Involvement of cAMP-response element binding protein-1 in arachidonic acid-induced vascular smooth muscle cell motility

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Abstract **In addition to their role in many vital cellular functions, arachidonic acid (AA) and its eicosanoid metabolites are involved in the pathogenesis of several diseases, including atherosclerosis and cancer. To understand the potential mechanisms by which these lipid molecules could influence the disease processes, particularly cardiovascular diseases, we studied AA's effects on vascular smooth muscle cell (VSMC) motility and the role of cAMP-response element binding protein-1 (CREB-1) in this process. AA exerted differential effects on VSMC motility; at lower doses, it stimulated motility, whereas at higher doses, it was inhibitory. AA-induced VSMC motility requires its conversion via the lipoxygenase (LOX) and cyclooxygenase (COX) pathways. AA stimulated the phosphorylation of extracellular signal-regulated kinases (ERKs), Jun N-terminal kinases (JNKs), and p38 mitogen-activated protein kinase (p38MAPK) in a time-dependent manner, and blockade of these serine/threonine kinases significantly attenuated AA-induced VSMC motility. In addition, AA stimulated CREB-1 phosphorylation and activity in a manner that was also dependent on its metabolic conversion via the LOX and COX pathways and the activation of ERKs and p38MAPK but not JNKs. Furthermore, suppression of CREB-1 activation inhibited AA-induced VSMC motility. 15(*S*)-Hydroxyeicosatetraenoic acid and prostaglandin $F_{2\alpha}$, the 15-LOX and COX metabolites of AA, respectively, that are produced by VSMC at lower doses, were also found to stimulate motility in these cells. Together, these results suggest that AA induces VSMC motility by complex mechanisms involving its metabolism via the LOX and COX pathways as well as the ERK- and p38MAPK-dependent and JNK-independent activation of CREB-1.**—Dronadula, N., F. Rizvi, E. Blaskova, Q. Li, and G. N. Rao. **Involvement of cAMP-response element binding protein-1 in arachidonic acid-induced vascular smooth muscle cell motility.** ***J. Lipid Res.*** **2006.** 47: **767–777.**

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All phospholipase A_2s catalyze the hydrolysis of the *sn*-2 fatty acyl ester bonds of phospholipids, generating a free fatty acid and a lysophospholipid (1, 2). Therefore, phospholipase A_2s play a rate-limiting role in the release of arachidonic acid (AA) from cellular phospholipids (1, 2). Once released, it is reincorporated into membrane phospholipids via esterification involving arachidonoyl-CoA synthase and arachidonoyl-lysophospholipid transferase or converted to eicosanoids by three major enzymatic pathways: cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 (CYP) epoxygenases (3, 4). AA metabolism via the COX, LOX, and CYP epoxygenase pathways produces prostaglandins, hydroperoxyeicosatetraenoic acids, and epoxyeicosatrienoic acids, respectively (3, 4). AA and its eicosanoid metabolites that were known to be involved in the regulation of vascular tone (4–6) have also been reported to play a role in many other cellular processes, such as angiogenesis, cell survival, chemotaxis, mitogenesis, and apoptosis (7–14). In addition, a role for phospholipase A_2s, AA, and its eicosanoid metabolites has been implicated in the pathogenesis of various inflammatory diseases, including atherosclerosis, cancer, and rheumatoid arthritis (4, 15–17). Despite the involvement of these lipid molecules in the regulation of cell growth and motility and thereby in the diseases mentioned above (4, 7–18), the underlying signaling mechanisms are still poorly understood.

cAMP-response element binding protein-1 (CREB-1) belongs to the basic leucine-zipper family of transcriptional factors that were shown to play an important role in gene regulation, particularly in response to cAMP (19, 20). In fact, a large body of data indicates that CREB-1 is in-

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; CREB-1, cAMP-response element binding protein-1; CYP, cytochrome P450; ERK, extracellular signal-regulated kinase; ETI, 5,8,11-eicosatriynoic acid; HETE, hydroxyeicosatetraenoic acid; JNK, Jun N-terminal kinase; LOX, lipoxygenase; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; p38MAPK, p38 mitogen-activated protein kinase; VSMC, vascular smooth muscle cell.

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volved in the regulation of a plethora of cellular functions, including cell survival, circadian rhythm, and neuronal plasticity (21–24). Its activity appears to be dependent on the phosphorylation of serine-133, which is catalyzed by various groups of serine/threonine kinases, such as calcium/calmodulin-dependent protein kinases, mitogen-activated protein kinases (MAPKs), protein kinase A, protein kinase G, and pp90 ribosomal S6 kinase (25–29). In addition, it is activated in response to a broad spectrum of stimulants in different cell types (19, 24). Vascular smooth muscle cell (VSMC) migration and proliferation are major contributing factors in the pathogenesis of vascular inflammatory diseases such as atherosclerosis and restenosis (18). Studies from several laboratories, including ours, have revealed the involvement of the ATF/CREB family of transcriptional factors in both the positive and negative regulation of VSMC growth and motility (30–36). To determine whether AA is a chemoattractant for VSMC and, if it is, what the underlying mechanisms are, we studied the effects of AA and the role of CREB-1 in VSMC motility. In this study, we show for the first time that AA induces VSMC motility by different mechanisms involving its metabolism via the LOX and/or COX pathways as well as the extracellular signal-regulated kinase (ERK)- and p38MAPK-dependent and Jun N-terminal kinase (JNK)-independent activation of CREB-1.

MATERIALS AND METHODS

Reagents

Aprotinin, indomethacin, PMSF, sodium orthovanadate, sodium deoxycholate, leupeptin, HEPES, and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO). AA, 15(*S*)-HETE, and $PGF_{2\alpha}$ were obtained from Cayman Chemical Co. (Ann Arbor, MI). 5,8,11-Eicosatriynoic acid (ETI), ketoconazole, and SKF525A were from Biomol (Plymouth Meeting, PA). Anti-ATF-1 (SC-270) mouse monoclonal antibody, anti-ATF-2 (SC-187X) rabbit polyclonal antibody, and anti-CREB-1 (SC-271X) mouse monoclonal antibody as well as the consensus CREB oligonucleotide, 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′ (SC-2504), were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-pCREB (9191S), anti-pERK1/2 (9181S), anti-pJNK1/2 (9251S), anti-pp38MAPK (9211S), and anti-CREB (9192S) antibodies were from Cell Signaling (Beverly, MA). CRE-luc plasmid was obtained from Stratagene (La Jolla, CA). The luciferase assay kit and T4 polynucleotide kinase were procured from Promega (Madison, WI). FuGENE 6 transfection reagent was obtained from Roche Molecular Biochemicals (Indianapolis, IN). [γ-^{32}P]ATP (3,000 Ci/mmol) was supplied by GE Healthcare (Piscataway, NJ).

