Evidence suggests that the arachidonic acid metabolite of 12-lipoxygenase, 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), not only mediates the effects of angiotensin II (AngII), but also has direct effects on hypertrophy and matrix protein production in vascular smooth muscle cells (VSMCs). This study is aimed at identifying the signaling pathways involved in these events. Treatment of porcine VSMCs with 12(S)-HETE led to the activation of Ras and p38 MAPK. It also stimulated phosphorylation, DNA-binding activity, and transactivation of the transcription factor cAMP response element (CRE)-binding protein. In addition, 12(S)-HETE induced transcription from a fibronectin promoter containing multiple CREs. AngII also induced transactivation of CRE-binding protein and transcription from the fibronectin promoter. A specific p38 MAPK inhibitor (SB202190) as well as a dominant-negative Ras mutant (Ras-N17) blocked both 12(S)-HETE and AngII effects. In addition, inhibitors of lipoxygenase also blocked AngII effects. Both 12(S)-HETE and AngII increased cellular hypertrophy with similar potency, and this was significantly blocked by SB202190. Stable overexpression of murine leukocyte-type 12/15-lipoxygenase in VSMCs increased the levels of cell-associated 12(S)-HETE as well as basal activity of both ERK and p38 MAPKs. Furthermore, these 12-lipoxygenase-overexpressing cells displayed significantly greater cellular hypertrophy relative to mock-transfected cells. These results show for the first time that oxidized lipids such as 12(S)-HETE can induce VSMC growth and matrix gene expression and mediate growth factor effects via activation of the Ras-MAPK pathway and key target transcription factors.

Growth factor- and cytokine-induced vascular smooth muscle cell proliferation, migration, hypertrophy, and matrix production have been shown to play important roles in the development of vascular disorders such as atherosclerosis, hypertension, and restenosis. Growth factors such as angiotensin II (AngII) and platelet-derived growth factor (PDGF) activate intracellular phospholipases, leading to the formation of arachidonic acid, which can be further metabolized by cyclooxygenases, cytochrome P-450 oxygenases, and lipoxygenases (1). Lipoxygenase (LO) action leads to the formation of oxidized lipids such as 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE). LOs and their products of arachidonic acid and linoleic acid metabolism have been shown to mediate growth factor effects in vascular smooth muscle cells (VSMCs) as well as responses to vascular injury. Lipoxygenases are classified as 5-, 8-, 12-, and 15-lipoxygenases based on their ability to insert molecular oxygen at the corresponding carbon atom of arachidonic acid. Three major isoforms of 12-LO have been cloned. They are platelet-type 12-LO, leukocyte-type 12-LO, and epidermis-type 12-LO. These proteins are products of distinct genes and vary in tissue distribution (2, 3).

Leukocyte 12-LO has been cloned from porcine and mouse leukocytes (4, 5). The presence of leukocyte-type 12-LO (also termed 12/15-LO) has also been demonstrated in various cell types and tissues, including VSMCs, adrenal cells, rat brain, and kidney (6–11). Studies from this and other laboratories (12–21) have implicated the leukocyte-type 12-LO pathway in the pathogenesis of atherosclerosis and restenosis. 12-LO expression was demonstrated in atherosclerotic lesions (12). In vascular and mononuclear cells, growth factors and cytokines as well as high glucose stimulate both the activity and expression of 12-LO (6, 13–15). Furthermore, expression of 12-LO protein and mRNA is markedly increased in neointima of balloon-injured rat carotid arteries, and pretreatment with LO inhibitors reduces the rate of neointimal thickening in this model (16, 17). We have demonstrated that treatment with a chimeric DNA-RNA hammerhead ribozyme targeted to cleave rat leukocyte-type 12-LO mRNA significantly reduces neointimal thickening in balloon-injured rat arteries (18). We have...
also recently shown that infection of porcine VSMCs (PVSMCs) with a recombinant adenovirus expressing a 12-LO ribozyme reduces PDGF-induced migration and glucose-induced fibronectin expression, thus providing further support for a functional role of 12-LO in growth factor-induced responses (19). The role of 12-LO in the pathology of atherosclerosis was further established when it was demonstrated that crossbreeding of apoE knockout mice with 12-LO knockout mice produces offspring with reduced rates of atherosclerosis (20, 21).

