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Norepinephrine-Induced Stimulation of p38 Mitogen-Activated Protein Kinase Is Mediated by Arachidonic Acid Metabolites Generated by Activation of Cytosolic Phospholipase A_2 in Vascular Smooth Muscle Cells

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ABSTRACT

p38 mitogen-activated protein kinase (MAPK) is activated by norepinephrine (NE) in the vasculature and is implicated in vascular smooth muscle hypertrophy, contraction, and cell migration. NE promotes influx of Ca^{2+} and activates cytosolic phospholipase A_2 (cPLA$_2$) in vascular smooth muscle cells (VSMC). The purpose of this study was to determine the contribution of cPLA$_2$-generated arachidonic acid (AA) and its metabolites to the activation of p38 MAPK measured by its phosphorylation, in response to NE in rabbit VSMC. NE-induced p38 MAPK activation was found to be mediated through the stimulation of α-1 and α-2 adrenergic receptors, was dependent on extracellular Ca^{2+}, and was attenuated by an inhibitor of cPLA$_2$ (pyrrolidine-1). Moreover, the cPLA$_2$ product, AA, activated p38 MAPK in VSMC. p38 MAPK activation elicited by NE was decreased significantly by the lipoxygenase (LO) inhibitor baicalein, and to a lesser extent by the cytochrome P450 inhibitor 17-octadecynoic acid, but was not affected by the cyclooxygenase inhibitor indomethacin. The LO metabolites of AA, namely 5(*S*)-hydroxyeicosatetraenoic acid (HETE), 12(*S*)-HETE, and 15(*S*)-HETE and the cytochrome P450 metabolite 20-HETE, activated p38 MAPK. NE-induced p38 MAPK stimulation was found to be independent of phospholipase D (PLD) activation in rabbit VSMC. Transactivation of the epidermal growth factor receptor (EGFR) by NE also did not contribute to p38 MAPK activation. These data suggest that cPLA$_2$-generated AA and its LO metabolites mediate NE-induced p38 MAPK stimulation in rabbit VSMC by a mechanism that is independent of PLD and EGFR activation.

Norepinephrine (NE), the principal neurotransmitter released from postganglionic sympathetic nerves, acts on adrenergic receptors (ARs) on postjunctional effector cells, including vascular smooth muscle cells (VSMC). Activation of α-ARs promotes vascular smooth muscle contraction, as well as proliferation, hypertrophy, and migration of VSMC (Nebigil and Malik, 1990; Hu et al., 1996; Nishio and Watanabe, 1997; Xin et al., 1997). Stimulation of both α-1 and α-2 ARs, which are coupled to pertussis toxin-sensitive $G_{i\alpha}$ proteins, increases Ca^{2+} influx through voltage-gated Ca^{2+} channels in VSMC (Nebigil and Malik, 1993). Elevation of intracellular Ca^{2+} results in activation of cytosolic phospholipase A_2 (cPLA$_2$) and phospholipase D (PLD), which release arachidonic acid (AA) from tissue phospholipids (Muthalif et al., 1996; Parmentier et al., 2001). In VSMC, AA is metabolized to prostaglandins by cyclooxygenase (COX), to 5(*S*)-hydroxyeicosatetraenoic acid (HETE), 12(*S*)-HETE, and 15(*S*)-HETE by lipoxygenase (LO) and to 20-HETE by NADPH-dependent cytochrome P450 (P450) (Larrue et al., 1983; Nebigil and Malik, 1990; Muthalif et al., 1998a). The LO and P450 metabolites, through activation of the Ras/mitogen-activated protein kinase kinase/ERK mitogen-activated protein kinase (MAPK) pathway, mediate NE-induced VSMC proliferation (Uddin et al., 1998).

p38 MAPK has been shown to be activated by multiple growth factors in many cell types, including VSMC (Kusu-

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ABBREVIATIONS: NE, norepinephrine; AR, adrenergic receptor; VSMC, vascular smooth muscle cells; cPLA$_2$, cytosolic phospholipase A_2; PLD, phospholipase D; AA, arachidonic acid; COX, cyclooxygenase; HETE, hydroxy-eicosatetraenoic acid; LO, lipoxygenase; P450, cytochrome P450; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; 17-ODYA, 17-octadecynoic acid; M199, medium 199; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation assay; TBST, Tris-buffered saline with Tween 20; DMSO, dimethyl sulfoxide; GPCR, G protein-coupled receptor.

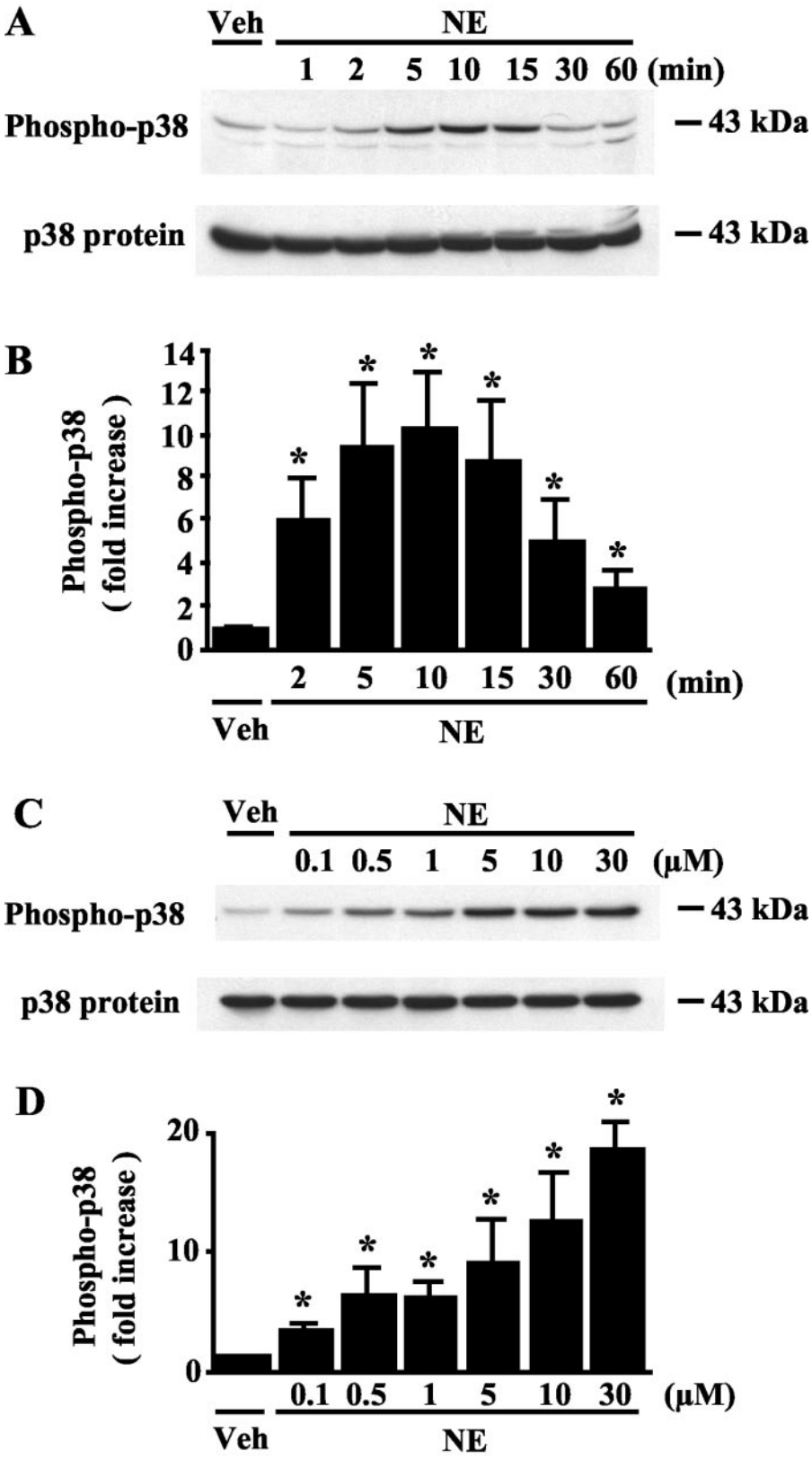

Fig. 1. Time course and concentration response of NE-induced p38 MAPK phosphorylation in rabbit VSMC. Confluent serum-deprived VSMC were treated with vehicle (Veh) or NE (10 μM) for different time intervals (A and B) or different concentrations of NE for 10 min (C and D). The proteins in cell lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, probed with anti-phospho-p38 MAPK antibody, and reprobed with anti-p38 MAPK antibody to confirm equal protein levels (representative blots A and C). The bar graphs (B and D) represent quantitation of the phospho-p38 MAPK protein bands by densitometric analysis. Results are expressed as mean ± S.E.M. of densitometric values of three to four independent experiments. *, denotes value significantly different from the corresponding value obtained in the presence of Veh ($P < 0.05$).