Cell culture

VSMCs were isolated from the thoracic aortae of 100–150 g male Sprague-Dawley rats by enzymatic dissociation as described previously (37). Cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a humidified 95% air and 5% CO_2 atmosphere. Cells were quiesced by incubating in serum-free DMEM for 72 h and used to perform the experiments unless stated otherwise.

Cell motility

Cell migration was performed using a modified Boyden chamber method. The cell culture inserts containing membranes of 10 mm in diameter and 8.0 μm pore size (Nalge Nunc International, Rochester, NY) were placed on a 24-well tissue culture plate (Costar, Corning, Inc.). The lower surface of the porous membrane was coated with 0.1% gelatin and left at 4°C overnight. The membrane was then incubated with 0.1% heat-inactivated BSA for 1 h at 37°C. VSMCs were quiesced for 72 h in serum-free DMEM, washed with PBS, trypsinized, and pelleted by centrifugation. Cells were resuspended in serum-free DMEM and seeded into the upper chamber at 1×10^5 cells/well. Vehicle or AA was added to the lower chamber at the indicated concentrations. Both the upper and lower chambers contained serum-free DMEM. After 5 h 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 min. The membrane was then stained with Giemsa-Wright stain for 10 min (Sigma Chemical Co.) and washed once each with 50% and 100% alcohol. Cells were counted in five randomly selected squares per well with a light microscope (model Eclipse 50i; Nikon, Japan) and are presented as number of migrated cells per field.

Electrophoretic mobility shift assay

After appropriate treatments, nuclear extracts were prepared from VSMCs as described previously (37). Protein-DNA complexes were formed by incubating 5 μg of nuclear protein in a total volume of 20 μl consisting of 15 mM HEPES, pH 7.9, 3 mM Tris-HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM PMSF, 1 mM dithiothreitol, 4.5 μg of BSA, 2 μg of poly(dI-dC), 15% glycerol, and 100,000 cpm of ^{32}P-labeled consensus CRE oligonucleotide probe for 30 min on ice. Protein-DNA complexes were resolved on a 4% polyacrylamide gel using 1× Tris-glycine-EDTA buffer (25 mM Tris-HCl, pH 8.5, 200 mM glycine, and 0.1 mM EDTA). Double-stranded oligonucleotides (CREB, 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′) were labeled with [γ-^{32}P]ATP using the T4 polynucleotide kinase kit as described in the supplier's protocol (Promega). To test the effect of KCREB on AA-induced CREB-DNA binding activity, cells were first transfected with an empty vector or KCREB plasmid DNA for 12 h in antibiotic and serum-free medium and quiesced for 48 h before they were subjected to treatments with AA, followed by nuclear extract preparation.

Reporter gene assay

VSMCs were plated evenly onto 60 mm dishes and grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. At ~80% confluence, medium was replaced with antibiotic, and serum-free medium and cells were transfected with 3 μg/dish CRE-luc plasmid (30) using FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche Molecular Biochemicals). Twelve hours after transfection, cells were quiesced for 48 h. Cells were then treated with and without AA (5 μM) in the presence and absence of the indicated pharmacological inhibitors for 6 h, and cell extracts were prepared. The cell extracts normalized for protein were assayed for luciferase activity using the Luciferase Assay System (Promega) and a Turner Luminometer (TD-20/20). To test the effect of KCREB on AA-induced CRE-luc activity, cells were cotransfected with CRE-luc plasmid DNA along with an empty vector or KCREB plasmid DNA and quiesced before they were subjected to treatments with AA, followed by measurement of the luciferase activity.

Western blot analysis

After appropriate treatments, VSMCs were rinsed with cold PBS and lysed in 250 μl of lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 100 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM sodium orthovanadate) on ice for 20 min. The cell lysates were scraped into 1.5 ml Eppendorf tubes and cleared by centrifugation at 12,000 rpm for 20 min at 4°C. Cell lysates containing equal amounts 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 Biosciences, Piscataway, NJ). After blocking in 10 mM Tris-HCl buffer, pH 8.0, containing 150 mM 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 HRP-conjugated secondary antibodies. The antigen-antibody complexes were detected using a chemiluminescence reageant kit (Amersham Biosciences).

Statistics

All experiments were repeated three times, and the data are presented as means ± SEM. The treatment effects were analyzed by Student's *t*-test.

RESULTS

Although a large body of data suggests that AA and its COX, LOX, and CYP epoxygenase metabolites are involved in various types of inflammatory diseases, such as atherosclerosis, cancer, and rheumatoid arthritis (4, 7–17), their mechanisms of action are less clear. To understand how AA, the precursor for eicosanoids, influences the pathogenesis of some of these diseases, we determined its effects on cell motility using a modified Boyden chamber method. As shown in **Fig. 1A**, AA exerted differential effects on VSMC motility; at lower doses, it stimulated motility, whereas at higher doses, it was inhibitory. Specifically, at 5 μM, AA increased VSMC motility by 3-fold compared with control, and at 40 μM, it was found to be inhibitory. Therefore, all experiments on VSMC motility described herein were performed using AA at 5 μM. To learn whether its metabolic conversion is required for the stimulation of VSMC motility, we next tested the effects of ETI and indomethacin, potent and specific inhibitors of the LOX and COX pathways, respectively (38, 39). ETI (25 μM) and indomethacin (20 μM) completely blocked AA-induced VSMC motility (Fig. 1B). Earlier studies have shown that the CYP epoxygenase products of AA, such as 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids, inhibit platelet-derived growth factor-BB-induced and serum-induced VSMC motility (40). To understand whether AA-induced VSMC motility requires its conversion via the CYP epoxygenases, we next studied the effects of SKF525A and ketoconazole, two potent and specific inhibitors of this enzymatic pathway (40, 41). Neither SKF525A (100 μM) nor ketoconazole (20 μM) inhibited AA-induced VSMC motility (Fig. 1C). These results indicate that AA metabolism via the LOX and COX pathways but not the CYP epoxygenase pathway is required for its effects on VSMC motility.