Lipoxygenases metabolize arachidonic acid to produce bioactive lipids such as 12(S)-HETE (2). Increased formation of LO products such as 12(S)-HETE by the biological agents involved in cardiovascular dysfunction has been reported in VSMCs, endothelial cells, and monocytes (6, 13–15, 22). 12(S)-HETE has direct effects like chemotaxis (23); changes in vascular tone (24); and production of vascular endothelial growth factor, a potent angiogenic agent (25). It can also induce monocyte binding to endothelial cells (22) and can mediate AngII-induced changes in neuronal K⁺ currents (26). We have also shown that 12(S)-HETE exhibits direct growth-promoting effects and increases levels of the key extracellular matrix protein fibronectin in PVSMCs (27). Furthermore, it also mediates the hypertrophic effects of AngII and the chemotactic effects of PDGF in the same model (13, 27). Thus, 12(S)-HETE may be involved in multiple events related to the development of hypertension and atherosclerosis. These diverse biological effects of 12(S)-HETE suggest that it can act as a distinct signaling molecule. However, signaling pathways and gene regulation mechanisms that mediate 12(S)-HETE-induced responses in VSMCs are not clear. In this study, we report that 12(S)-HETE activates the Ras-MAPK pathway, which is essential for the activation of multiple transcription factors, including cAMP response element-binding protein (CREB). Furthermore, 12(S)-HETE could also induce transcription from a fibronectin promoter containing CRE-binding sites. The effects of 12(S)-HETE were similar to those of AngII, and LO activation could also mediate AngII effects. In addition, we report that stable overexpression of 12-LO in VSMCs increases activation of MAPKs and cellular hypertrophy.

EXPERIMENTAL PROCEDURES

Materials—12(S)-Hydroxyeicosatetraenoic acid (12(S)-HETE), 12(R)-hydroxyeicosatetraenoic acid (12(R)-HETE), and the LO inhibitor cinnaamy1-4,4-dihydroxy-ar-cyanocinnamate (CDC) were purchased from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). Inhibitors for p38 MAPK (SB202190), MEK (PD98059), and Ras farnesyltransferase (FPT III) were from Calbiochem. Protease inhibitor mixture (Complete) was from Roche Molecular Biochemicals. Gel shift oligonucleotides for CREB and SP1 were from Chemicon. Protease inhibitor mixture (Complete) was from Roche Molecular Biochemicals. Gel shift oligonucleotides for CREB and SP1 were from Chemicon. Protease inhibitor mixture (Complete) was from Roche Molecular Biochemicals. Gel shift oligonucleotides for CREB and SP1 were from Chemicon.

RESULTS

Preparation of Nuclear Extracts—Nuclear extracts were prepared as described previously (29). Briefly, after the stimulation with 12(S)-HETE, cells were washed once with 1 ml of PBS, harvested, and spun down at 4 °C. Cell pellets were resuspended in nuclear extraction buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and protease inhibitors) and then lysed in nuclear extraction buffer A containing 0.1% Nonidet P-40. Cell lysates were centrifuged, and nuclei were resuspended in nuclear extraction buffer B (10 mM Hepes, pH 7.9, 0.42 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 25% glycerol, and protease inhibitors). After a 30-min incubation on ice, they were centrifuged at 20,000 × g for 15 min at 4 °C, and the supernatant was used as nuclear extract.

Gel Shift Assays—DNA binding of nuclear proteins was performed as described previously (29). Synthetic oligonucleotides containing consensus DNA-binding sequences for transcription factors CREB and SP1 were used as probes in gel shift assays. Gels were dried, and DNA-protein complexes were visualized on a PhosphorImager. Quantitation of radioactivity in each complex was carried out using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA).

Immunoblotting—Immunoblotting of the cell lysates was performed as described before (11, 30) with the following modifications. Protein bands on Western blots were visualized using Supersignal reagents, and images were scanned using a Molecular Science GS-800 densitometer, and protein bands were quantified with QuantiTide One software (Bio-Rad).

Transient DNA Transfections and Luciferase Assays—PVSMCs were split 24 h prior to transfection to obtain 70% confluence in 12-well plates and transfected with Lipotaxi for 6 h in serum-free medium as described by the manufacturer. Cells were allowed to recover overnight in serum-containing medium and stimulated with 12(S)-HETE or AngII (1 μM each) for 6 h. Then, the cells were lysed in reporter lysis buffer, and luciferase assays were performed as described by the manufacturer. Transfection efficiency was normalized to β-galactosidase activity. In some experiments, cells were pretreated with inhibitors of p38 MAPK (SB202190, 1–5 μM), ERK1/2 (PD98059, 10–50 μM), and 12-LO (CDC, 10 μM) for 30 min prior to 12(S)-HETE addition.

Stable Overexpression of 12-LO—Rat VSMCs (A10 cell line) were cotransfected with an expression vector for mouse leukocyte-type 12-LO (m12-LO) or the empty expression vector pcDNA3.1 using Lipotaxi. Transfected cells were grown in the presence of Geneticin (G418) at a concentration of 400 μg/ml until resistant colonies formed. These colonies were cloned using cloning cylinders, and then individual colonies were tested for 12-LO expression by immunoblotting with anti-12-LO antibody. Clones expressing high amounts of 12-LO were used in further experiments.

