inflammatory reactions at doses as low as 1 fmol (12). 12(*R*)-HETrE is present in human tears, and its levels are many fold higher in tears from inflamed eyes (7). This latter finding suggests that this eicosanoid is relevant to human pathophysiology. It should be noted that 12(*R*)-HETrE is a potent chemoattractant, whereas VEGF is not (11). Thus, 12(*R*)-HETrE could be critical to the activity of VEGF not only by inducing its expression in the injured tissue and its vascular surroundings but also by providing the chemoattractant activity for polymorphonuclear cells, a major source of VEGF. This relationship could also be significant in the generation of chronic inflammation, a major clinical problem, because neovascularization is necessary to maintain chronic inflammation (36).

FIG. 8. **12(*R*)-HETrE stimulation of capillary-like network formation in RLME cells.** *A*, quiesced subconfluent RLME cells plated in a 12-well culture dish (1×10^5 cells/well) precoated with Matrigel were incubated with various concentrations of 12(*R*)-HETrE (1 pM to 10 nM) for 16 or 24 h. Photographs were taken at $\times 10$ magnification. Maximal response was obtained at 24 h after the addition of 12(*R*)-HETrE. Capillary length was measured by image analysis using Image Pro software as described under "Experimental Procedures." Results of capillary length after 24 h as a function of 12(*R*)-HETrE concentrations are the mean ± S.E.; $n = 3$; *, $p < 0.05$ from control untreated cells. *B*, stereospecificity of 12(*R*)-HETrE effect on capillary-like network formation. Quiesced subconfluent RLME cells plated in a 12-well culture dish precoated with Matrigel were incubated with 12(*R*)-HETrE (0.1 nM) or 12(*S*)-HETrE (10 nM) for 24 h. Photographs were taken at $\times 10$ magnification. Capillary length was measured by image analysis using Image Pro software as described under "Experimental Procedures." Results of capillary length are the mean ± S.E.; $n = 3$; *, $p < 0.05$ from control untreated cells. *C*, inhibition of 12(*R*)-HETrE stimulated capillary-like tube formation by anti-VEGF-specific antibody. Quiesced subconfluent RLME cells were plated in 12-well culture dishes precoated with Matrigel and incubated with 12(*R*)-HETrE (0.1 nM) or 12(*R*)-HETrE (0.1 nM) plus anti-VEGF-specific antibodies (1:1000) for 24 h. Photographs were taken at $\times 10$ magnification. Capillary length was measured by image analysis using Image Pro software as described under "Experimental Procedures." Results of capillary length are the mean ± S.E.; $n = 3$; *, $p < 0.05$ from control.
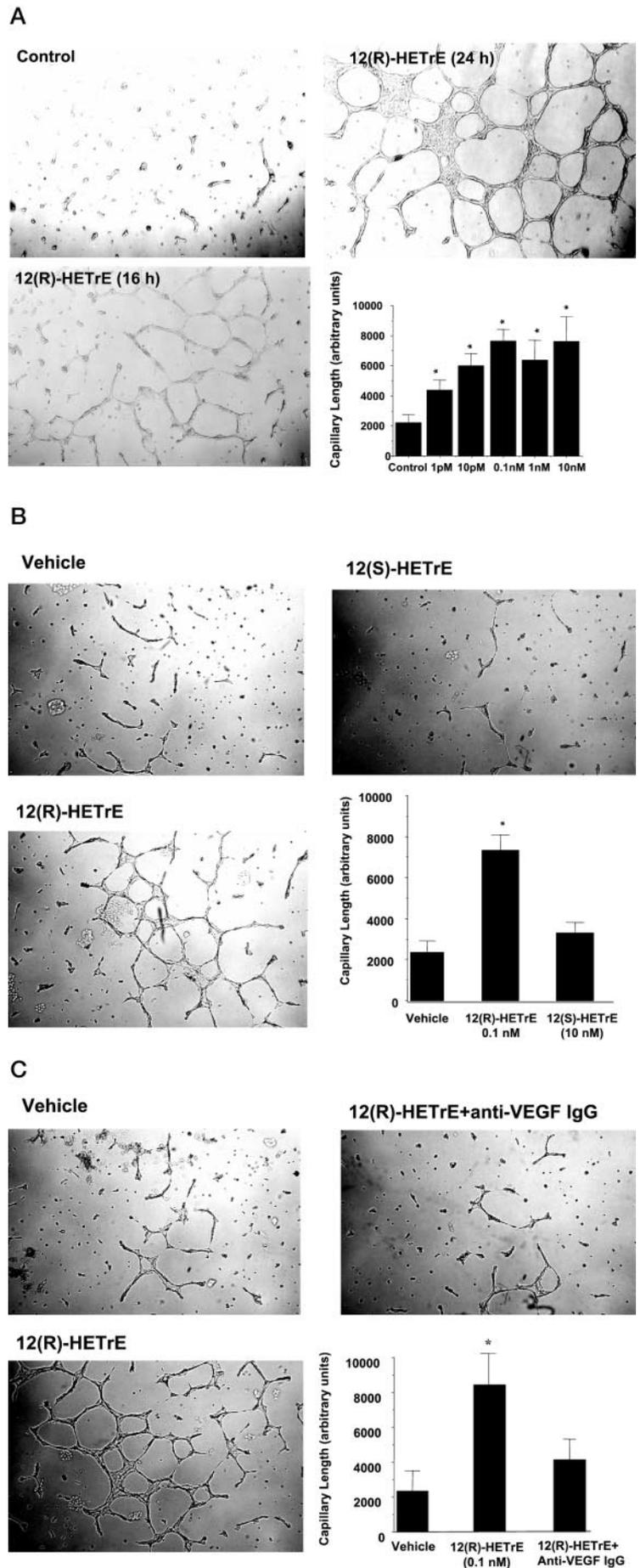
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Eicosanoid Regulation of Vascular Endothelial Growth Factor Expression and Angiogenesis in Microvessel Endothelial Cells

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