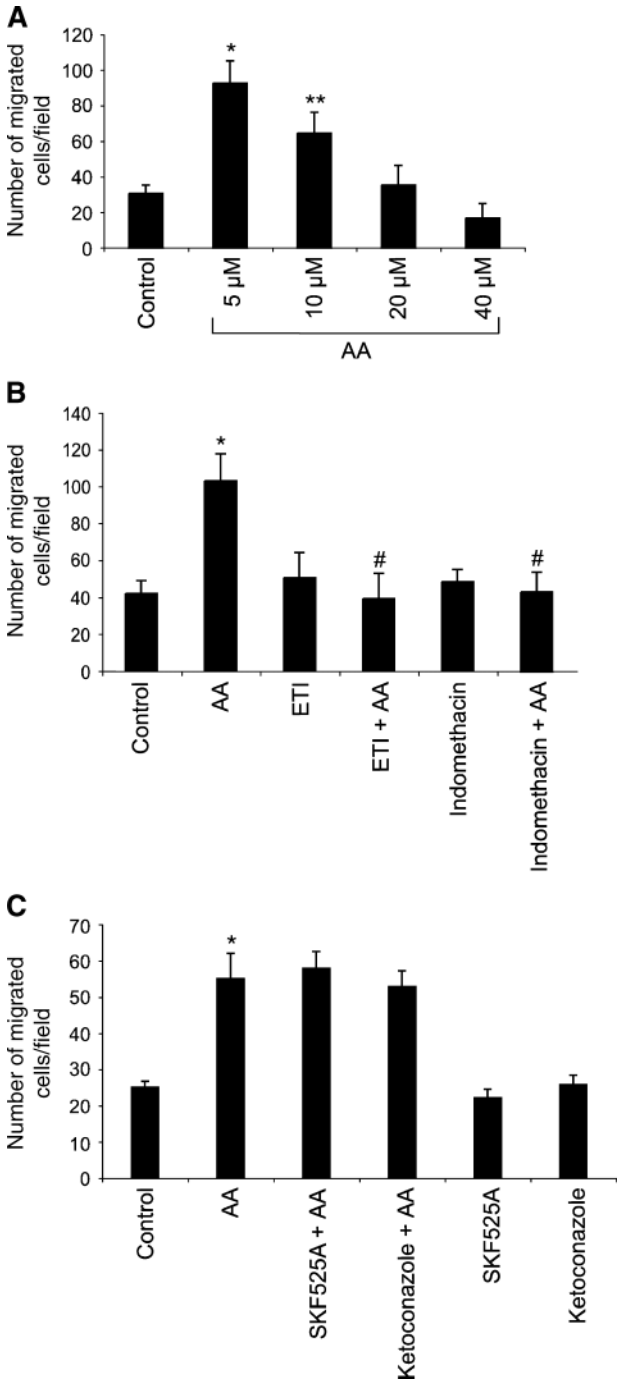

Fig. 1. Arachidonic acid (AA) alone, and by its conversion via the lipoxygenase (LOX) and/or cyclooxygenase (COX) pathways but not the cytochrome P450 (CYP) epoxygenase pathway, stimulates vascular smooth muscle cell (VSMC) motility. A: Quiescent VSMCs were placed in the upper chamber of the transwell at 1×10^5 cells/well. Vehicle or AA at the indicated concentrations was added to the lower chamber of the transwell, and after 5 h of incubation at 37°C, cells that migrated to the lower side of the membrane were fixed in methanol, stained with Giemsa-Wright stain, washed with alcohol, and counted by light microscopy. B, C: Conditions were as described for A except that AA (5 μM) was added to the lower chamber with and without ETI (25 μM), indomethacin (20 μM), ketoconazole (20 μM), or SKF525A (100 μM), and cell motility was measured as described. Data are presented as means ± SEM. * $P < 0.001$ versus control; ** $P < 0.01$ versus control; # $P < 0.01$ versus AA alone. ETI, 5,8,11-eicosatriynoic acid.

We reported previously that AA activates ERKs and JNKs in VSMCs (42, 43). To understand the signaling mechanisms underlying AA-induced VSMC motility, we next tested the role of the three major groups of MAPKs: ERKs, JNKs, and p38MAPK. Quiescent VSMCs were treated with and without AA (5 μM) for the indicated times, and cell extracts were prepared. An equal amount of protein from control and each treatment was analyzed by Western blotting for the phosphorylation of ERKs, JNKs, and p38MAPK using their phosphospecific antibodies. AA stimulated the phosphorylation of ERKs, JNKs, and p38MAPK in a time-dependent manner, with near maximum effects of activation for the former two at 10 min and for the latter one at 1 min (**Fig. 2A**). We next tested the effect of ETI and indomethacin on AA-induced MAPK activation. Quiescent VSMCs were treated with and without AA (5 μM) in the presence or absence of ETI (25 μM) or indomethacin (20 μM) for 10 min, and cell extracts were prepared and analyzed by Western blotting for the phosphorylation of ERKs, JNKs, and p38MAPK as described above. ETI, while having no effect on p38MAPK phosphorylation, blocked the AA-induced phosphorylation of ERKs and JNKs by 30–40% (Fig. 2B). Likewise, indomethacin, while having no effect on p38MAPK phosphorylation, attenuated the AA-induced phosphorylation of ERKs and JNKs (Fig. 2B). However, indomethacin at lower doses was found to have no effect on AA-induced ERK and JNK activation in our previous studies (42, 43). These results suggest that although AA alone can activate p38MAPK, its metabolism via the LOX and/or COX pathways appears to be needed at least partially for its effects on the phosphorylation of ERKs and JNKs. To test the role of MAPKs in AA-induced VSMC motility, we next studied the effects of PD98059 (50 μM), SB203580 (10 μM), and SP600125 (10 μM), potent and