Incorporation of L[3H]Leucine—Serum-depleted VSMCs were incubated overnight with [3H]leucine and 12(S)-HETE or AngII at the indicated concentrations, and radioactivity in trichloroacetic acid-insoluble material precipitated with trichloroacetic acid as described previously (14). 12(S)-HETE Assay—Rat VMSCs (A10 cell line) transfected with m12-LO or pcDNA3.1 were serum-depleted, and cell pellets were hydrolyzed to release cell-associated HETEs as described previously (6). 12(S)-HETE was then extracted, and levels were quantitated using a specific radioimmunoassay as described previously (6, 31).

Data Analysis—Results are expressed as means ± S.E. of multiple experiments. Paired Student’s t tests were used to compare two groups.
and analysis of variance with Dunnet’s post-test was used for multiple groups using PRISM software (GraphPad Software for Science, San Diego, CA). Statistical significance was detected at \( p < 0.05 \) level.

RESULTS

Activation of p38 MAPK by 12(S)-HETE—To evaluate the signaling responses of 12(S)-HETE in PVSMCs, we examined the activation of key MAPKs. Serum-depleted VSMCs were treated with 12(S)-HETE (0.1 \( \mu \text{M} \)) for 5, 10, 30, and 60 min, and MAPK activation was determined by immunoblotting with phospho-specific antibodies that recognize only the activated kinase. As shown in Fig. 1A, p38 MAPK activation could be seen within 5 min after stimulation with 12(S)-HETE and remained elevated until 30 min as detected by increases in the levels of the phospho-p38 MAPK band. When the same blot was stripped and probed with an antibody to non-phosphorylated p38 MAPK, the results showed that an equal amount of p38 MAPK was present in all the lanes (Fig. 1B). Immunoblotting with anti-phospho-ERK antibodies showed that 12(S)-HETE did not significantly activate ERK1/2 (Fig. 1C). 12(S)-HETE also did not activate a third MAPK, JNK (data not shown). These results demonstrate that 12(S)-HETE is a potent activator of p38 MAPK (but not ERK1/2 or JNK) in VSMCs.

Activation of Ras by 12(S)-HETE—We next examined whether 12(S)-HETE can activate the small GTP-binding protein Ras, a key common upstream activator of MAPKs. Quiescent VSMCs were left untreated or were pretreated with an inhibitor of Ras farnesylation (FPT III, 10 \( \mu \text{M} \)) and then stimulated for 10 min either with vehicle alone or with 0.1 \( \mu \text{M} \) 12(S)-HETE or 12(R)-HETE, which is not a lipoxygenase product. Cells were then stained with anti-Ras antibody followed by Texas Red-conjugated secondary antibody, and Ras translocation to the plasma membrane was examined in a confocal microscope. There was peaked increase in the translocation of Ras to the plasma membrane in cells stimulated with 12(S)-HETE (Fig. 2C) compared with those treated with vehicle (Fig. 2A). In contrast, 12(R)-HETE had no effect on translocation (Fig. 2B). Furthermore, 12(S)-HETE-induced Ras translocation to the plasma membrane was abolished by pretreatment with FPT III (Fig. 2F). These results demonstrate that, in PVSMCs, 12(S)-HETE induces translocation of Ras to the membrane, a process that can result in the activation of Ras.

Phosphorylation of CREB in 12(S)-HETE-stimulated PVSMCs—We next examined whether 12(S)-HETE can activate the transcription factor CREB, a key downstream target of the MAPK pathway, particularly p38 MAPK (32). In addition, CREB is a key player in transcription of the matrix protein fibronectin (33–36). Serum-depleted PVSMCs were treated for 5, 10, 30, and 60 min with 12(S)-HETE (0.1 \( \mu \text{M} \)). The cell lysates were then immunoblotted with anti-phospho-CREB antibody that recognizes only CREB protein phosphorylated at Ser133 in the transactivation domain. As shown in Fig. 3A, phosphorylation of CREB was increased at 5 min and remained elevated for 30 min. In contrast, the levels of non-phosphorylated CREB remained unchanged, as shown in Fig. 3B. These results demonstrate that 12(S)-HETE stimulates CREB phosphorylation in PVSMCs.

