Suppression of smooth-muscle α-actin expression by platelet-derived growth factor in vascular smooth-muscle cells involves Ras and cytosolic phospholipase A_2

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Platelet-derived growth factor (PDGF), which is a potent mitogen for vascular smooth-muscle cells (VSMC), also inhibits the expression of specific smooth-muscle proteins, including smooth-muscle α-actin (SM-α-actin), in these cells. The goal of this study was to identify signalling pathways mediating these distinct effects. In rat aortic VSMC, PDGF caused a rapid activation of Ras and Raf, leading to the activation of mitogen-activated protein kinases (ERKs). Cells stably transfected with constitutively active Ras (H-Ras) expressed low levels of SM-α-actin protein. Arginine vasopressin, which stimulated SM-α-actin promoter activity in wild-type cells or controls (Neo; transfected with a plasmid lacking an insert), failed to do so in cells transiently expressing H-Ras. The effects of Ras on suppression of SM-α-actin expression were not mediated by the Raf/ERK pathway, since cells stably expressing constitutively active Raf (BxB-Raf) had normal levels of SM-α-actin protein, and stimulation of SM-α-actin promoter activity by vasopressin was unaffected in cells transiently expressing BxB-Raf. Furthermore a specific inhibitor of ERK activation had no effect on SM-α-actin expression. Exposure of wild-type VSMC to PDGF, or stable expression of Ras but not Raf, also resulted in constitutive increases in prostaglandin E_2 production and cytosolic phospholipase A_2 ($cPLA_2$) activity, which was mediated by an increased expression of $cPLA_2$ protein. Transient expression of $cPLA_2$ in wild-type VSMC inhibited the stimulation of SM-α-actin promoter activity by vasopressin. These results suggest that PDGF-induced inhibition of SM-α-actin expression is mediated through a Ras-dependent/Raf independent pathway involving the induction of $cPLA_2$ and eicosanoid production.

INTRODUCTION

Platelet-derived growth factor (PDGF) is a potent mitogen for vascular smooth-muscle cells (VSMC) [1]. In addition to its mitogenic effects in these cells, PDGF has been shown to regulate the expression of muscle-specific genes such as the smooth-muscle isoform of α-actin (SM-α-actin). PDGF decreased basal steady-state levels of SM-α-actin protein and blocked the increases in SM-α-actin protein caused by exposure to vasoconstrictors such as angiotensin II or arginine vasopressin (AVP) [2]. We have recently shown that these effects are mediated through the regulation of the SM-α-actin promoter, and have defined two CArG elements that are critical for both vasoconstrictor stimulation and PDGF suppression of promoter activity [3]. Expression of SM-α-actin has been used as a marker for the phenotypic states of VMSC. Developmentally, SM-α-actin expression increases as VSMC undergo differentiation from an embryonic synthetic phenotype to the contractile phenotype characteristic of adult vessels [4,5]. Thus, in addition to its growth-promoting role in these cells, PDGF might also regulate VSMC differentiation. Although a number of post-receptor pathways mediating the mitogenic effects of PDGF have been described, the events leading to the suppression of SM-α-actin expression are not well defined.

In VSMC, PDGF has been shown to activate the extracellular signal-regulated protein kinases (ERKs), members of the mitogen-activated protein kinase (MAP kinase) family [6–8]. Activation of ERKs has been shown to be sufficient for cell proliferation in other cell types [9]. Tyrosine kinase receptors such as the PDGF receptor regulate ERKs through a pathway involving the sequential activation of the low-molecular-mass G-protein Ras, the proto-oncogene serine kinase Raf-1, and the dual-specificity MAP kinase kinase (MEK-1) [10–12]. The goal of the present study was to determine whether elements of the Ras/Raf pathway have a role in the suppression of SM-α-actin expression by PDGF, by examining the effects of expressing constitutively active forms of these proteins in VSMC. We report here that the expression of constitutively active Ras mimics the effects of PDGF to suppress SM-α-actin levels; however, these effects do not seem to be mediated through the activation of Raf-1, but instead involve the Ras-mediated activation of additional effectors.

MATERIALS AND METHODS

Materials

Trypsin/EDTA and Eagle's minimal essential medium (MEM) were from Gibco (Grand Island, NY, U.S.A.). Monoclonal antibody against SM-α-actin was obtained from Boehringer

Abbreviations used: AVP, arginine vasopressin; CAT, chloramphenicol acetyltransferase; $cPLA_2$, cytosolic phospholipase A_2; ERK, extracellular signal-regulated protein kinase; FCS, fetal calf serum; GST, glutathione S-transferase; JNK, cJun N-terminal kinase; MAP, mitogen-activated protein; MEK-1, MAP kinase kinase; MEM, minimal essential medium; Neo, neomycin-resistant, transfected with plasmid lacking an insert; PDGF, platelet-derived growth factor; PGE_2, prostaglandin E_2; SAPK, stress-activated protein kinase; SM-α-actin, smooth-muscle α-actin; VSMC, vascular smooth-muscle cells.