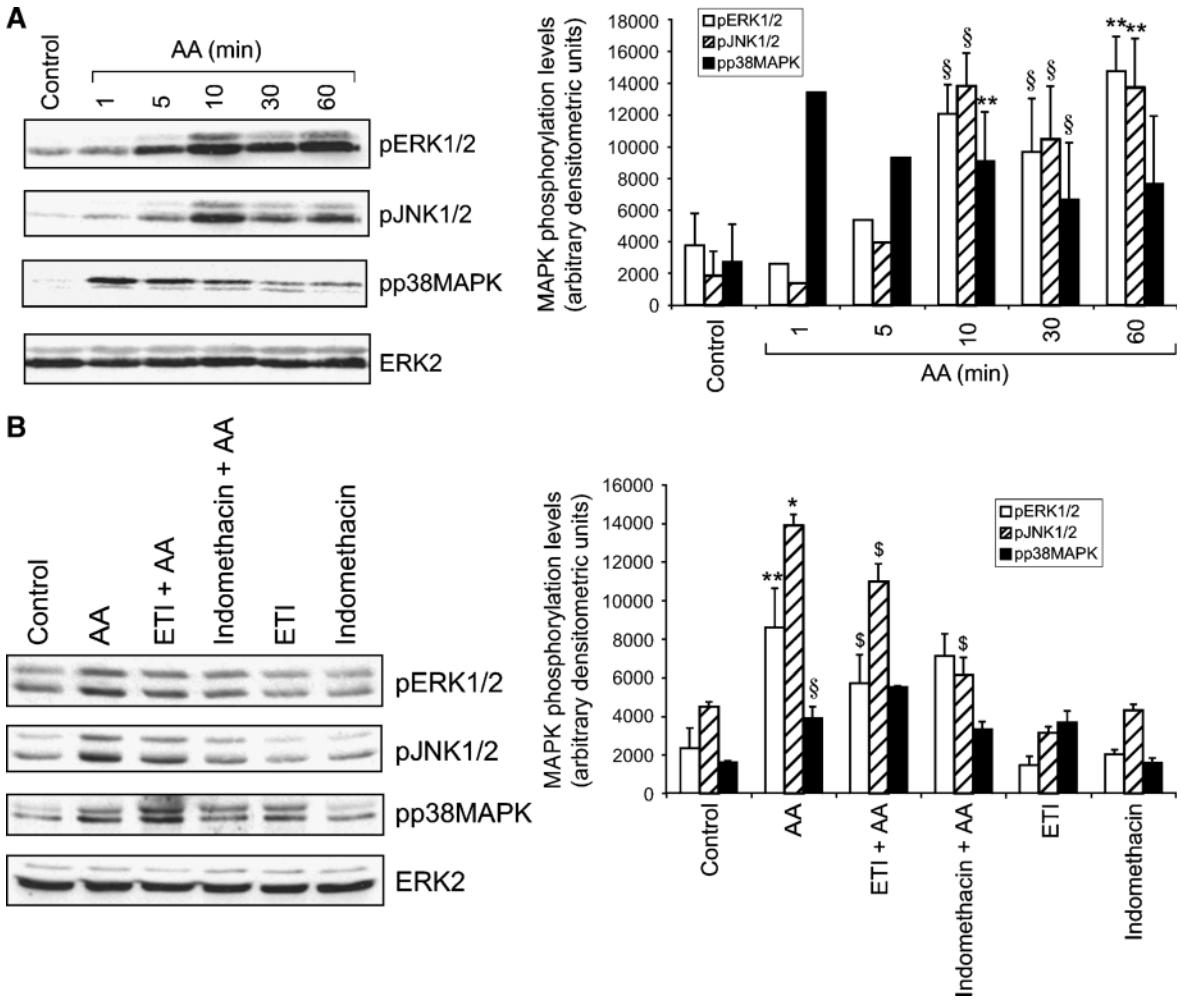

Fig. 2. AA alone, and by its conversion via the LOX and/or COX pathways, activates extracellular signal-regulated kinases (ERKs), Jun N-terminal kinases (JNKs), and p38 mitogen-activated protein kinase (p38MAPK) in VSMCs. A: Quiescent VSMCs were treated with and without AA (5 μM) for the indicated times, and cell extracts were prepared. An equal amount of protein from control and each treatment was analyzed by Western blotting for the phosphorylation of ERKs, JNKs, and p38MAPK using their phosphospecific antibodies. B: Conditions were as described for A except that cells were treated with and without AA (5 μM) in the presence or absence of ETI (25 μM) or indomethacin (20 μM) for 10 min, and cell extracts were prepared and analyzed for the phosphorylation of ERKs, JNKs, and p38MAPK using their phosphospecific antibodies. For lane loading controls, the same blots were reprobed with total anti-ERK2 antibodies. The bar graphs represent the quantitative analysis of three independent experiments. Data are presented as means ± SEM. * $P < 0.001$ versus control; ** $P < 0.01$ versus control; § $P < 0.05$ versus control; $^{\$}$ $P < 0.05$ versus AA alone.

specific inhibitors of MAP/ERK kinase 1/2 (MEK 1/2), p38MAPK, and JNKs, respectively (44–46). All three inhibitors attenuated VSMC motility by >60% (**Fig. 3**). These findings clearly suggest that AA-induced VSMC motility requires the activation of all three major groups of MAPKs.

One recent study reported that CREB-1 plays a role in tumor necrosis factor-α-induced VSMC motility (34). In addition, other studies have reported that MAPKs, particularly ERKs, JNKs, and p38MAPK, via phosphorylating serine-133, modulate CREB-1 activity (26, 27, 29). Therefore, to address whether AA-stimulated MAPKs target CREB-1 to modulate VSMC motility, a time-course effect of this fatty acid on CREB-1 phosphorylation was determined. AA (5 μM) stimulated CREB-1 phosphorylation on serine-133 in a time-dependent manner (**Fig. 4A**). A maximum 3-fold increase in the phosphorylation of CREB-1 occurred at 10 min, and these increases were sustained at least until 1 h of AA treatment. To determine whether AA-induced CREB-1 phosphorylation requires its conversion via the LOX and/or COX pathways, we next tested the effect of ETI and indomethacin. ETI (25 μM) and indomethacin (20 μM) suppressed AA-induced CREB-1 phosphorylation by 30% and 40%, respectively (Fig. 4B). This finding suggests that AA alone and by its conversion via the LOX and/or COX pathways stimulates CREB-1 phosphorylation in VSMCs. To test the role of MAPKs in AA-induced CREB-1 phosphorylation, we next determined the effects of PD98059, SB203580, and SP600125. As shown in Fig. 4C, PD98059 and SB203580 but not SP600125 significantly inhibited AA-induced CREB-1 phosphorylation, a finding that suggests that AA induces CREB-1 phosphorylation via the involvement of ERKs and p38MAPK.

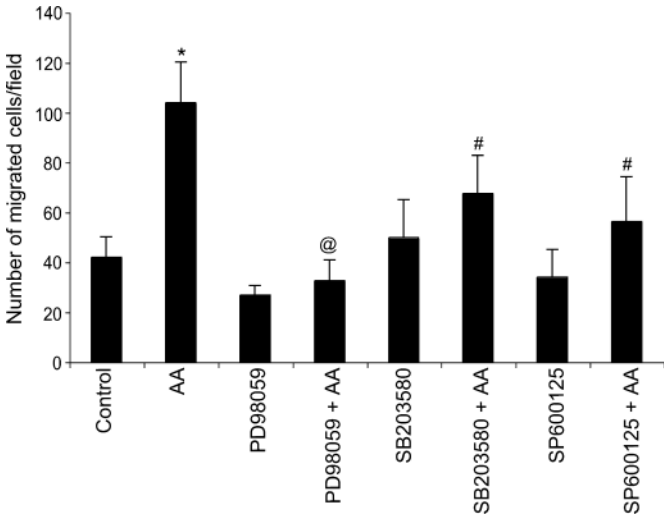

Fig. 3. AA-induced VSMC motility requires the activation of ERKs, JNKs, and p38MAPK. Quiescent VSMCs were placed in the upper chamber of the transwell at 1×10^5 cells/well. Vehicle or AA (5 μM) was added to the lower chamber of the transwell with and without PD98059 (50 μM), SB203580 (10 μM), or SP600125 (10 μM), and after 5 h of incubation at 37°C, cells that migrated to the lower side of the membrane were fixed in methanol, stained with Giemsa-Wright stain, washed with alcohol, and counted by light microscopy. Data are presented as means ± SEM. * $P < 0.001$ versus control; @ $P < 0.001$ versus AA treatment alone; # $P < 0.01$ versus AA treatment alone.