Activation of p38 MAPK and CREB by 12(S)-HETE, but Not by 12(R)-HETE—To test the specificity of 12(S)-HETE signaling, VSMCs were stimulated with either 12(S)-HETE or 12(R)-HETE for 5 min. The cell lysates were then analyzed for the activation of p38 MAPK and CREB by immunoblotting with phospho-specific antibodies. 12(S)-HETE stimulated activation of both p38 MAPK (Fig. 4A, second lane) and CREB (C, second lane) compared with controls (A and C, lanes 1). In contrast, 12(R)-HETE had no effect on the activation of either p38 MAPK (Fig. 4A, third lane) or CREB (C, third lane). Subsequently, the same blots were stripped and probed with anti-p38 MAPK or anti-CREB antibody recognizing both phosphorylated and non-phosphorylated forms. The results show that 12(S)-HETE did not alter the p38 MAPK (Fig. 4D) and CREB (Fig. 4D) protein levels, indicating that equal amounts of proteins were loaded in all lanes. Thus, 12(S)-HETE (but not 12(R)-HETE) was able to stimulate p38 MAPK and CREB in VSMCs; and hence, the effects are specific to the LO product.

Involvement of p38 MAPK in 12(S)-HETE-induced CREB Phosphorylation—To investigate the upstream kinase(s) involved in the phosphorylation of CREB and to determine whether CREB is a target of 12(S)-HETE-induced p38 MAPK activation, we tested the effect of MAPK inhibitors on CREB phosphorylation. PVSMCs were pretreated with the p38 MAPK inhibitor SB202190 (1–5 \( \mu \text{M} \)) or the MEK inhibitor PD98059 (10–50 \( \mu \text{M} \)) for 30 min prior to stimulation with 12(S)-HETE for 5 min. The cell lysates were then immunoblotted with anti-phospho-CREB antibodies, and the results are shown in Fig. 5 (A and C). Phosphorylation of CREB was inhibited by SB202190 at as low as 1 \( \mu \text{M} \). In contrast, the MEK inhibitor PD98059 had no effect on CREB phosphorylation even at 50 \( \mu \text{M} \).
p38 MAPK antibody. As shown in Fig. 5B, 1 µM SB202190 blocked p38 MAPK activation, in contrast to PD98059, which had no effect on p38 MAPK activation at 50 µM. These results demonstrate that p38 MAPK mediates 12(S)-HETE-induced phosphorylation of CREB in PVSMCs.

Stimulation of CREB DNA-binding Activity by 12(S)-HETE—Serum-depleted PVSMCs were stimulated with 12(S)-HETE for 15 min to 2 h, and nuclear extracts were prepared. Nuclear extracts were analyzed for DNA-binding activity by gel shift assay using 32P-labeled double-stranded oligonucleotides containing consensus DNA-binding sequences for CREB (Fig. 6A) and the constitutively active transcription factor SP1 (Fig. 6B). Radioactivity in the DNA-protein complexes was determined using a PhosphorImager and is shown as -fold over control in Fig. 6C. The DNA-binding activity of CREB was increased by 12(S)-HETE within 15 min, peaked at 30 min, and continued to remain elevated until 120 min (Fig. 6A). However, in the same nuclear extracts, 12(S)-HETE had no effect on SP1 DNA-binding activity, indicating the specificity of 12(S)-HETE for the activation of CREB DNA binding. These results demonstrate that 12(S)-HETE can activate the DNA-binding activity of CREB in PVSMCs.

Transcriptional Activation of CREB by 12(S)-HETE—To determine whether 12(S)-HETE-induced CREB phosphorylation and DNA-binding activity lead to actual transcription events, we evaluated the involvement of 12-LO and its product 12(S)-HETE in the transcriptional activation of CREB. The transcriptional activation of CREB was analyzed using the PathDetect Trans-Reporting system. This system consists of a reporter plasmid (pFR-Luc) in which the expression of the luciferase reporter gene is driven by a minimal promoter containing upstream yeast GAL4 DNA-binding sites and an expression vector with the CREB transactivation domain fused to the yeast GAL4 DNA-binding domain (pFA2-CREB). Cotransfection and subsequent activation of CREB-GAL4 fusion protein by an upstream signal transduction pathway stimulates expression of the reporter gene luciferase. Thus, luciferase activity can be used as a direct measure of transcriptional activation of CREB. PVSMCs were cotransfected with pFR-Luc and pFA2-CREB, and the cells were stimulated with 0.1 µM 12(S)-HETE or AngII. The latter has been shown to activate the 12-LO pathway (6). Luciferase activity in cell lysates was then determined as described under “Experimental Procedures.” The results show that luciferase activity was 3-fold higher in
PVS MCs stimulated with 12(S)-HETE (Fig. 7A) and that Ang II treatment increased luciferase activity by 7-fold (Fig. 7B) compared with untreated cells. Thus, both 12(S)-HETE and Ang II were able to induce transcriptional activation of CREB in PVS MCs.