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Mannheim (Germany). PDGF-BB, polyclonal rabbit anti-rat MAP kinase R2(erk1-CT) and polyclonal anti-Ras antibodies were from UBI (Lake Placid, NY, U.S.A.). The catalytically inactive MEK-1 and the Y13-259 anti-Ras antibody were gifts from Gary L. Johnson (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO, U.S.A.). The rabbit antibody to the C-terminus of Raf-1 (C-12) was obtained from Santa Cruz Biotech (Santa Cruz, CA, U.S.A.). The MEK inhibitor PD90859 was a gift from Dr. Alan Saltiel (Parke Davis, Ann Arbor, MI, U.S.A.). [γ-^{32}P]ATP was from Dupont–New England Nuclear (Boston, MA, U.S.A.) and ^{125}I-protein A from ICN (Irvine, CA, U.S.A.). The Bradford protein assay kit was purchased from Bio-Rad (Richmond, CA, U.S.A.).

Culture and transfections of VSMC

Rat aortic VSMC were isolated and cultured as previously described in detail [13,14]. Briefly, aortas were resected from male Sprague–Dawley rats (200–300 g) and cleaned of adventitia and connective tissue. The vessels were chopped and incubated at 37 °C for 2 h in Eagle's MEM containing 2.4 mg/ml collagenase. The resulting cell suspension was plated on 35 mm dishes and grown in Eagle's MEM containing 1 mM L-glutamine, 2 g/l $NaHCO_3$, 100 i.u./ml penicillin, 100 μg/l streptomycin and 10 % (v/v) fetal calf serum (FCS) at 37 °C in a humidified air/CO_2 (19:1) atmosphere. Cells were passaged with trypsin/EDTA.

The human T24 H-Ras cDNA (Gly-12 to Val) was inserted at the *Hin*dIII site of the retroviral expression vector pMV7 [15]. The vector was packaged into replication-defective retrovirus in GP + E-86 cells [16]. Similarly, a cDNA encoding constitutively active Raf-1 (BxB-Raf) [17] was inserted into pLXSN [18] and packaged in GP + E-86 cells. Polybrene (8 μg/ml) was added to the retrovirus-containing medium from the GP + E-86 packaging cells and filtered before a 24 h incubation with subconfluent monolayers of rat VSMC. The infected VSMC were replated and selected for G418 resistance conferred by the neomycin resistance gene encoded within pMV7 and pLXSN. Individual colonies were expanded and screened for expression of Ras and BxB-Raf by immunoblotting. Control cell lines (Neo, neomycin-resistant, transfected with plasmid lacking an insert) were selected by infecting cells with virus lacking a cDNA insert. In indicated cases pools of transfected cells were studied instead of individual clones.

Assay of ERK activity

Cells were incubated in serum-free medium for 20 h before the addition of agonists. Cells were stimulated by 100 nM PMA, 100 nM AVP or 20 ng/ml PDGF for 5 min at 37 °C. After stimulation, cells were rinsed three times with ice-cold PBS and then solubilized with 250 μl of lysis buffer [50 mM β-glycerophosphate (pH 7.2)/100 μM Na_3VO_4/2 mM $MgCl_2$/1 mM EGTA/0.5 % Triton X-100/1 mM dithiothreitol). Lysates were centrifuged at 12000 ***g*** for 10 min and supernatants were matched for protein. MAP kinase activity was assayed by phosphorylation of myelin basic protein, with a modification of the method previously described [7,8]. The assay was performed at 30 °C for 15 min in a final volume of 40 μl containing 12.5 mM $MgCl_2$, 50 μg/ml PKI, 1 mg/ml myelin basic protein and 50 μM [γ-^{32}P]ATP. Reactions were terminated by the addition of 10 μl of 25 % (w/v) trichloroacetic acid. Portions (25 μl) of the acidified reaction mixtures were spotted on 1.5 cm × 1.5 cm phosphocellulose paper (Whatman P-81). After four washes in 75 mM phosphoric acid and one wash in acetone, ^{32}P-labelled protein was counted by liquid scintillation. MAP kinase activity was expressed as pmol of ^{32}P/min per mg of protein. To confirm the specificity of this assay, p42/44 MAP kinases were immunoprecipitated from cell lysates with anti-ERK-1, and anti-ERK-2 antibodies and activity were measured in the immunoprecipitate as described previously [19].