hara et al., 1998; Ushio-Fukai et al., 1998). p38 MAPK mediates vascular contraction through the phosphorylation of its substrate, heat shock protein 27 (Yamboliev et al., 2000). p38 MAPK has also been implicated in hypertrophy and migration of VSMC (Ushio-Fukai et al., 1998; Hedges et al., 1999). NE promotes contraction of rat mesenteric arteries through p38 MAPK activation, which is dependent on Ca^{2+} influx (Ohanian et al., 2001). However, the Ca^{2+}-dependent signaling mechanisms that mediate NE-induced p38 MAPK activation are unexplored. It has been reported that AA as

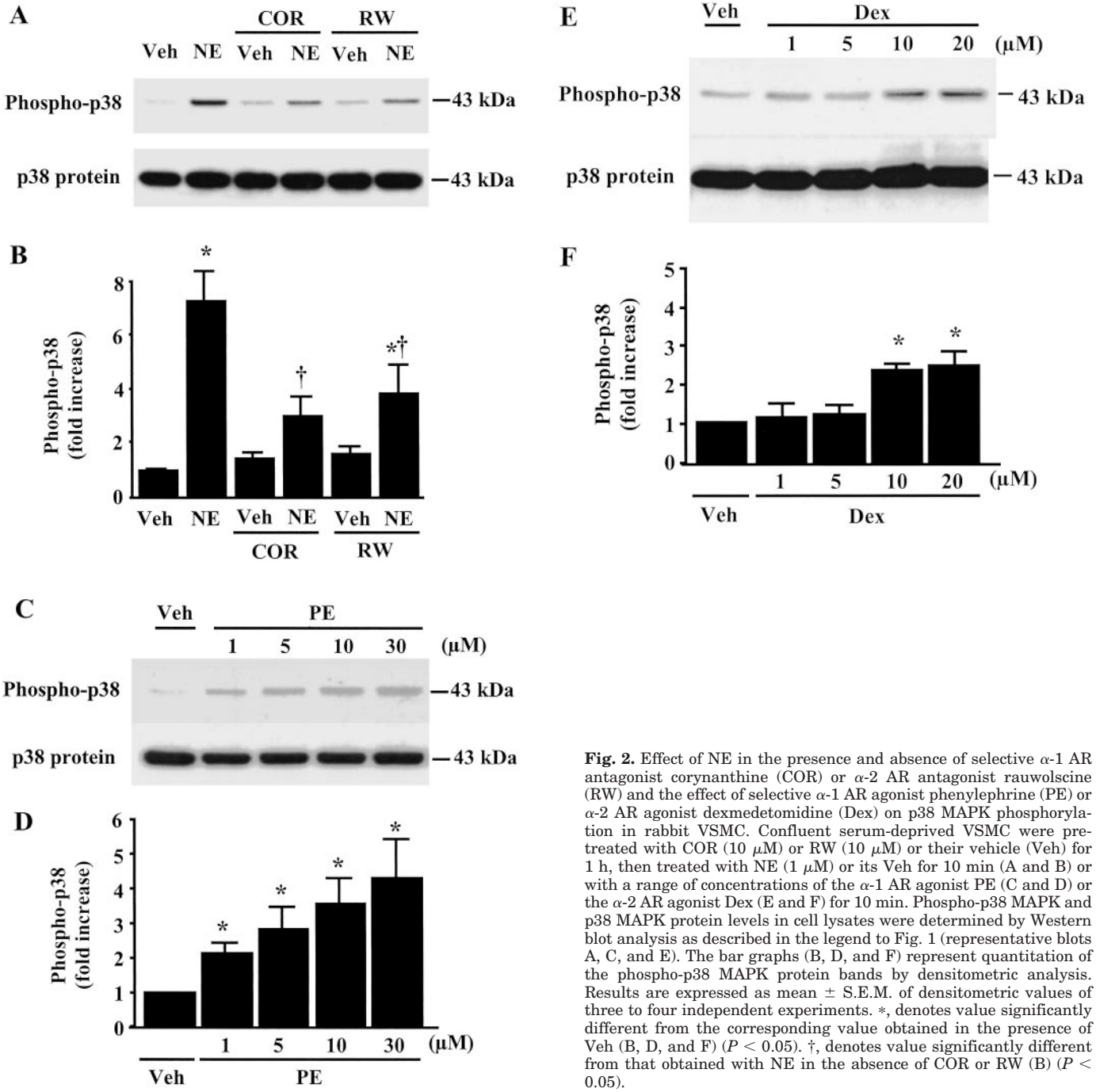

Fig. 2. Effect of NE in the presence and absence of selective α-1 AR antagonist corynanthine (COR) or α-2 AR antagonist rauwolscine (RW) and the effect of selective α-1 AR agonist phenylephrine (PE) or α-2 AR agonist dexmedetomidine (Dex) on p38 MAPK phosphorylation in rabbit VSMC. Confluent serum-deprived VSMC were pretreated with COR (10 μM) or RW (10 μM) or their vehicle (Veh) for 1 h, then treated with NE (1 μM) or its Veh for 10 min (A and B) or with a range of concentrations of the α-1 AR agonist PE (C and D) or the α-2 AR agonist Dex (E and F) for 10 min. Phospho-p38 MAPK and p38 MAPK protein levels in cell lysates were determined by Western blot analysis as described in the legend to Fig. 1 (representative blots A, C, and E). The bar graphs (B, D, and F) represent quantitation of the phospho-p38 MAPK protein bands by densitometric analysis. Results are expressed as mean ± S.E.M. of densitometric values of three to four independent experiments. *, denotes value significantly different from the corresponding value obtained in the presence of Veh (B, D, and F) ($P < 0.05$). †, denotes value significantly different from that obtained with NE in the absence of COR or RW (B) ($P < 0.05$).

well as its metabolites 12(*S*)-HETE and 11,12-epoxyeicosatrienoic acid activate p38 MAPK in some cell types (Paine et al., 2000; Alexander et al., 2001; Fleming et al., 2001; Reddy et al., 2002). Whether AA and its metabolites mediate the activation of p38 MAPK by a physiological agonist has not been investigated. We therefore postulated that NE might activate p38 MAPK in VSMC through generation of metabolites of AA released via activation of $cPLA_2$ and/or PLD. Stimulation of α-2 ARs overexpressed in Chinese hamster ovary cells has been shown to transactivate the epidermal growth factor receptor (EGFR) (Pierce et al., 2001). EGF activates p38 MAPK in VSMC; moreover, EGFR transactivation has been reported to mediate p38 MAPK activation by angiotensin II (Eguchi et al., 2001). This led us to hypothesize that NE, by acting on endogenously expressed ARs in VSMC, transactivates the EGFR, which in turn activates p38 MAPK. To test these hypotheses, we have investigated the phosphorylation-dependent activation of p38 MAPK in response to NE in the presence of inhibitors of $cPLA_2$, PLD, AA metabolism, and EGFR tyrosine kinase.

We now report that AA and its metabolites 5(*S*)-HETE, 15(*S*)-HETE, and 20-HETE activate p38 MAPK in VSMC. Moreover, NE-induced p38 MAPK activation is mediated by $cPLA_2$, but not PLD-dependent release of AA and generation of its metabolites, primarily via LO [5(*S*)-, 12(*S*)-, and 15(*S*)-HETE)] and to a lesser extent, P450 (20-HETE). NE transactivates EGFR in rabbit VSMCs; however, this does not contribute to NE-induced p38 MAPK activation.

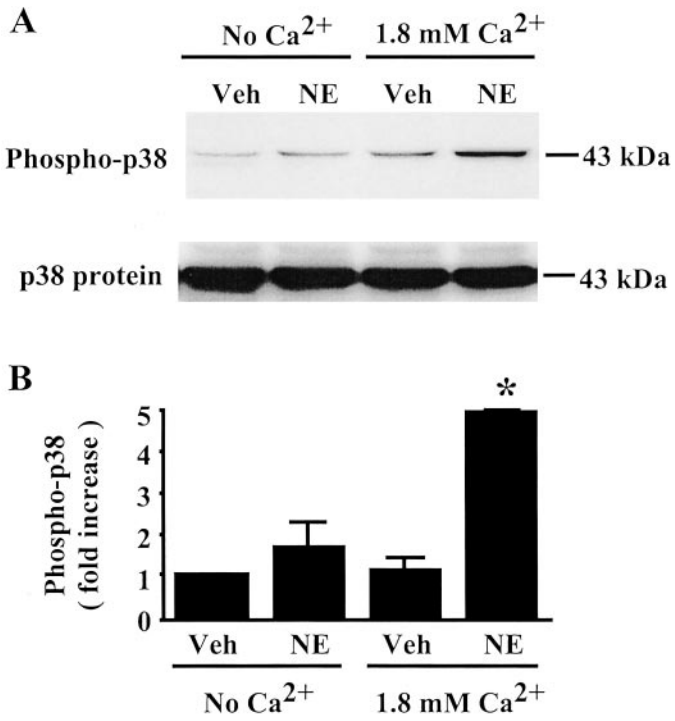

Fig. 3. Effect of removal of extracellular Ca^{2+} on NE-induced p38 MAPK phosphorylation in rabbit VSMC. Cells were treated with NE (10 μM) in HBSS without Ca^{2+}, or HBSS with 1.8 mM Ca^{2+} for 10 min. Phospho-p38 MAPK and p38 MAPK protein levels in cell lysates were determined by Western blot analysis as described in the legend to Fig. 1. A representative blot is shown in A. The bar graph (B) represents quantitation of the phospho-p38 MAPK protein bands by densitometric analysis. Similar results were obtained in three independent experiments. *, denotes value significantly different from the corresponding value obtained in the absence of Ca^{2+} ($P < 0.05$).