Furthermore, the dose-response study depicted in Fig. 7C shows that 12(S)-HETE effects were optimal at 0.1 μM.

We then examined whether lipoygenase activation is involved in Ang II-induced CREB transcriptional activation. PVS MCs transfected with pFR-Luc and pFA-CREB were pretreated with the 12-LO inhibitor CDC (10 μM) for 30 min prior to stimulation with Ang II, and then the luciferase activity of the lysates was determined. The LO inhibitor CDC completely abolished Ang II-induced CREB activation (Fig. 7B, bars CD and CD/AII), demonstrating the involvement of 12-LO in these events.

**Signaling Events in 12(S)-HETE-induced Transcriptional Activation of CREB**—Next, we determined the role of MAPK activation in 12(S)-HETE transcriptional activation by 12(S)-HETE and Ang II. PVS MCs transiently transfected with pFR-Luc and pFA-CREB were pretreated with 5 μM SB202190 or 50 μM PD98059 (inhibitors of p38 MAPK and MEK, respectively) for 30 min. The cells were then stimulated with 0.1 μM 12(S)-HETE or Ang II for 6 h, and luciferase activity was determined. The results in Fig. 7A show that the p38 MAPK inhibitor (SB202190) completely blocked 12(S)-HETE-induced transcriptional activation of CREB. In contrast, the MEK inhibitor (PD98059) had no effect in these experiments. In the case of Ang II, SB202190 significantly blocked CREB activation, whereas PD98059 showed only a slight but significant inhibitory effect (Fig. 7B). These results demonstrate for the first time that p38 MAPK is involved in the transcriptional activation of CREB by 12(S)-HETE and Ang II in PVS MCs.

**Activation of Fibronectin Promoter in PVS MCs—12(S)-HETE** has been shown to increase the levels of the matrix protein fibronectin in PVS MCs as determined by an enzyme-linked immunosorbent assay (27). However, it is not known whether it is regulated at the level of transcription. Because 12(S)-HETE activated CREB, a key transcription factor involved in fibronectin expression, we tested if it can induce transcription from a reporter plasmid containing a minimal fibronectin promoter (+59 to −510) fused to a luciferase reporter gene. The fibronectin promoter in this construct (pFN510-Luc) contains three CRE-binding sites at positions −170, −260, and −415 and has been shown to be induced by forskolin, serum, and Ang II (33–36). PVS MCs were transiently transfected with pFN510-Luc and stimulated with 12(S)-HETE for 6 h, and luciferase activity was determined. As shown in Fig. 8, 12(S)-HETE stimulated transcription from pFN510-Luc by 3-fold, which is similar to that induced by Ang II (3.5-fold).

Pretreatment of PVS MCs with the p38 MAPK inhibitor SB202190 completely inhibited both 12(S)-HETE- as well as Ang II-induced activation of the fibronectin promoter. Thus, 12(S)-HETE was able to induce transcription of a fibronectin

**FIG. 7. Transcriptional activation of CREB by 12(S)-HETE and Ang II.** PVS MCs were transiently transfected with pFR-Luc and pFA-CREB and stimulated with 0.1 μM 12(S)-HETE (A), 0.1 μM Ang II (AII; B), or the indicated concentrations of 12(S)-HETE (C) for 6 h. Luciferase activity was determined. In some experiments, cells were pretreated with the LO inhibitor CDC (10 μM), the p38 MAPK inhibitor SB202190 (SB; 5 μM), or the ERK inhibitor PD98059 (PD; 50 μM) prior to stimulation with agonists. Data represent means ± S.E. of at least three experiments performed in triplicate. *, p < 0.001 versus control (C); **, p < 0.001, and ***, p < 0.05 versus 12(S)-HETE or Ang II. RLU, relative luminescence units.

**FIG. 8. Stimulation of transcription from the fibronectin promoter by 12(S)-HETE.** PVS MCs were transiently transfected with the reporter plasmid pFN510-Luc, which contains the luciferase gene under the control of the minimal fibronectin promoter (-510) with three CRE-binding sites in the upstream sequences. The cells were then stimulated with 0.1 μM 12(S)-HETE or Ang II (AII) in the presence or absence of 5 μM SB202190 (SB) for 6 h, and luciferase activity was determined. In some experiments, an expression vector for Ras-N17, a dominant-negative mutant of Ras (DRas), was also co-transfected. Data represent means ± S.E. of at least three experiments performed in triplicate. *, p < 0.001 versus control (C); **, p < 0.001 versus 12(S)-HETE or Ang II.
promoter construct containing CREs in a p38 MAPK-dependent manner. Because 12(S)-HETE also activates Ras, pFNS510-Luc was cotransfected with a dominant-negative Ras mutant (Ras-N17), and luciferase activity was determined after stimulation with 12(S)-HETE or AngII. The results show that Ras-N17 could inhibit the effects of both 12(S)-HETE and AngII on transcription from FN510-Luc (Fig. 8). These results demonstrate that the Ras-p38 MAPK pathway plays an important role in 12(S)-HETE- as well as AngII-induced transcription from the fibronectin promoter.