To determine whether AA-induced CREB-1 phosphorylation leads to an increase in its DNA binding activity, quiescent VSMCs were treated with and without AA (5 μM) for the indicated times, and nuclear extracts were prepared. An equal amount of nuclear protein was analyzed for CRE DNA binding activity using ^{32}P-labeled consensus CRE oligonucleotide as a probe. As shown in **Fig. 5A**, AA induced protein-CRE DNA binding activity, as revealed by two discrete bands, in a concentration- and time-dependent manner, with a near maximum effect at 1 h of treatment by a dose of 5 μM. To identify whether AA-induced protein-CRE DNA complexes contain CREB-1, supershift electrophoretic mobility shift assay was performed. Anti-CREB-1 antibodies caused the supershift of both of the bands, resulting in the S2 complex, whereas anti-ATF-1 antibodies led to the disappearance of the lower band and thereby caused the formation of the S1 complex that apparently comigrated with the upper band (Fig. 5B). On the other hand, anti-ATF-2 antibodies supershifted the upper band and caused the formation of the S3 complex. These results clearly show that AA-induced protein-CRE DNA complexes contain CREB-1, ATF-1, and ATF-2 and that the former forms heterodimers with the latter two molecules (Fig. 5B). To confirm these results further, quiescent VSMCs that were transfected with an empty vector or a dominant negative CREB-1 (KCREB) (30) plasmid DNA were treated with and without AA (5 μM), and nuclear extracts were prepared and analyzed for protein-CRE DNA binding activity. Forced expression of KCREB inhibited AA-induced protein-CRE DNA binding activity, suggesting that these complexes contain CREB-1 (Fig. 5C). Consistent with their effects on CREB-1 phosphorylation, ETI, indomethacin, PD98059, and SB203580 also blocked AA-induced protein-CRE DNA binding activity (Fig. 5D, E). SP600125 had little or no effect on protein-CRE DNA binding activity induced by AA.

To confirm the role of LOX, COX, ERKs, JNKs, and p38MAPK on the AA activation of CREB-1, VSMCs were transfected first with a reporter gene (CRE-luc) plasmid DNA in which luciferase transcription is driven by the CRE motif, quiesced, and treated with and without AA (5 μM) in the presence or absence of ETI (25 μM), indomethacin (20 μM), PD98059 (50 μM), SB203580 (10 μM), or SP600125 (10 μM) for 6 h, and luciferase activity was measured. As expected with their effects on protein-CRE DNA binding activity, ETI, indomethacin, PD98059, and SB203580 suppressed AA-induced CRE-dependent luciferase activity as well (**Fig. 6A, B**). Consistent with its lack of effect on protein-CRE DNA binding activity, SP600125 also failed to suppress CRE-dependent luciferase activity induced by AA (Fig. 6B). To obtain additional evidence for the ability of AA to stimulate CREB-1 transcriptional transactivation activity, VSMCs were cotransfected with CRE-luc plasmid DNA with and without an empty vector or KCREB plasmid DNA, quiesced, and treated with and without AA (5 μM) for 6 h, and luciferase activity was determined. As shown in Fig. 6C, KCREB blocked both basal

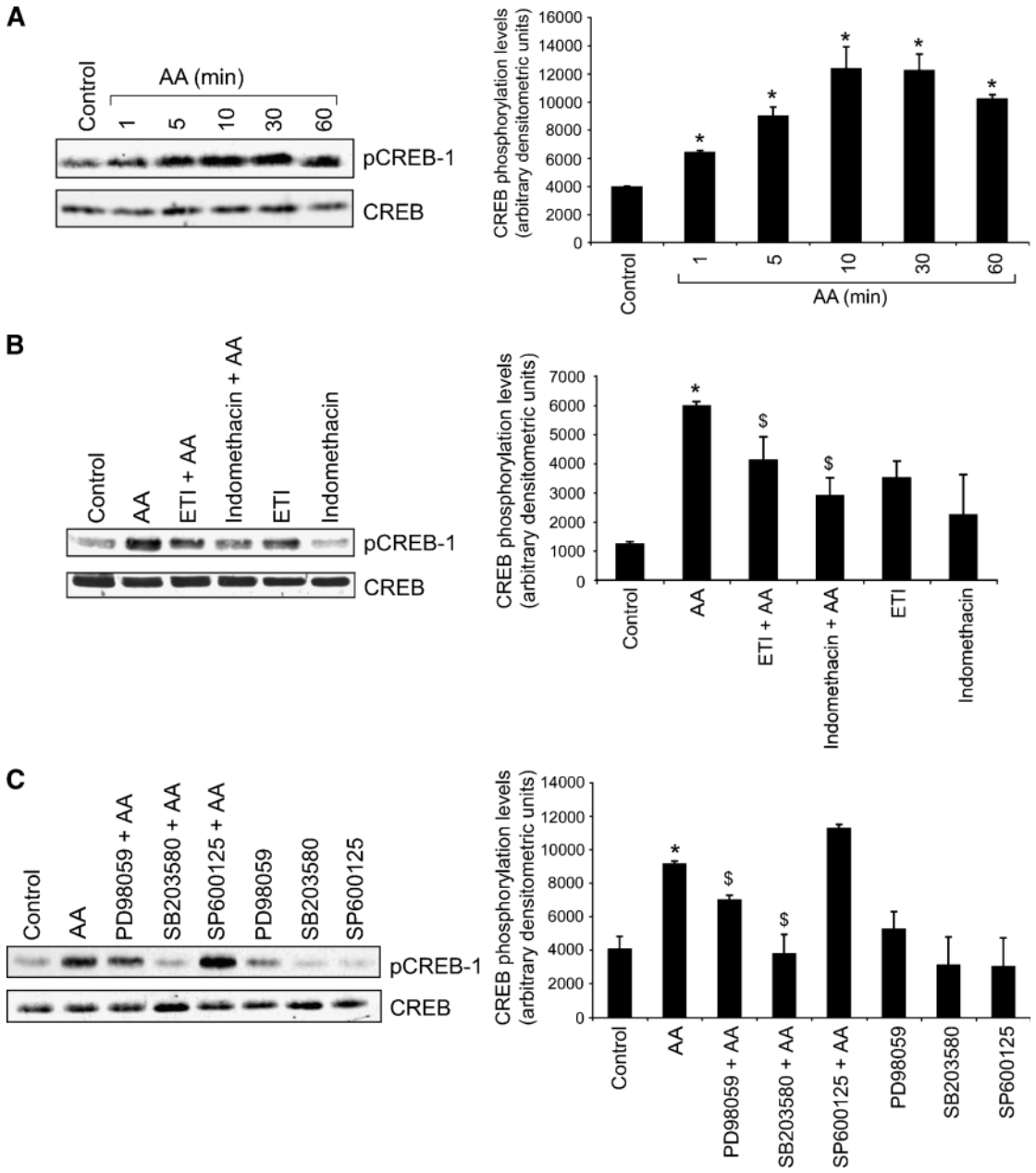

Fig. 4. AA stimulates the phosphorylation of CREB-1 in a LOX-, COX-, ERK-, and p38MAPK-dependent and JNK-independent manner in VSMCs. Quiescent VSMCs were treated with and without AA (5 μM) for the indicated times (A) or for 10 min in the presence or absence of ETI (25 μM), indomethacin (20 μM), PD98059 (50 μM), SB203580 (10 μM), or SP600125 (10 μM) (B, C), and cell extracts were prepared. An equal amount of protein from control and each treatment was analyzed by Western blotting for pCREB-1 using its phosphospecific antibodies. For lane loading controls, the blots were reprobed with total anti-CREB-1 antibodies. The bar graphs represent the quantitative analysis of three independent experiments. Data are presented as means ± SEM. * $P < 0.001$ versus control; $^{\$}$ $P < 0.05$ versus AA alone.

and AA-induced luciferase activity. To test the role of CREB-1 in AA-induced VSMC motility, we next studied the effect of KCREB. Forced expression of KCREB substantially blocked AA-induced VSMC motility (**Fig. 7**).