Materials and Methods

Norepinephrine HCl, corynanthine HCl, phenylephrine HCl, rauwolscine HCl, 1-butanol, 2-butanol, and antibodies against α-smooth muscle actin and smooth muscle myosin were purchased from Sigma-Aldrich (St. Louis, MO); AG1478 was from Calbiochem (San Diego, CA); dexmedetomidine-HCl was a gift from Orion Pharma (Turku, Finland); and pyrrolidine-1 was a gift from Dr. Michael Gelb (University of Washington, Seattle, WA). Baicalein and 17-octadecynoic acid (17-ODYA) were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA); AA, 5(*S*)-, 12(*S*)-, 15(*S*)-, and 20-HETE were from Cayman Chemicals (Ann Arbor, MI); antibody against phospho-p38 MAPK was from Cell Signaling Technology Inc. (Beverly, MA); anti-phospho-EGFR (PY1068) was from BioSource International (Camarillo, CA); antibodies against p38 MAPK α and EGFR were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phosphotyrosine (4G10) was from Upstate Biotechnology (Lake Placid, NY); and protein A agarose was from Invitrogen (Carlsbad, CA). Medium 199 (M199), Hanks' balanced salt solution (HBSS), and fetal bovine serum were purchased from Cellgro (Herndon, VA).

Methods

VSMC Isolation and Culture. Male New Zealand White rabbits (1–2 kg) were used (Myrtle's Rabbitry, Franklin, TN) and the thoracic aorta was isolated as described previously (Muthalif et al., 1996) according to a protocol approved by the Animal Care and Use Committee of the University of Tennessee (Memphis, TN). VSMC were isolated by enzymatic digestion, by a modification of the method of Campbell and Campbell (1993). The outer layers of fat were removed. The endothelium was peeled off and the aorta incubated in 10 ml of a solution containing 35 mg of collagenase for 30 min. This was followed by removal of the outer adventitia. The remaining smooth muscle was finely minced and subjected to enzymatic digestion in 10 ml of a solution containing 5 mg of elastase, 20 mg of trypsin inhibitor, and 10 mg of bovine serum albumin for 1.5 h, and then 35 mg of collagenase was added. When enzymatic digestion was complete, cells were centrifuged, washed with medium, and plated in dishes, with a change of medium on the subsequent day. VSMC were grown in Medium 199 containing 10% fetal bovine serum, penicillin (100 units/ml), and amphotericin B (0.25 μg/ml). The characteristic spindle-shaped morphology of rabbit VSMC as well as the presence of the smooth muscle cell markers, α-smooth muscle actin, and smooth muscle myosin were confirmed (Appendix 1). Rat VSMC were isolated and cultured in a similar manner. Both rabbit and rat VSMC were used for experiments from passages 3 to 7.

Protocol 1. The first series of experiments was performed to determine the time course and concentration response of NE-induced p38 MAPK phosphorylation. Dual phosphorylation of p38 MAPK on Thr180/Tyr 182 is required for its activation (Enslen et al., 2000). Therefore, antibody against doubly phosphorylated p38 MAPK was used to detect phosphorylation of p38 MAPK in response to NE, and used as an index of its activation. Stock solutions of NE-HCl were freshly prepared in water on the day of the experiment. Confluent VSMC were serum-deprived for 2 to 3 days, followed by treatment with NE at different concentrations and for different time intervals. Cells were washed thrice with cold PBS, lysed using radioimmunoprecipitation assay (RIPA) buffer [containing 50 mM Tris-HCl, 1% Igepal, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, the phosphatase inhibitors sodium orthovanadate (1 mM) and sodium fluoride (1 mM), and the protease inhibitors aprotinin and leupeptin (10 μg/ml each); pH of RIPA buffer was 7.4]. The lysate was sonicated followed by centrifugation at 14,000 rpm. The amount of protein in the supernatant was estimated by the Bradford method, followed by Western blot analysis to measure phosphorylation of p38 MAPK.

Western Blot Analysis. Equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked with 5% fat-free milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h, washed three times with TBST, followed by overnight incubation with a primary antibody against phosphorylated p38 MAPK or phosphorylated EGFR, in TBST containing 5% bovine serum albumin. The following day, blots were washed three times with TBST for 5 min each, incubated with secondary antibody at room temperature for an hour, again followed by three 5-min washes with TBST. Phosphorylated proteins were detected by chemiluminescence using the ECL Plus kit (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). To confirm that samples contained equal levels of protein, blots were stripped and reprobed with antibody recognizing unphosphorylated and phosphorylated p38 MAPK or EGFR.

Protocol 2. To determine whether extracellular Ca^{2+} is required for NE-induced p38 MAPK activation, VSMC were treated with NE (10 μM) in HBSS in the presence or absence of Ca^{2+}. Serum-deprived VSMC were washed with HBSS with or without Ca^{2+} and treated with NE in the same medium for 10 min. This was followed by washing with PBS, lysis with RIPA buffer, and Western blotting to measure p38 MAPK phosphorylation as described above.

Protocol 3. The third series of experiments was performed to determine the contribution of α-1 and α-2 ARs to NE-induced p38 MAPK activation. Serum-deprived VSMC were pretreated with the α-1 AR antagonist corynanthine (10 μM) or the α-2 AR antagonist rauwolscine (10 μM) for 1 h, followed by treatment with 1 μM NE for 10 min. To confirm the contribution of α-1 and α-2 ARs to p38 MAPK activation, VSMC were treated with various concentrations of the selective α-1 AR agonist phenylephrine or the selective α-2 AR agonist dexmedetomi-