Role of p38 MAPK Activation in Mediating Growth-promoting Effects of 12(S)-HETE in VSMCs—We tested if 12(S)-HETE can induce [3H]leucine incorporation as an index of hypertrophy in VSMCs. Serum-depleted PVSMCs were stimulated with 12(S)-HETE (0.01–0.1 μM), and [3H]leucine incorporation was determined as described under “Experimental Procedures.” As shown in Fig. 9A, 12(S)-HETE significantly increased [3H]leucine incorporation into VSMCs even at 0.01 μM. Furthermore, the p38 MAPK inhibitor SB202190 (5 μM) completely abolished the 12(S)-HETE (0.1 μM)-induced [3H]leucine incorporation in PVSMCs (Fig. 9B). Similar results were obtained in AngII-stimulated PVSMCs, i.e., AngII (0.1 μM)-stimulated [3H]leucine incorporation was blocked by the p38 MAPK inhibitor (Fig. 9B). Thus, p38 MAPK appears to mediate the growth-promoting effects of both 12(S)-HETE and AngII in VSMCs.

12-LO Overexpression Increases Cellular Hypertrophy—Because 12(S)-HETE has growth-promoting effects in VSMCs, we hypothesized that overexpression of 12-LO in VSMCs would confer increased growth properties. We therefore examined the consequences of 12-LO overexpression in VSMCs. To this end, we used the VSMC line A10, which is derived from the thoracic aorta of the DB1X embryonic rat, possesses many of the properties characteristic of VSMCs, and is amenable to stable transfection. m12-LO cDNA was cotransfected with pcDNA3.1, and G418-resistant colonies were screened for the overexpression of 12-LO as described under “Experimental Procedures” and are represented as picograms/10^5 cells. Results are expressed as means ± S.E. *p < 0.05 versus 12(S)-HETE or AngII.

**Fig. 9.** Stimulation of hypertrophic responses by 12(S)-HETE. A, stimulation of [3H]leucine incorporation by 12(S)-HETE in PVSMCs. Cells were stimulated with the indicated concentrations of 12(S)-HETE for 24 h in the presence of [3H]leucine, and radioactivity incorporated into trichloroacetic acid-insoluble protein was determined by liquid scintillation counting. Results are expressed as means ± S.E. *, p < 0.001 versus control (n = 3). B, inhibition of 12(S)-HETE- or AngII-stimulated [3H]leucine incorporation by the p38 MAPK inhibitor. PVSMCs were pretreated with SB202190 (5 μM), and then 12(S)-HETE (0.1 μM)- or AngII (0.1 μM)-stimulated [3H]leucine incorporation into trichloroacetic acid-insoluble protein was determined by liquid scintillation counting. The results represent means ± S.E. of at least three experiments performed in triplicate. *, p < 0.001 versus control; **, p < 0.01 versus 12(S)-HETE or AngII.

**Fig. 10.** Overexpression of 12-LO in A10 VSMCs. Rat VSMCs (A10 cell line) were cotransfected with the m12-LO expression vector, and pcDNA3.1- and G418 (400 μg/ml)-resistant colonies were isolated as described under “Experimental Procedures.” A, shown are the 12-LO protein levels in mock-transfected cells (pcDNA cells) and in 12-LO-transfected cells (m12-LO cells). The first lane has authentic porcine leukocyte 12-LO protein. B, VSMCs plated on chamber slides were stained with anti-12-LO antibody to detect the expression levels of 12-LO in mock-transfected cells (pcDNA cells) or in VSMCs overexpressing m12-LO (m12-LO cells). IgG represents m12-LO cells stained with normal rabbit IgG. C, 12(S)-HETE levels were determined by radioimmunoassay in pcDNA and m12-LO cells as described under “Experimental Procedures” and are represented as picograms/10^5 cells. Results are expressed as means ± S.E. *, p < 0.01 (n = 3).
12-LO antibody (16) also showed strong staining in m12-LO cells compared with pcDNA cells (Fig. 10B). The two cell lines showed no differences in morphology or rate of cell proliferation (data not shown). The cell-associated 12(S)-HETE levels were 3-fold greater in m12-LO cells compared with pcDNA cells (Fig. 10C).