Previously, we have shown that VSMCs upon exposure to AA for 5 min produce 15(*S*)-hydroxyeicosatetraenoic acid (15(*S*)-HETE) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) as the major eicosanoids (42). Because AA-induced VSMC motility is sensitive to inhibition of the LOX and COX pathways, we were intrigued to learn whether 15(*S*)-HETE and $PGF_{2\alpha}$ are the mediators of VSMC motility induced by this fatty acid. To address this, we studied the effects of 15(*S*)-HETE and $PGF_{2\alpha}$ on VSMC motility. Both 15(*S*)-HETE and $PGF_{2\alpha}$ stimulated VSMC motility (**Fig. 8A, B**). At lower doses, they both were found to be more efficacious in stimulating VSMC motility than at higher concentrations.

DISCUSSION

The important finding of this study is that AA alone, and by its conversion via the LOX and/or COX pathways and

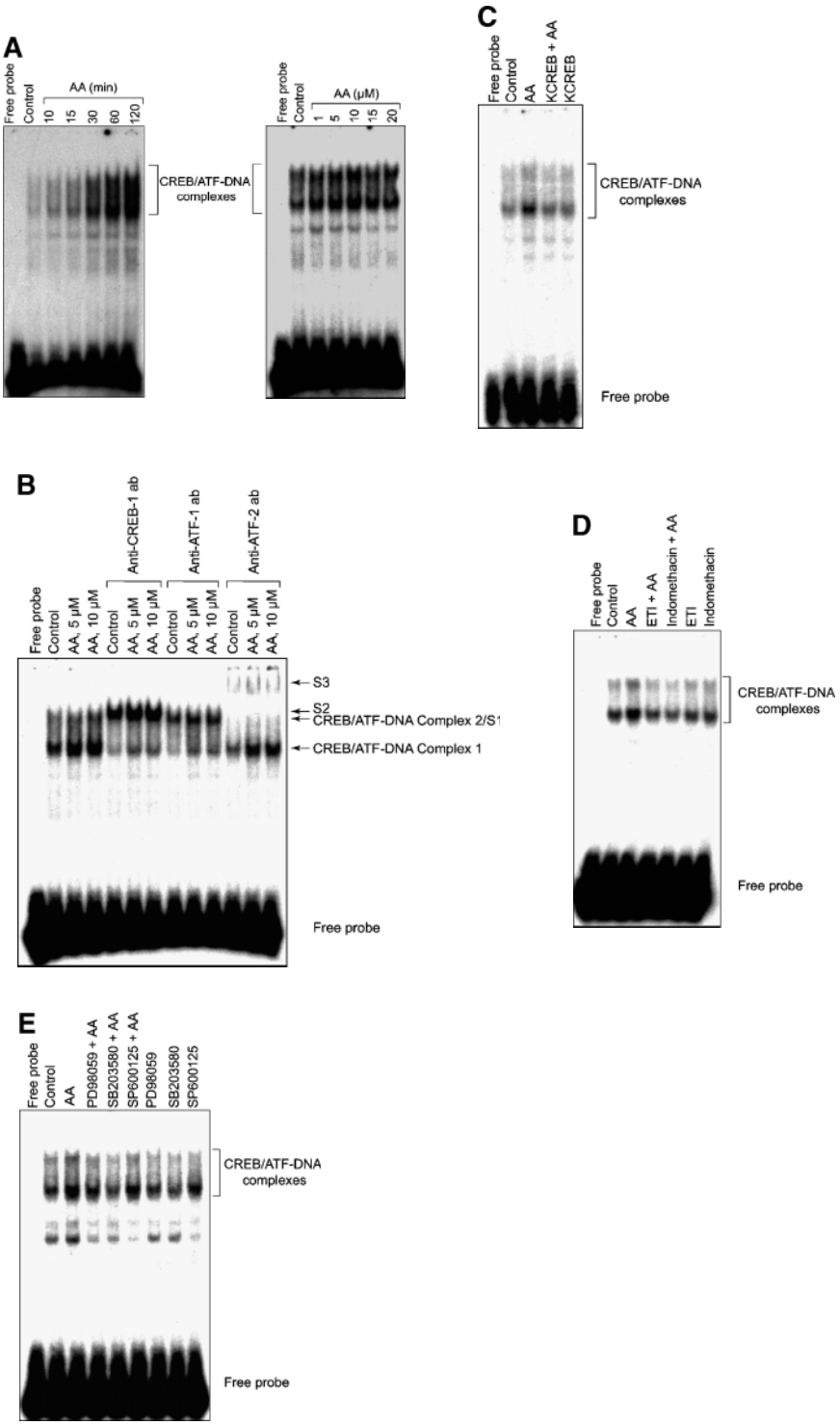

Fig. 5. AA stimulates protein-CRE DNA binding activity in VSMCs in a LOX-, COX-, ERK-, and p38MAPK-dependent and JNK-independent manner. A: Quiescent VSMCs were treated with and without 5 μM AA for the indicated times (left panel) or with and without the indicated doses of AA for 1 h (right panel), and nuclear extracts were prepared. Nuclear extracts containing an equal amount of protein from control and each treatment were analyzed for protein-CRE DNA binding activity using ^{32}P-labeled consensus CRE oligonucleotide as a probe. B: Conditions were as described for A (right panel) except that anti-CREB-1, anti-ATF-1, or anti-ATF-2 antibody was added to the protein-DNA reaction mix 20 min after the start of the reaction and incubated on ice for 3 h before it was analyzed by PAGE. C: VSMCs were transfected first with an empty vector or KCREB plasmid DNA and quiesced before they were subjected to treatment with and without AA (5 μM) for 1 h and analysis for protein-CRE DNA binding activity. D, E: Quiescent VSMCs were treated with and without AA (5 μM) in the presence or absence of ETI (25 μM), indomethacin (20 μM), PD98059 (50 μM), SB203580 (10 μM), or SP600125 (10 μM) for 1 h, and nuclear extracts were prepared and analyzed for protein-CRE DNA binding activity. S1, S2, and S3 represent supershifts 1, 2, and 3, respectively.