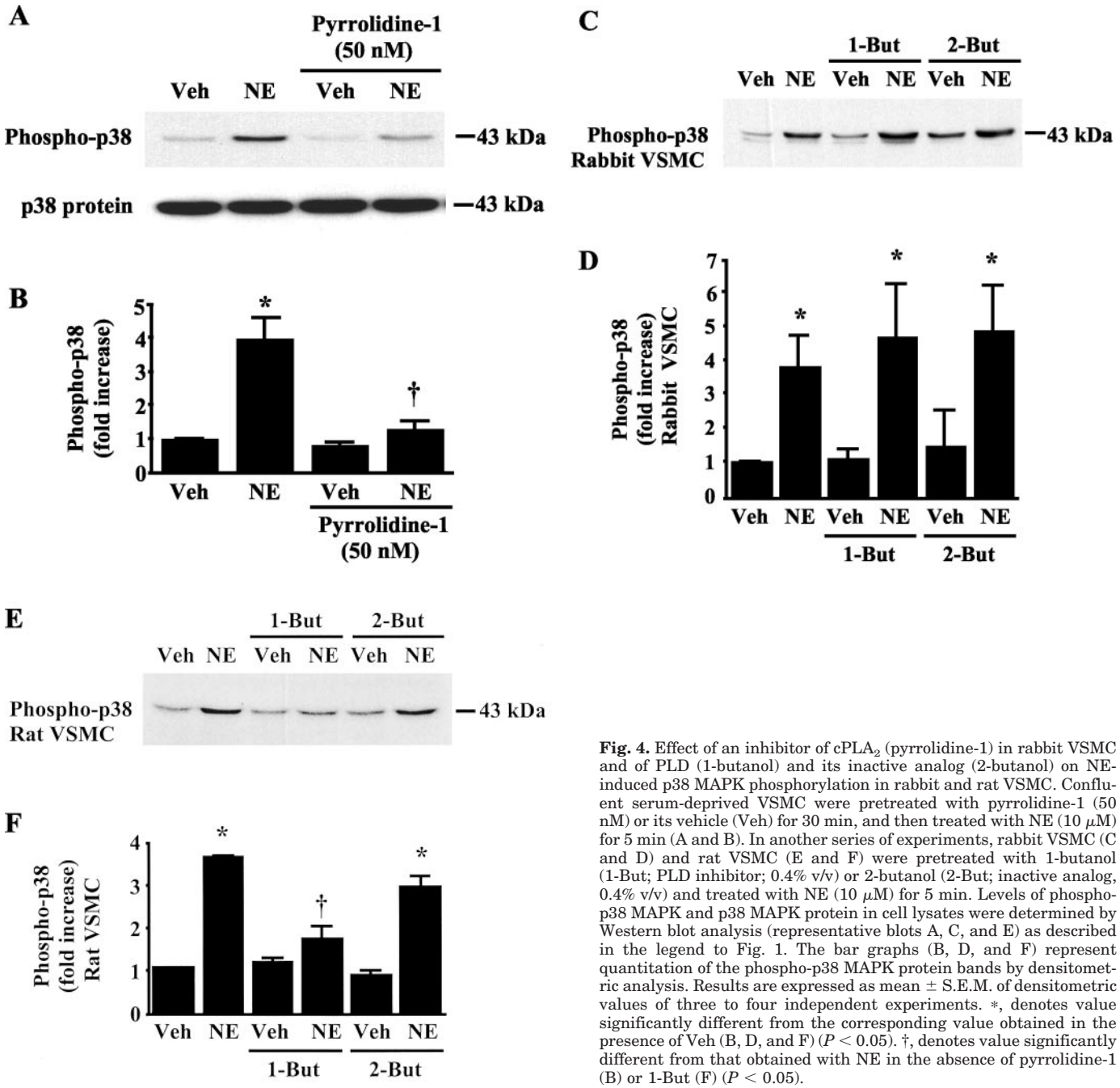

Fig. 4. Effect of an inhibitor of $cPLA_2$ (pyrrolidine-1) in rabbit VSMC and of PLD (1-butanol) and its inactive analog (2-butanol) on NE-induced p38 MAPK phosphorylation in rabbit and rat VSMC. Confluent serum-deprived VSMC were pretreated with pyrrolidine-1 (50 nM) or its vehicle (Veh) for 30 min, and then treated with NE (10 μM) for 5 min (A and B). In another series of experiments, rabbit VSMC (C and D) and rat VSMC (E and F) were pretreated with 1-butanol (1-But; PLD inhibitor; 0.4% v/v) or 2-butanol (2-But; inactive analog, 0.4% v/v) and treated with NE (10 μM) for 5 min. Levels of phospho-p38 MAPK and p38 MAPK protein in cell lysates were determined by Western blot analysis (representative blots A, C, and E) as described in the legend to Fig. 1. The bar graphs (B, D, and F) represent quantitation of the phospho-p38 MAPK protein bands by densitometric analysis. Results are expressed as mean ± S.E.M. of densitometric values of three to four independent experiments. *, denotes value significantly different from the corresponding value obtained in the presence of Veh (B, D, and F) ($P < 0.05$). †, denotes value significantly different from that obtained with NE in the absence of pyrrolidine-1 (B) or 1-But (F) ($P < 0.05$).

dine. Stock solutions of all the adrenergic agonists and antagonists were freshly prepared in water on the day of the experiment.

Protocol 4. To determine the contribution of $cPLA_2$-derived AA to NE-induced p38 MAPK activation, cells were pretreated for 30 min with the specific $cPLA_2$ inhibitor pyrrolidine-1 (Ghomashchi et al., 2001). Pyrrolidine-1 (50 nM) has been shown to abolish NE-induced AA release in these cells (Muthalif et al., 2001). Pyrrolidine-1 (10 mM stock solution in DMSO) was stored at −20°C and the final solution of pyrrolidine-1 (50 nM in M199) used in our experiments contained a negligible amount of DMSO (0.0005%). NE can also release AA by activating PLD in VSMC (Parmentier et al., 2001). To determine the involvement of PLD-derived AA in p38 MAPK activation, VSMC were pretreated for 30 min with 1-butanol (0.4% v/v), which reacts with the PLD product phosphatidic acid by the transphosphatidylation reaction, thereby preventing PLD-dependent accumulation of phosphatidic acid. 2-Butanol, which does not inhibit PLD activity, was used as a control. To determine the activation of p38 MAPK by AA, VSMC were also treated with varying concentrations of exogenous AA. To investigate the possible contribution of AA metabolites, cells were pretreated with inhibitors of LO (baicalein; 5 μM), P450 (17-ODYA; 5 μM), or COX (indomethacin; 10 μM) for 1 h. Previous studies from our laboratory have established the effectiveness of these inhibitors at these concentrations in VSMC (Muthalif et al., 1998b; Parmentier et al., 2001). Baicalein and ODYA were dissolved in DMSO and ethanol (final concentration of vehicle, 0.1%), respectively. Pretreatment of VSMC with these agents was followed by exposure to NE (10 μM) for 5 min. Then the cells were washed with cold PBS and lysed by sonication in RIPA buffer. p38 MAPK phosphorylation was determined by Western blot analysis as described above. To confirm the involvement of the LO and P450 metabolites in p38 MAPK activation, VSMC were treated with the products of these enzymatic pathways, namely, 5(*S*)-, 12(*S*)-, 15(*S*)-, and 20-HETE

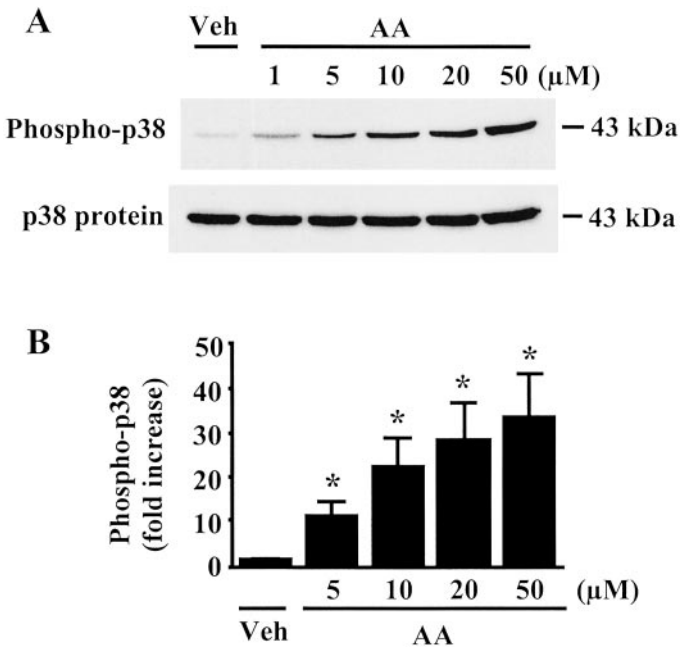

Fig. 5. Effect of exogenous AA on p38 MAPK phosphorylation in rabbit VSMC. Cells were treated with different concentrations of AA (5–50 μM) or its vehicle (A and B). Phospho-p38 MAPK and p38 MAPK protein levels in cell lysates were determined by Western blot analysis as described in the legend to Fig. 1. A representative blot is shown in A. The bar graph (B) represents quantitation of the phospho-p38 MAPK protein bands by densitometric analysis. Results are expressed as mean ± S.E.M. of densitometric values of four independent experiments. *, denotes value significantly different from the corresponding value obtained in the presence of Veh ($P < 0.05$).

(0.5–1 μM). For experiments with HETEs, the solvent ethanol was evaporated under a stream of N_2 gas and the HETEs were redissolved in M199. The cell lysates were prepared as described in protocol 1 and p38 MAPK phosphorylation was measured by Western blot analysis as described above.

Protocol 5. This series of experiments was performed to determine whether NE transactivates the EGFR in VSMCs and whether this contributes to NE-induced p38 MAPK activation. Autophosphorylation of EGFR at tyrosine 1068 in response to growth factor stimulation is required for MAPK activation (Rojas et al., 1996); this was therefore used as an index of EGFR activation by NE. Confluent, serum-deprived VSMC were treated with NE (10 μM) for different time intervals, and subsequent steps to prepare cell lysates were performed as described in protocol 1. Phosphorylated EGFR was visualized by Western blot analysis as described above, using anti-phospho-EGFR antibody. The effect of the EGFR kinase inhibitor AG1478 (500 nM) on NE-induced p38 MAPK phosphorylation was determined. As a positive control, to verify the efficacy of the inhibitor, the effect of the same concentration of AG1478 on EGF-induced p38 MAPK phosphorylation was determined. Cells were pretreated with AG1478, followed by treatment with NE (10 μM) or EGF (100 ng/ml), processed as described in protocol 1, and p38 MAPK phosphorylation was measured by Western blot analysis as described above.