Because 12-LO products could induce activation of MAPK, phospho-MAPK levels were determined by immunoblotting in pcDNA and m12-LO cells. The results shown in Fig. 11A show that the basal activity of p38 MAPK was increased in m12-LO cells compared with pcDNA cells. Furthermore, after serum stimulation, p38 MAPK activity was higher in m12-LO cells compared with pcDNA cells (Fig. 11, A and D). Reprobing of the stripped membrane with anti-p38 MAPK antibody showed equal amounts of total p38 MAPK in all lanes (Fig. 11C). The basal levels of phospho-ERK1/2 were also elevated in m12-LO cells, but very little difference was observed in serum-stimulated activation of ERK1/2 (Fig. 11, B and E).

To determine whether 12-LO overexpression can alter cellular hypertrophy, we compared [3H]leucine incorporation in m12-LO and pcDNA cells. m12-LO cells showed a small but significantly higher rate of [3H]leucine incorporation compared with pcDNA cells. Furthermore, after serum stimulation, ERK1/2 activation was dose-dependently enhanced in m12-LO cells compared with pcDNA cells (Fig. 10C). The cell-associated 12(S)-HETE levels were 3-fold higher in m12-LO cells compared with pcDNA cells. Furthermore, after serum stimulation, p38 MAPK activity was higher in m12-LO cells compared with pcDNA cells (Fig. 10C). The cell-associated 12(S)-HETE levels were 3-fold higher in m12-LO cells compared with pcDNA cells (Fig. 10C).

DISCUSSION

Oxidized lipids have been shown to have important biological effects. Biologically active lipids can induce potent inflammatory, chemotactic, and growth-promoting effects in various vascular and mononuclear cells and thus play a major role in the development of atherosclerotic disease. However, much less is known about the specific signaling and molecular mechanisms by which these lipids lead to the expression of key genes involved in the pathology of atherosclerosis. We recently showed that oxidized lipid products of arachidonic acid metabolism via the LO pathway can have growth- and matrix-inducing effects in VSMCs (27). Furthermore, the LO pathway can mediate the growth-promoting effects of AngII as well as the chemotactic effects of PDGF (13, 18, 27). In this study, we report for the first time that 12(S)-HETE can lead to cellular growth and fibronectin transcription as well as mediate the effects of AngII via activation of the Ras-MAPK pathway and CREB transcription factor activation. We also showed that CREB transactivation plays a key role in AngII effects. This study demonstrates that bioactive lipids such as these LO products can directly initiate intracellular signaling and gene expression.

We observed that 12(S)-HETE could lead to the activation of Ras as well as p38 MAPK, but not ERK1/2 or JNK MAPKs. In contrast, the stereoisomer 12(R)-HETE, which is not an LO product, did not have these stimulatory effects, indicating specificity for the LO pathway. Although there is evidence in other cell types for a 12(S)-HETE receptor (37, 38), it is not clear from this study whether the effects of 12(S)-HETE on Ras-MAPK activation are mediated by a specific receptor. Because 12(S)-HETE did not directly induce oxidant stress in these cells, additional unknown mechanisms for MAPK activation may be operative.

* M. A. Reddy, P.-R. Thimmalapura, L. Lanting, J. L. Nadler, S. Fatima, and R. Natarajan, unpublished data.
12(S)-HETE (but not 12(R)-HETE) also induced the phosphorylation and activation of the CREB transcription factor. The p38 MAPK inhibitor SB202190 blocked this effect. 12(S)-HETE also led to increased CREB (but not SP1) DNA binding as assessed by electrophoretic mobility shift assays. AngII could also induce CREB phosphorylation at Ser133. Because CREB is a key target of p38 MAPK (32) and also plays a role in fibroblast transfection (33–36), we were interested in examining whether 12(S)-HETE and AngII could lead to transcriptional activation of CREB using an *in vivo* trans-reporting assay. We found that both AngII as well as 12(S)-HETE led to significant increases in CREB transactivation. Furthermore, AngII-induced CREB transactivation was attenuated by an LO inhibitor, CDC. Moreover, a p38 MAPK inhibitor (but not an ERK pathway inhibitor) significantly blocked both 12(S)-HETE- as well as AngII-induced effects. To determine the functional significance of these observations, we then examined whether 12(S)-HETE could induce fibroblast transcription by performing studies using the luciferase reporter gene under the control of the minimal fibroblast promoter (−510) with three CRE-binding sites in the upstream sequence. We noted that both AngII and 12(S)-HETE could induce expression of the luciferase gene to a similar extent and that a p38 MAPK inhibitor as well as a dominant-negative Ras mutant blocked the effects of both. This study suggests the involvement of the Ras-p38 MAPK-CREB pathway in 12(S)-HETE- and AngII-induced fibroblast transcription. Furthermore, AngII effects may be mediated, at least in part, by the LO pathway.