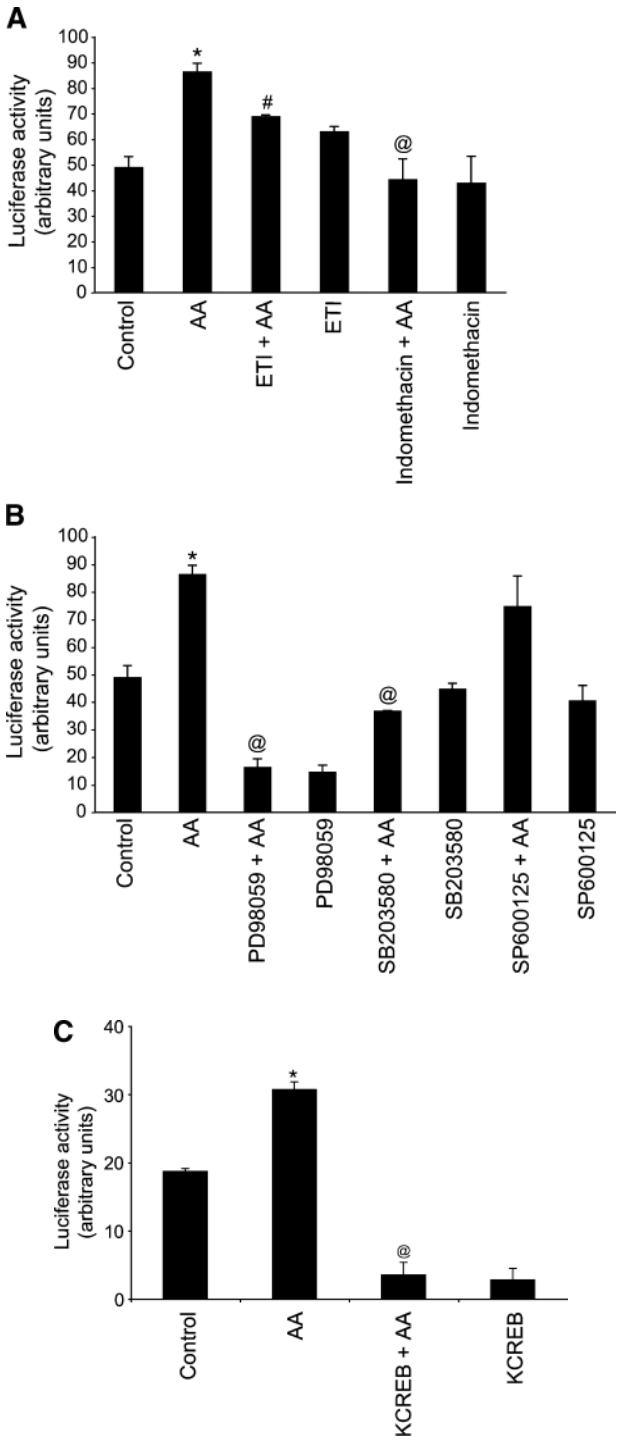

Fig. 6. AA stimulates CRE-dependent luciferase reporter gene activity in VSMCs in a LOX-, COX-, ERK-, and p38MAPK-dependent and JNK-independent manner. A, B: Quiescent VSMCs that were transfected with CRE-luc plasmid DNA and quiesced were treated with and without AA (5 μM) in the presence or absence of ETI (25 μM), indomethacin (20 μM), PD98059 (50 μM), SB203580 (10 μM), or SP600125 (10 μM) for 6 h, and cell extracts were prepared. Cell extracts containing an equal amount of protein from control and each treatment were assayed for luciferase activity. C: Cells were cotransfected with CRE-luc along with an empty vector or KCREB plasmid DNA and quiesced before they were subjected to the indicated treatment for 6 h and analysis for CRE-luciferase activity. Data are presented as means ± SEM. * $P < 0.001$ versus control; @ $P < 0.001$ versus AA treatment alone; # $P < 0.01$ versus AA treatment alone.

through ERK- and p38MAPK-dependent CREB-1 activation, induces VSMC motility. Besides this, VSMC motility induced by AA alone or by its conversion via the LOX and/or COX pathways exhibits a requirement for the activation of JNKs. The following observations support these conclusions. *1*) AA at lower doses increases VSMC motility, whereas at higher concentrations it was found to be inhibitory. *2*) AA-induced VSMC motility was dependent at least partially on its conversion via the LOX and/or COX pathways and the activation of ERKs, JNKs, and p38MAPK. *3*) AA stimulated CREB-1 transcriptional transactivation activity in a manner that is also dependent on its conversion via the LOX and/or COX pathways and activation of ERKs and p38MAPK. *4*) Dominant negative mutant-mediated suppression of CREB-1 inhibited AA-induced VSMC motility. *5*) 15(*S*)-HETE and $PGF_{2\alpha}$, the 15-LOX and COX metabolites of AA that were produced by VSMCs, stimulated the motility of these cells. It was noted that inhibition of either LOX or COX pathways completely suppresses AA-induced VSMC motility. This finding indicates cross-talk between the LOX and COX pathways in the modulation of VSMC motility. Although a large body of data suggests that AA and its COX, LOX, and CYP epoxygenase metabolites play a role in the regulation of growth and motility in various cell types, including VSMCs, the underlying mechanisms were not well defined (7–17). Based on our previous (42, 43) and present findings, it is clear that AA activates all three major groups of MAPKs in VSMCs. Although the activation of ERKs and JNKs by AA requires its conversion, at least partially, via the LOX and/or COX pathways, its stimulation of p38MAPK appears to occur independent of its metabolism to eicosanoids. However, because the inhibition of ERKs, JNKs, and p38MAPK substantially reduced AA-induced VSMC motility, it is likely that AA with and without its conversion via the LOX and/or COX pathways induces VSMC motility. Many studies have also reported a role for ERKs, JNKs, and p38MAPK in the regulation of cell motility in different cell types in response to various chemoattractants (47–51).

CREB-1 plays an important role in gene regulation, and its activity appears to be dependent on the phosphorylation of serine-133 (19, 24). Several types of serine/threonine kinases, including calcium/calmodulin-dependent protein kinases, MAPKs, protein kinase A, protein kinase G, and pp90 ribosomal S6 kinase, have been reported to phosphorylate CREB-1 on serine-133 (25–29). The findings that AA-induced CREB-1 serine-133 phosphorylation, DNA binding, and reporter gene activities were sensitive to inhibition by PD98059 and SB203580 suggest that both ERKs and p38MAPK mediate AA's effects on CREB-1 activation. Furthermore, the fact that the inhibition of the LOX and/or COX pathways attenuates AA-induced phosphorylation of only ERKs but not p38MAPK suggests that AA alone, and by its metabolism via the LOX and/or COX pathways, activates CREB-1. These results, together with the observation that KCREB, the dominant negative mutant of CREB-1, suppresses VSMC motility, indicate that AA stimulates VSMC motility via ERK- and p38MAPK-dependent activation of CREB-1.