Data Analysis. Images obtained by chemiluminescence on Biomax MR film were scanned, and densitometric analysis was performed using NIH Image 1.62. This program was developed at the U.S. National Institutes of Health and is available on the Internet at http://rsb.info.nih.gov/nih-image/. Values from three to four independent experiments were used to plot graphs representative of the densitometric analysis. Data were analyzed by one-way analysis of variance and Student's t test was used to determine the difference between two groups. In the case of significant difference in standard deviation between two groups, a nonparametric test was used. P values less than 0.05 were considered to be statistically significant.

Results

NE Activates p38 MAPK in VSMC through α-1 and α-2 ARs in a Ca^{2+}-Dependent Manner. NE caused phosphorylation of p38 MAPK in a time-dependent manner, initiated as early as 2 min and peaking at 10 min at levels of approximately 10-fold over basal, in rabbit VSMC. The phosphorylation then decreased to a level slightly above basal level at 60 min (Fig. 1, A and B). NE-induced phosphorylation of p38 MAPK was concentration-dependent (Fig. 1, C and D). The effect was detected at 100 nM and increased to a maximum (10- to 20-fold over basal) at concentrations between 10 and 30 μM. NE-induced p38 MAPK phosphorylation was inhibited by the selective α-1 and α-2 AR antagonists corynanthine and rauwolscine, respectively (Fig. 2, A and B). The selective α-1 AR agonist phenylephrine and the selective α-2 AR agonist dexmedetomidine also increased p38 MAPK phosphorylation in a concentration-dependent manner (Fig. 2, C and D, and E and F, respectively). Activation of p38 MAPK by NE is dependent on extracellular Ca^{2+} in the vasculature (Ohanian et al., 2001), and ERK activation is mediated through a Ca^{2+}-dependent pathway in VSMC (Muthalif et al., 1996). We therefore investigated whether extracellular Ca^{2+} is required for NE-induced p38 MAPK activation in VSMC. In the absence of extracellular Ca^{2+}, NE failed to promote phosphorylation of p38 MAPK (Fig. 3, A and B).

$cPLA_2$-Generated AA, but not PLD-Generated AA, Mediates NE-Induced p38 MAPK Activation. To determine the contribution of $cPLA_2$-mediated AA release to NE-induced p38 MAPK activation, VSMC were pretreated with a $cPLA_2$ inhibitor, pyrrolidine-1, followed by treatment with NE and detection of p38 MAPK phosphorylation by Western blot analysis. Pyrrolidine-1, at a concentration of 50 nM attenuated NE-induced p38 MAPK phosphorylation (Fig. 4, A and B). This concentration of pyrrolidine-1 has been shown to abolish NE-induced AA release in these cells (Muthalif et al., 2001). Because NE-induced PLD activation can also mediate AA release, the contribution of PLD to p38 MAPK activation was determined by examining the effect of 0.4% (v/v) 1-butanol, which has been shown to inhibit NE-induced PLD activity and AA release by approximately 70%, at this concentration (Parmentier et al., 2001). NE-induced p38 MAPK phosphorylation was not affected by 1-butanol in rabbit VSMC (Fig. 4, C and D), but was inhibited by 1-butanol in rat VSMC (Fig. 4, E and F), suggesting that it is independent of PLD activation in the former, but is mediated in part, by PLD in the latter. Exogenous AA activated p38 MAPK in a concentration-dependent manner in rabbit VSMC (Fig. 5, A and B).

AA Metabolites Generated Primarily via LO Mediate NE-Induced p38 MAPK Activation. AA is metabolized by COX, LO, and P450 in VSMC. The effect of inhibitors of these pathways and potential LO and P450 metabolites on p38 MAPK activation was tested. The LO inhibitor baicalein attenuated NE-induced p38 MAPK activation. The P450 inhibitor 17-ODYA tended to attenuate p38 MAPK activation by a modest amount, but the effect failed to reach statistical significance due to variability between batches of cells (Fig. 6, A–C). The COX inhibitor indomethacin did not alter NE-

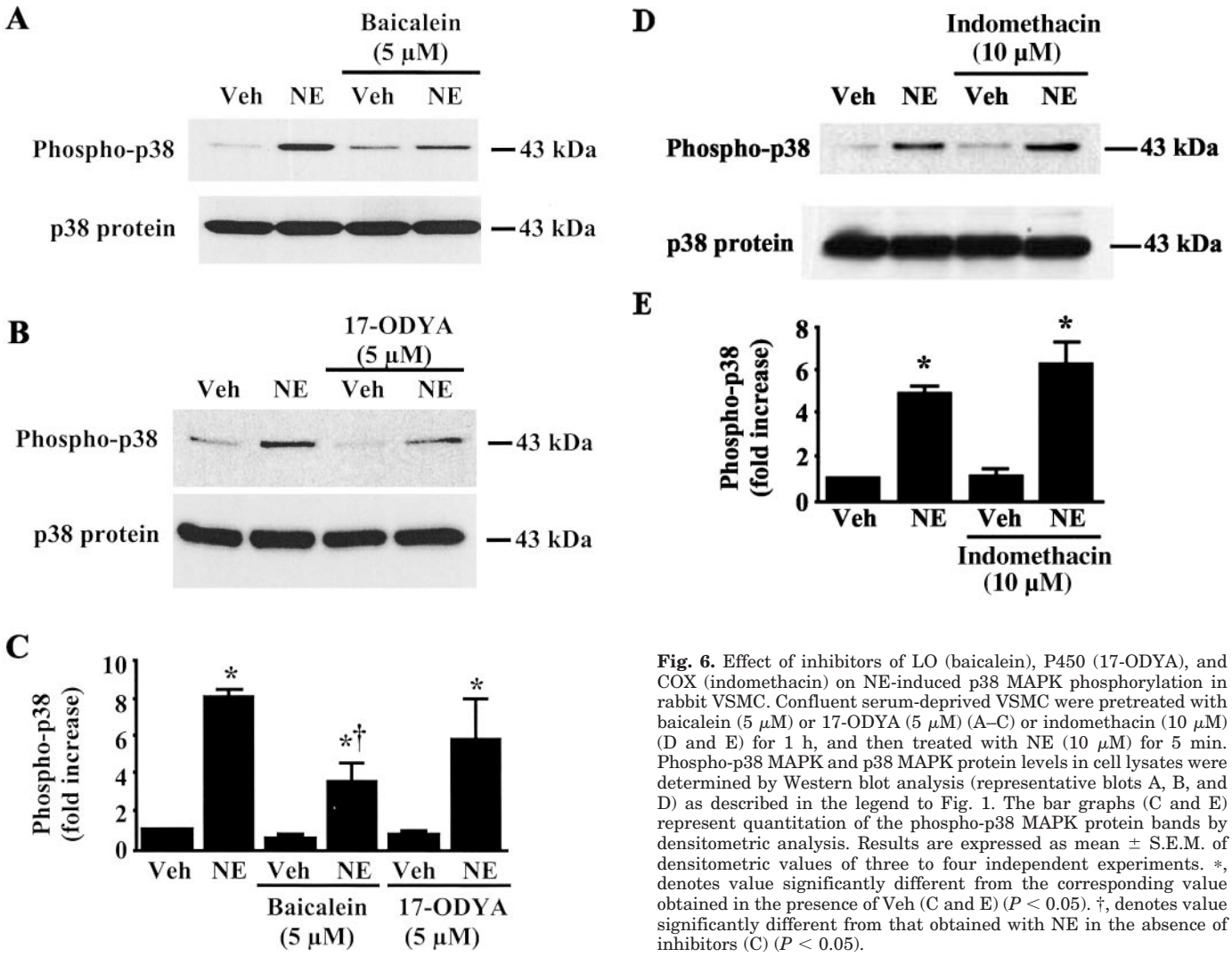

Fig. 6. Effect of inhibitors of LO (baicalein), P450 (17-ODYA), and COX (indomethacin) on NE-induced p38 MAPK phosphorylation in rabbit VSMC. Confluent serum-deprived VSMC were pretreated with baicalein (5 μM) or 17-ODYA (5 μM) (A–C) or indomethacin (10 μM) (D and E) for 1 h, and then treated with NE (10 μM) for 5 min. Phospho-p38 MAPK and p38 MAPK protein levels in cell lysates were determined by Western blot analysis (representative blots A, B, and D) as described in the legend to Fig. 1. The bar graphs (C and E) represent quantitation of the phospho-p38 MAPK protein bands by densitometric analysis. Results are expressed as mean ± S.E.M. of densitometric values of three to four independent experiments. *, denotes value significantly different from the corresponding value obtained in the presence of Veh (C and E) ($P < 0.05$). †, denotes value significantly different from that obtained with NE in the absence of inhibitors (C) ($P < 0.05$).

induced p38 MAPK activation (Fig. 6, D and E). In an additional series of experiments, the effect of 5(*S*)-, 12(*S*)-, 15(*S*)-, and 20-HETE on p38 MAPK activity was examined. All these agents increased p38 MAPK phosphorylation; 12(*S*)- and 15(*S*)-HETE were more potent than 5(*S*)- and 20-HETE in causing p38 MAPK activation (Fig. 7, A–D). This suggests that NE-induced p38 MAPK activation depends primarily on LO-catalyzed metabolism of AA.