Further functional evidence was obtained from our results showing that 12(S)-HETE could lead to cellular hypertrophy and that the p38 MAPK inhibitor could block the hypertrophic effects of both AngII and 12(S)-HETE. Recently, Ushio-Fukai et al. (39) showed that oxidant stress and the activation of both the ERK and p38 MAPK pathways might mediate the hypertrophic effects of AngII. Also, based on our *in vitro* data, we tested our hypothesis that 12-LO overexpression should lead to increased MAPK activation and cellular hypertrophy by creating A10 VSMC lines stably overexpressing 12-LO (m12-LO cells). The basal activation levels of both p38 and ERK were increased in these m12-LO cells compared with mock-transfected cells (pcDNA cells). Serum-induced p38 MAPK activation was also greater in m12-LO cells relative to pcDNA cells. Furthermore, m12-LO cells displayed increased \(^{[3]H}\)leucine incorporation relative to pcDNA cells. Taken together, these results demonstrate the key role played by 12-LO activation in leading to MAPK activation and cellular growth in VSMCs, properties that implicate it in the pathology of atherosclerosis and hypertension. Interestingly, although 12(S)-HETE did not directly lead to significant ERK1/2 phosphorylation, the basal levels of phospho-ERK1/2 were greater in m12-LO cells relative to pcDNA cells. This is not entirely surprising because 12-LO overexpression can also lead to the formation of biologically active metabolites other than 12(S)-HETE that may activate ERK1/2. We have also recently shown that these 12-LO-overexpressing cells (m12-LO cells) have a greater rate of nitric oxide consumption than the control cells (pcDNA cells). This suggests that LOs may contribute to vascular dysfunction not only by the bioactivity of their lipid products, but also by serving as a catalytic sink for nitric oxide in the vasculature (40). These studies provide additional support for the important role of vascular and mononuclear 12-LO in vascular disorders.

12(S)-HETE can lead to cellular effects and gene regulation by various mechanisms. HETEs can activate protein kinase C directly or indirectly by incorporating into membrane phospholipids, which then generate HETE-containing diacylglycerol species to activate protein kinase C (41, 42). It is possible that, in this study, protein kinase C activation was also involved upstream of p38 MAPK. Earlier studies have indicated that 12(S)-HETE can activate members of the MAPK pathway in fibroblasts and VSMCs (43–45). However, p38 MAPK activation was not evaluated in those studies. Cardiac fibroblasts overexpressing 12-LO have increased p38 MAPK, which seems to mediate the increased growth in these cells (46). Lipoxigenase products can modulate calcium currents in VSMCs (47) and also induce oxidant stress and inflammatory gene expression (48, 49). In cancer cells, 12(S)-HETE has been shown to induce multiple signaling events through a potential receptor-mediated mechanism (37). Our initial unpublished experiments did not support the presence of specific 12(S)-HETE receptors on VSMCs. Hence, the specific mechanisms by which oxidized lipids such as 12(S)-HETE lead to Ras-MAPK activation and intracellular signaling are not yet clear. Novel membrane perturbation mechanisms may be involved, and these will be the focus of future studies.

The regulation of pathological gene expression by lipids such as 12(S)-HETE could be relevant to cardiovascular and diabetic complications. Increased levels of 12(S)-HETE have been observed in hypertensive and diabetic patients (50, 51). The LO pathway has been implicated in the oxidation of low density lipoprotein to its atherogenic form and in the pathogenesis of atherosclerosis (52–54). Overexpression of 15-LO in fibroblasts can lead to augmented low density lipoprotein oxidation (55). We recently demonstrated increased 12-LO expression in a swine model of diabetes-induced atherosclerosis under hyperlipemic and hyperglycemic states (12). Furthermore, 12-LO expression was increased in a rat carotid artery model of balloon injury and restenosis (16). In this model, neointimal thickening could be significantly attenuated by a novel ribozyme directed to rat leukocyte-type 12-LO. This ribozyme was also effective in reducing PDGF-induced migration and fibronectin expression in rat VSMCs (18). Adenoviral vector-mediated delivery of a ribozyme to PVSMS is similarly effective in PVSMS and also attenuates high glucose-induced adhesion of monocytes to endothelial cells (19). These results, coupled with the recent observations that crossingbreeding 12-LO-deficient mice with apoE-deficient mice greatly reduces the rates of atherosclerosis (20, 21), underscore the key role played by LO products such as 12(S)-HETE in the development of cardiovascular complications. Hence, therapeutic modalities and novel ribozyme technology to block 12-LO and the formation of its products such as 12(S)-HETE may be beneficial for diabetic and vascular diseases.

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