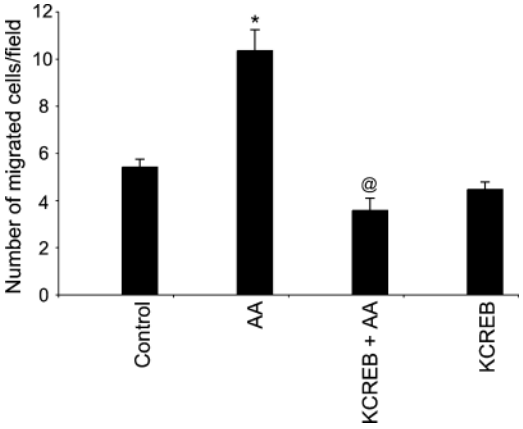

Fig. 7. Blockade of CREB-1 activation inhibits AA-induced VSMC motility. VSMCs were transfected with an empty vector or KCREB plasmid DNA and quiesced. Quiescent VSMCs were then placed in the upper chamber of the transwell at 1×10^5 cells/well. Vehicle or AA was added to the lower chamber of the transwell, and after 5 h of incubation at 37°C, cells that migrated to the lower side of the membrane were fixed in methanol, stained with Giemsa-Wright stain, washed with alcohol, and counted by light microscopy. Data are presented as means ± SEM. * $P < 0.001$ versus control; @ $P < 0.001$ versus AA treatment alone.

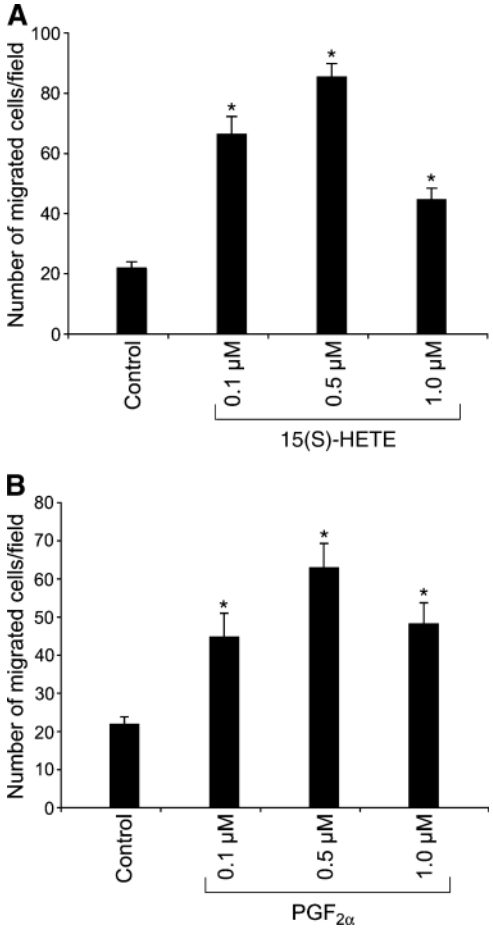

Fig. 8. 15(*S*)-Hydroxyeicosatetraenoic acid (15(*S*)-HETE) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulate VSMC motility. Quiescent VSMCs were placed in the upper chamber of the transwell at 1×10^5 cells/well. Vehicle, 15(S)-HETE, or $PGF_{2\alpha}$ at the indicated concentrations was added to the lower chamber of the transwell, and after 5 h of incubation at 37°C, cells that migrated to the lower side of the membrane were fixed in methanol, stained with Giemsa-Wright stain, washed with alcohol, and counted by light microscopy. Data are presented as means ± SEM. * $P < 0.001$ versus control.

Because the inhibition of JNKs attenuates AA-induced VSMC motility but not CREB-1 activation, it is likely that CREB-1-independent mechanisms that are subject to modulation by JNKs are also involved in the mediation of AA and its eicosanoid chemotactic effects in this cell type.

CREB-1 has been shown to be involved in both the positive and negative regulation of growth in many cell types, including VSMCs (30–33, 35, 36, 52–54). Similarly, the involvement of CREB-1 in both the positive and negative regulation of VSMC motility has been reported (34–36). These differential roles of CREB-1 in the positive and negative regulation of VSMC growth and motility may reflect its coupling to various signaling pathways targeting either the stimulation or suppression of growth and/or motility and the prevalence of the responsiveness of these events to various external cues. In this respect, this study demonstrates that ERK- and p38MAPK-dependent CREB-1 activation by AA leads to VSMC motility. Based on these observations, it is also apparent that CREB-1 is one of the target transcriptional factors involved in the regulation of VSMC motility by AA and its eicosanoid metabolites.

It was shown that COX-2, which is implicated in the progression of several diseases, including atherosclerosis (55) and cancer (56), is regulated by CREB-1 (57). Previously, we reported that VSMCs upon exposure to AA produce $PGF_{2\alpha}$ (42). In addition, here we found that $PGF_{2\alpha}$ stimulates VSMC motility. Based on these observations, it can be inferred that AA, via the activation of CREB-1 and thereby COX-2 expression, may facilitate its conversion to $PGF_{2\alpha}$, which in turn triggers VSMC motility. A similar situation can take place in the AA-15(*S*)-HETE axis, leading to the stimulation of VSMC motility. A role for phospholipase A_2s has been demonstrated in the pathogenesis of atherosclerosis (15, 16), and increased expression of COX-2 was observed in atherosclerotic lesions (58). Likewise, increased levels of HETEs have been reported in human atherosclerotic plaques (17). In view of these in vivo observations and the present results, it is likely that CREB-1 is an important player in the pathogenesis of vascular diseases such as atherosclerosis, particularly in conditions in which its initiation is linked to AA, eicosanoids, and inflammation. Because CREB-1 appears to be involved in the regulation of COX-2 expression (57) and the COX-2-prostaglandin axis has been implicated in colon cancer (10, 56), it is also likely that CREB-1 plays a role in the progression of this disease as well.

Leukotriene C_4 synthase, which produces leukotriene C_4 and is implicated in several inflammatory and allergic disorders, contains a CREB/ATF binding motif in its promoter region (59). Leukotriene C_4 has been shown to play an important role in the regulation of cell migration (60). Therefore, in addition to the mechanisms described above,

the other potential signaling by which CREB-1 may be involved in vascular diseases is via its possible role in the conversion of AA to leukotriene C_4 by the 5-LOX to 5-HETE to leukotriene C_4 synthase axis. Leukotriene C_4, in turn, may influence the growth and/or motility of VSMCs. Several reports have indicated an association of the 5-LOX-leukotriene C_4-multidrug resistance protein axis with the progression of prostate cancer (61–63). In this regard, CREB-1, via the induction of expression of leukotriene C_4 synthase and thereby leukotriene C_4 production, may also be involved in the progression of prostate cancer. Future studies are required to address whether CREB-1 is involved in the conversion of AA to HETEs and/or prostaglandins in VSMCs.

In summary, the results presented here demonstrate for the first time that AA at lower doses, either alone or by its conversion via the LOX and/or COX pathways and the ERK- and p38MAPK-dependent and JNK-independent activation of CREB-1, induces VSMC motility.

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