Transactivation of EGFR by NE Does Not Contribute to Its Action on p38 MAPK Activation. Autophosphorylation of EGFR at tyrosine 1068 is required for EGFR-mediated MAPK activation (Rojas et al., 1996). The effect of NE on phosphorylation of EGFR was determined using an antibody recognizing phospho-Y1068 EGFR. The specificity of this antibody has been determined using 1068 Y-F EGFR mutants (Erik Schaefer, personal communication). NE caused detectable autophosphorylation of EGFR within 2 min, reaching maximal level by 5 min. EGFR phosphorylation in response to NE was, however, smaller in magnitude than that induced by its own ligand EGF (100 ng/ml) (Fig. 8, A and B). The specificity of the antibody was further confirmed using a competing PY1068-EGFR phosphopeptide. However, immunoprecipitation of EGFR from VSMC lysates, followed by immunoblotting with pan-PY4G10 antibody, revealed that treatment of VSMC with EGF, but not NE, elicited an increase in total tyrosine phosphorylation of EGFR (data not shown).

To determine the contribution of EGFR transactivation to NE-induced p38 MAPK activation, we studied the effect of the EGFR kinase inhibitor AG1478 on NE- as well as EGF-induced p38 MAPK activation. AG1478, at a concentration of 500 nM, significantly inhibited EGF-induced p38 MAPK activation, but did not attenuate NE-induced p38 MAPK activation (Fig. 8, C and D). This indicates that EGFR does not mediate NE-induced p38 MAPK activation in rabbit VSMC.

Discussion

The present study demonstrates that NE activates p38 MAPK by stimulation of both α-1 and α-2 ARs in rabbit VSMC. Activation of p38 MAPK elicited by NE is dependent upon extracellular Ca^{2+} and is mediated by $cPLA_2$, but not PLD-dependent AA release and generation of LO metabolites, 5(*S*)-, 12(*S*)-, and 15(*S*)-HETE, and is independent of EGFR transactivation. The p38 MAPK/heat shock protein 27 pathway is known to modulate smooth muscle force (Yamboliev et al., 2000). p38 MAPK has been shown to be activated by NE in rat mesenteric arteries, and this has been impli-

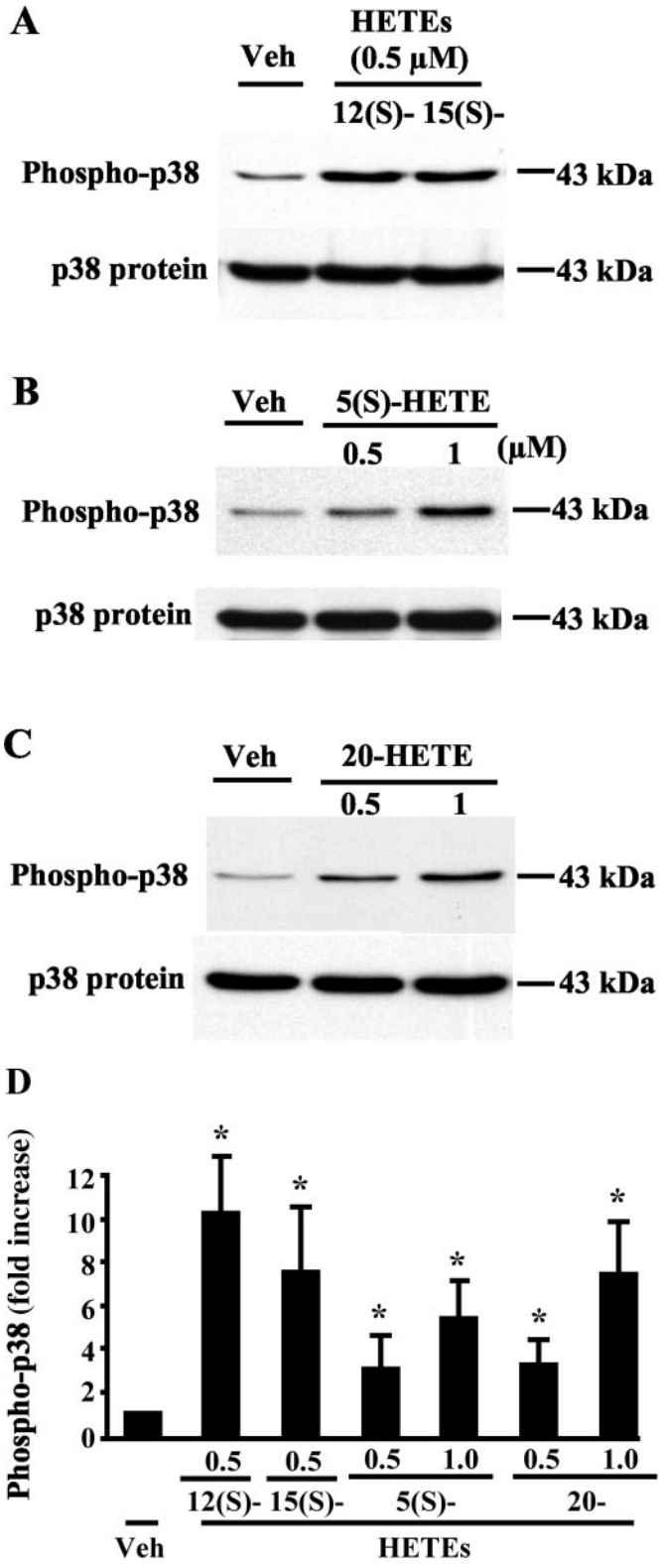

Fig. 7. Effect of exogenous HETEs on p38 MAPK phosphorylation in rabbit VSMC. Cells were treated with 5(*S*)-, 12(*S*)-, 15(*S*)-, or 20-HETE or their vehicle for 5 min. Phospho-p38 MAPK and p38 MAPK protein levels in cell lysates were determined by Western blot analysis (representative blots A–C) as described in the legend to Fig. 1. The bar graph (D) represents quantitation of the phospho-p38 MAPK protein bands by densitometric analysis. Results are expressed as mean ± S.E.M. of densitometric values of three to four independent experiments. *, denotes value significantly different from the corresponding value obtained in the presence of Veh (D) ($P < 0.05$).

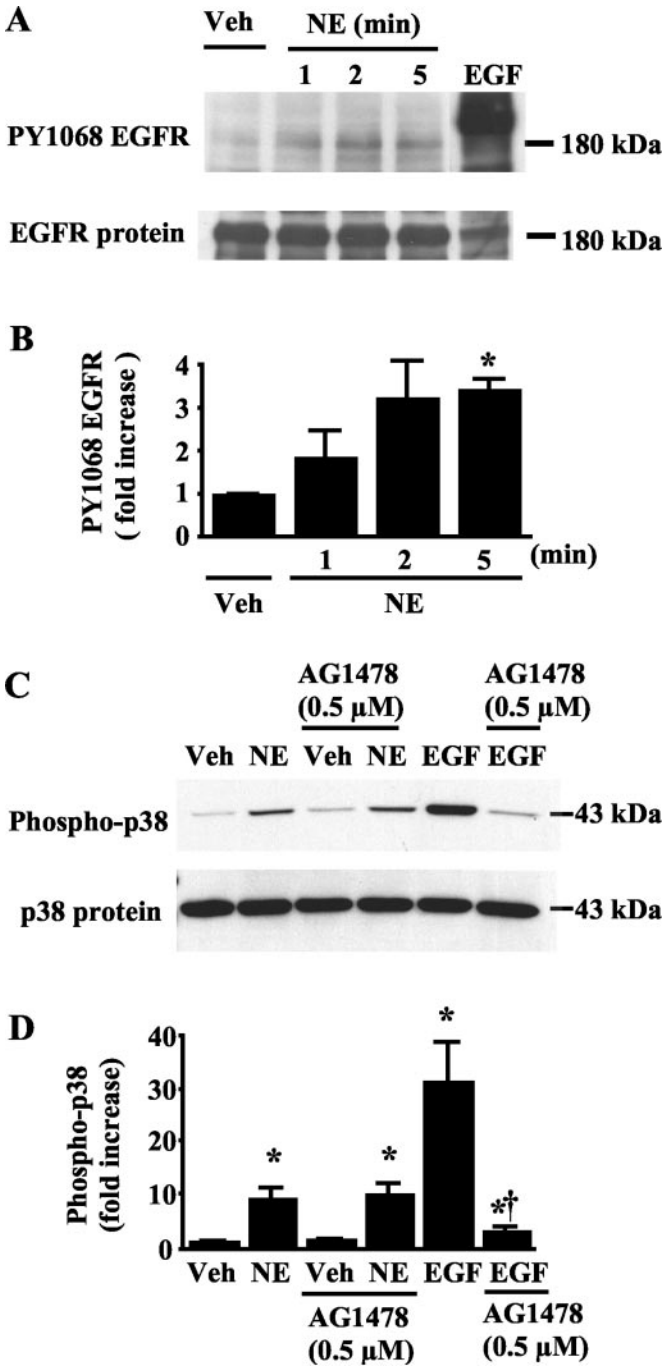

Fig. 8. Effect of NE on phosphorylation of EGFR and effect of EGFR kinase inhibitor AG1478 on NE-induced p38 MAPK phosphorylation in rabbit VSMC. Confluent serum-deprived VSMC were treated with vehicle or NE (10 μM) for the specified periods of time, or 100 ng/ml EGF (2 min; positive control in last lane) (A and B). Phospho-EGFR (PY1068) levels in cell lysates were determined by Western blot analysis as described in the legend to Fig. 1. Each blot was stripped and reprobed with anti-EGFR antibody to confirm equal protein levels (representative blot A). VSMC were also pretreated with AG1478 (500 nM) for 30 min, and then treated with NE (10 μM) or EGF (100 ng/ml) for 5 min (C and D). Phospho-p38 MAPK and p38 MAPK protein levels in cell lysates were determined by Western blot analysis (representative blot C) as described in the legend to Fig. 1. The bar graphs represent quantitation of the phospho-EGFR (B) and phospho-p38 MAPK (D) protein bands by densitometric analysis. Results are expressed as mean ± S.E.M. of densitometric values of three to four independent experiments. *, denotes value significantly different from the corresponding value obtained in the presence of Veh (B and D) ($P < 0.05$). †, denotes value significantly different from that obtained with EGF in the absence of AG1478 (D) ($P < 0.05$).

cated in vascular contraction (Ohanian et al., 2001). In the present study, NE caused activation of p38 MAPK in a concentration-dependent manner in rabbit VSMC. Although NE produces maximal contraction at 1 μM in the rabbit aorta (Nebigil and Malik, 1990), we found that NE elicits maximal p38 MAPK activation in the range of 5 to 10 μm in rabbit VSMC. A plausible explanation for this may be the decrease of AR number during culture of VSMC (Faber et al., 2001). This may necessitate higher concentrations of agonist in VSMC to produce the same level of stimulation as in the whole aorta. Both α-1 and α-2 ARs mediate NE-induced Ca^{2+} influx and eicosanoid synthesis (Nebigil and Malik, 1990). NE-induced p38 MAPK activation also seems to be mediated by stimulation of both α-1 and α-2 ARs, because it was inhibited by the selective α-1 and α-2 AR antagonists corynanthine and rauwolscine, respectively. Supporting this conclusion was our finding that phenylephrine, a selective α-1 AR agonist, and dexmedetomidine, a selective α-2 AR agonist, increased p38 MAPK activity in a concentration-dependent manner. This is the first evidence of α-2 AR-mediated activation of p38 MAPK in any cell type and may be specific for VSMC, in view of a previous report that NE failed to activate p38 MAPK in α-2 AR-transfected PC12 cells (Williams et al., 1998). α-1- and α-2 AR stimulation with phenylephrine and dexmedetomidine, respectively, has been found to promote VSMC migration (Nishio and Watanabe, 1997; Richman and Regan, 1998). Our finding that both these agents activate p38 MAPK raises the possibility that the p38/heat shock protein 27 pathway, which is critical for VSMC migration (Hedges et al., 1999), mediates α-1 and α-2 AR-induced migration.

NE increases cytosolic Ca^{2+} in VSMC by α-1 and α-2 AR-mediated influx of extracellular Ca^{2+}, and release of intracellular Ca^{2+} via α-1 AR-mediated generation of inositol trisphosphate (Heagerty and Ollerenshaw, 1987; Nebigil and Malik, 1993). NE-induced p38 MAPK activation was inhibited by depletion of extracellular Ca^{2+}, suggesting that influx of extracellular Ca^{2+} stimulates p38 MAPK activation. Increased cytosolic Ca^{2+} also causes phosphorylation and activation of $cPLA_2$ in VSMC (Muthalif et al., 1996). p38 MAPK has been implicated in phosphorylation and activation of $cPLA_2$ in platelets and HeLa cells (Borsch-Haubold et al., 1998). However, a previous study from our laboratory has ruled out the involvement of p38 MAPK in NE-induced activation of $cPLA_2$ in VSMC (Fatima et al., 2001). On the contrary, this study provides evidence that NE-induced p38 MAPK activation is dependent upon $cPLA_2$ activity, because the inhibitor of $cPLA_2$, pyrrolidine-1 (Ghomashchi et al., 2001), attenuated NE-induced p38 MAPK activation. Activation of $cPLA_2$ promotes hydrolysis of tissue phospholipids and releases AA, which has been reported to activate p38 MAPK in a breast carcinoma cell line, and in rabbit proximal tubule cells (Paine et al., 2000; Alexander et al., 2001). We found that AA also activates p38 MAPK in VSMC. Our finding that the LO inhibitor baicalein and to a lesser extent, the P450 inhibitor 17-ODYA, but not the COX inhibitor indomethacin, attenuated NE-induced p38 MAPK activation, suggests that AA metabolite(s) derived mainly via the LO pathway mediate NE-induced p38 MAPK activation. Supporting our finding is the report that an LO metabolite of AA, 12(*S*)-HETE activates p38 MAPK in porcine aortic VSMC (Reddy et al., 2002). The present study shows that, in addition to 12(*S*)-HETE, other LO metabolites of AA, 5(*S*)- and 15(*S*)-HETE generated in rabbit VSMC (Larrue et al., 1983) also cause activation of p38 MAPK. 20-HETE, which is derived from AA through the P450 pathway, also increased p38 MAPK activity, but was less potent than 12(*S*)- and 15(*S*)-HETE.

Previous studies from our laboratory have shown that $cPLA_2$ mediates PLD activation through generation of AA metabolites in response to NE in VSMC (Parmentier et al., 2001). PLD activation promotes breakdown of phospholipids to phosphatidic acid, which in turn is hydrolyzed by phosphatidate phosphohydrolase to diacylglycerol that is subsequently metabolized by diacylglycerol lipase to release AA (Parmentier et al., 2001). This raises the possibility that PLD-mediated AA release also contributes to NE-induced p38 MAPK activation. PLD has been reported to mediate p38 MAPK activation in neutrophil-like HL-60 cells (Bechoua and Daniel, 2001). However, in the present study, 1-butanol, which inhibits NE-induced PLD-mediated AA release (Parmentier et al., 2001), failed to alter NE-induced p38 MAPK activation in rabbit VSMC. The specificity of NE-induced p38 MAPK activation by metabolites of AA generated via $cPLA_2$ but not PLD, could be due to cellular compartmentalization of AA and/or its metabolites in rabbit VSMC. PLD has been reported to mediate insulin-induced ERK activation in rat-1 fibroblasts (Rizzo et al., 1999), suggesting an important contribution of PLD to MAPK activation in these cells. Indeed, surprisingly, we found that in rat VSMC, NE-induced p38 MAPK activation is mediated through PLD activation. This finding suggests differential contribution of PLD to p38 MAPK activation in rabbit and rat VSMC.

An emerging paradigm in G protein-coupled receptor (GPCR) signaling is the stimulation of cellular effects by GPCR agonists through transactivation of tyrosine kinase receptors such as EGFR (Gschwind et al., 2001). For example, the increase in p38 MAPK activity elicited by angiotensin II and thrombin has been reported to be mediated through EGFR transactivation in rat VSMC (Eguchi et al., 2001; Kanda et al., 2001a). We found that NE caused transactivation of EGFR in rabbit VSMC, resulting in its phosphorylation at Y1068. However, the EGFR kinase inhibitor AG1478, at a concentration that significantly decreased EGF-induced p38 MAPK phosphorylation, failed to attenuate NE-induced p38 MAPK phosphorylation. Therefore, it seems that NE- induced p38 MAPK activation is independent of EGFR transactivation in rabbit VSMC. This is similar to the report that platelet-activating factor-induced EGFR transactivation does not contribute to p38 MAPK activation by this agonist in epidermal cells (Marques et al., 2002), but is in contrast to the report of Eguchi et al. (2001), which demonstrated EGFR-dependent p38 MAPK activation in rat VSMC. To reconcile the differences between these findings, the contribution of EGFR to the activation of p38 MAPK by NE and angiotensin II was compared in rabbit and rat VSMC using the EGFR kinase inhibitor AG1478. In agreement with the report of Eguchi et al. (2001), AG1478 abolished angiotensin II-induced p38 MAPK activation by 96% in rat VSMC; angiotensin II-induced phosphorylation of p38 MAPK in rabbit VSMC was also inhibited (31%) by AG1478. In contrast, AG1478 did not attenuate the phosphorylation of p38 MAPK by NE in either rabbit or rat VSMC (S. Kalyankrishna and K. U. Malik, unpublished observations). This suggests that

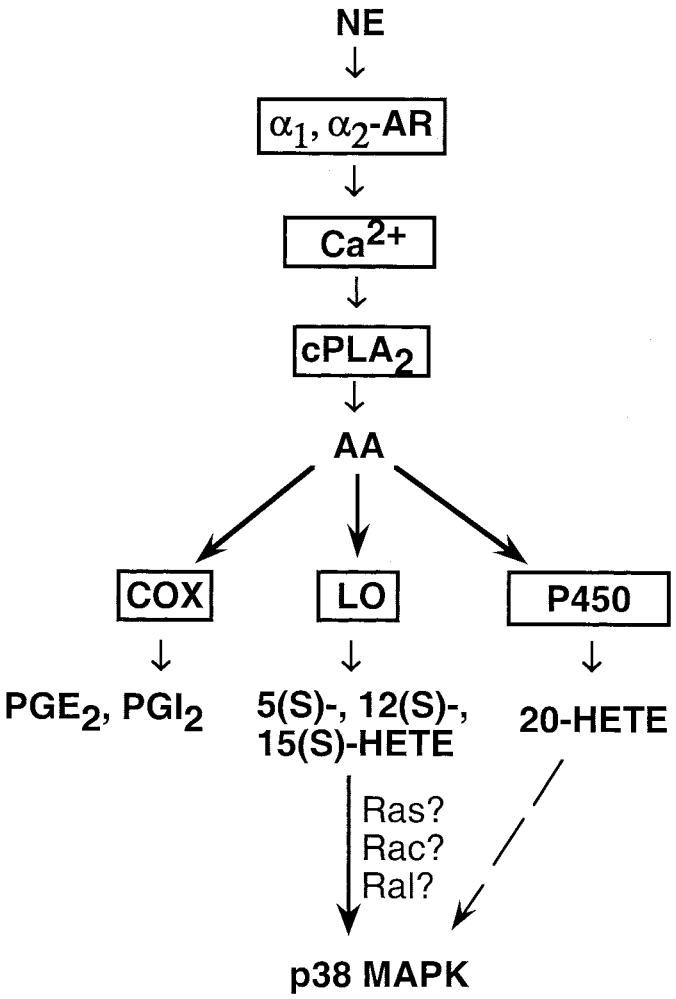

Fig. 9. Proposed model for activation of p38 MAPK by NE in rabbit VSMC. NE, by stimulating α-1 and α-2 ARs, promotes Ca^{2+}-dependent $cPLA_2$-mediated generation of LO metabolites of AA, which results in p38 MAPK activation. The P450 metabolites of AA also have a minor contribution (dotted line) to NE-induced p38 MAPK activation.

the contribution of EGFR transactivation to the regulation of p38 MAPK and perhaps other downstream signaling molecules by GPCRs may vary depending on the agonist and also between VSMC of different species.

We have unearthed significant differences in signaling between the VSMC of rat and rabbit. The PLD and EGFR signaling pathways seem to have a greater contribution to p38 MAPK activation in rat VSMC, compared with rabbit VSMC. In this respect, it is to be noted that rabbit VSMC maintain their contractile state for greater periods of time in culture than rat VSMC, which have an earlier onset of transformation to the synthetic phenotype (Campbell and Campbell, 1993). We are currently extending our studies to correlate variations in signaling pathways with phenotypic differences in rat and rabbit VSMC.

In conclusion, our study demonstrates that $cPLA_2$-dependent generation of LO metabolites of AA, 5(*S*)-HETE, 12(*S*)-HETE, and 15(*S*)-HETE, contribute to NE-induced activation of p38 MAPK by a mechanism that is independent of EGFR transactivation (Fig. 9). The mechanism by which 5(*S*)-, 12(*S*)-, and 15-HETE activate p38 MAPK in VSMC is not known. 12(*S*)-HETE has been shown to activate Rac and Cdc42 GTPases, which in turn activate downstream signaling molecules p21-activated kinase (PAK1) and MKK3/6 (Wen et al., 2000; Yamauchi et al., 2001). 12(*S*)-HETE and 20-HETE have also been reported to activate Ras GTPase in VSMC (Muthalif et al., 1998a; Reddy et al., 2002). Moreover, Ras has been shown to mediate p38 MAPK activation caused by thrombin and low-density lipoprotein in VSMC (Kanda et al., 2001b; Zhu et al., 2001). Because activation of Ras can also increase the activity of Rac (Scita et al., 1999) and Ral (Ouwens et al., 2002), it is possible that HETEs generated through LO in response to NE also increase p38 MAPK activity by activating Rac, Cdc42, and/or Ral via Ras. Acti-

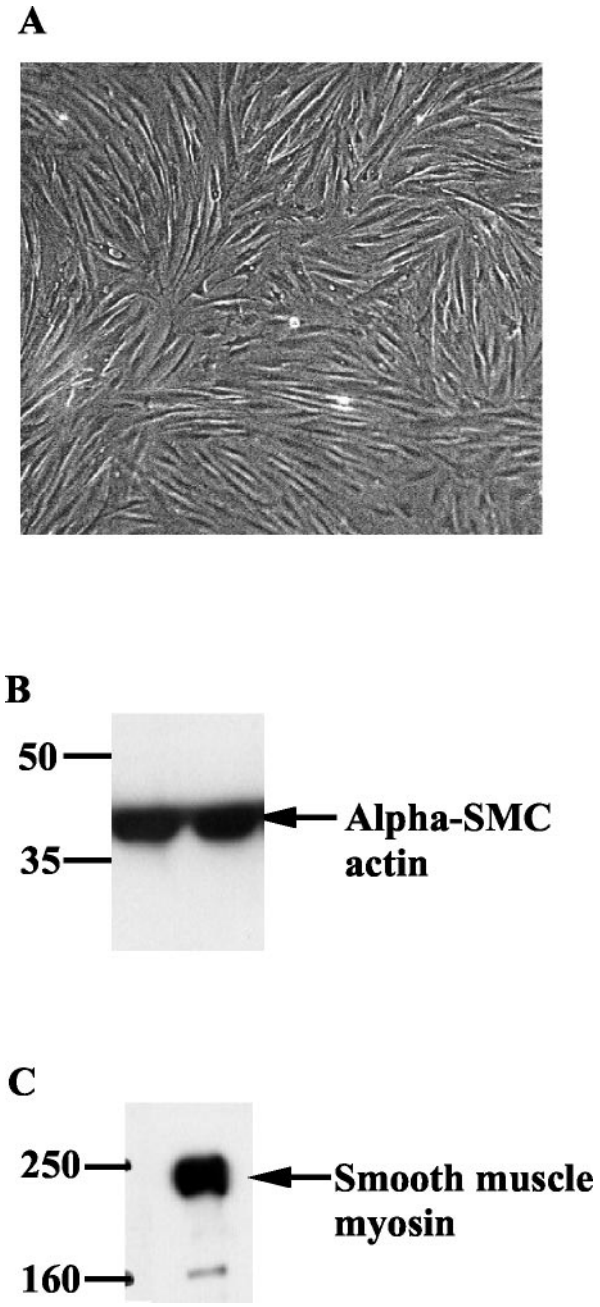

Fig. 10. Characterization of the phenotype of cultured rabbit aortic VSMC. The morphology of confluent rabbit VSMC was observed by light microscopy (A). Expression of the VSMC marker proteins, α-smooth muscle cell (SMC) actin (B) and smooth muscle myosin (C) was determined by Western blot analysis of VSMC lysates.

vation of p38 MAPK by AA metabolites may have important consequences. 12(*S*)-HETE-induced VSMC hypertrophy is mediated by p38 MAPK in VSMC (Reddy et al., 2002). The mechanism by which HETEs activate p38 MAPK via small G proteins and the possible contribution of p38 MAPK to other vascular effects of HETEs, such as migration and contraction, are under current investigation.

Appendix 1

Rabbit VSMC exhibit a spindle-shaped morphology characteristic of contractile VSMC. This morphology was maintained even at the latest passage, i.e., seventh passage VSMC that were used for experiments.

Rabbit VSMC exhibit the presence of α-smooth muscle actin and smooth muscle myosin, as determined by Western Blot analysis. This provides evidence that the cells have maintained their smooth muscle cell phenotype and have not dedifferentiated into a non-smooth muscle phenotype under our culture conditions (Fig. 10).

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