Determination of Ras activity

Activation of Ras was determined by analysing the ratio of GTP to GDP bound to immunoprecipitated Ras [20,21]. Cells grown on 100 mm dishes were serum-starved in phosphate-free medium and labelled with 30 μCi/ml of [^{32}P]P_i for 5 h. Cells were then stimulated for 5 min with PDGF. After this stimulation, cells were lysed in lysis buffer [Tris/HCl-buffered saline (pH 7.5)/16 mM $MgCl_2$/1 % NP-40/10 μg/ml leupeptin/0.1 mg/ml aprotinin] containing 200 ng/ml Y13-259 anti-Ras antibody. The lysates were centrifuged at 12000 ***g*** for 10 min. Supernatants were incubated on ice for 1 h. Agarose beads coupled to goat anti-(rat IgG) were added to lysates and incubated for a further 1 h. The beads were washed eight times in lysis buffer without protease inhibitors or antibody. Ras was eluted from the beads by boiling for 3–5 min in boiling solution (0.2 % SDS/2 mM EDTA/2 mM dithiothreitol). Guanine nucleotides were resolved by poly(ethyleneimine)–cellulose TLC with 0.75 M KH_2PO_4, pH 3.4. GTP and GDP were detected by co-migration of unlabelled standards. The ratio of [^{32}P]GTP/[^{32}P]GDP was quantified by densitometry.

Assay of cJun N-terminal kinase (JNK) activity

The activity of JNK/stress-activated protein kinase (JNK/SAPK) was determined essentially as described previously [22]. Cells were stimulated for 15 min and lysed in MAP kinase lysis buffer. After a 5 min microcentrifugation at 10000 ***g***, aliquots of the soluble extracts containing 400 μg of protein were incubated for 2 h at 4 °C with glutathione S-transferase (GST)-conjugated cJun(1–79) adsorbed on glutathione–agarose as described [22]. The GST–cJun(1–79) beads were washed four times by repetitive centrifugation in 20 mM Hepes (pH 7.7)/50 mM NaCl, 2.5 mM $MgCl_2$/0.1 mM EDTA/0.05 % Triton X-100 and then incubated for 20 min at 30 °C in 40 μl of 50 mM β-glycerophosphate (pH 7.6)/0.1 mM Na_3VO_4/10 mM $MgCl_2$/20 μM [γ-^{32}P]ATP (25000 c.p.m./pmol). The reactions were terminated with 10 μl of SDS/PAGE sample buffer, boiled and subjected to SDS/PAGE [10 % (w/v) gel]. The GST–cJun(1–79) polypeptides were identified in Coomassie-stained gels and excised, then counted in a scintillation counter.

Immunoblotting

Cells were washed twice with PBS and lysed in ERK lysis buffer. Extracts matched for protein were separated by SDS/PAGE [23]. Proteins were transferred electrophoretically to Immobilon-P. Ras proteins were probed with a polyclonal anti-Ras antibody followed by ^{125}I-Protein-A. MAP kinase was detected with a polyclonal antibody specific for p42/44 MAP kinase followed by ^{125}I-Protein-A. SM-α-actin was detected with a specific monoclonal antibody against SM-α-actin followed by ^{125}I-protein-A. Levels of cytosolic phospholipase A_2 (cPLA_2) were detected with a polyclonal antibody raised against a recombinant fragment of the cPLA_2 molecule [24], followed by ^{125}I-protein A. ^{125}I-labelled protein bands were detected by autoradiography with Kodak-X p-Omat RP film.

Measurement of SM-α-actin promoter activity

Cells were transiently co-transfected with 15 μg of the pCAT-α-actin construct (where CAT represents chloramphenicol acetyltransferase) containing 713 base pairs of the 5′ region previously described [3] [pCATACT(−713/52)], along with 5 μg of H-Ras (inserted into pCMV5), BxB-Raf (inserted into pLXSN) or $cPLA_2$ cDNA [25]. Vector lacking an insert was used as a control. Each transfection also received 5 μg of CMV-β-galactosidase vector (Clontech, Palo Alto, CA, U.S.A.) to normalize for transfection efficiency. Cell suspension (100 μl) in full culture medium containing 2×10^7 cells/ml was transfected by electroporation with a geneZAPPER (IBI). The cells were then plated in culture medium with 10 % (v/v) FCS for 18 h. At this time the cells were placed in Eagle's MEM with 0.2 % (v/v) FCS and incubated with or without AVP for 72 h. Duplicate samples were electroporated for each treatment.

After exposure to the hormones, cells were harvested by trypsinization and cell pellets frozen at −20 °C. The CAT activity of the cell lysates was measured with a modification of the thin-layer chromatographic method as described by Gorman et al. [26]. CAT activity was normalized to the β-galactosidase activity [27] present in the same sample and was calculated as pmol of chloramphenicol acetylated/h per m-unit of β-galactosidase.

Measurement of prostaglandin E_2 (PGE_2) production and PLA_2 activity

Cells were incubated for 15 min in the absence or presence of agonist. Medium was then removed and PGE_2 production was determined with a radioimmunoassay kit (Amersham). Cell pellets were saved to determine protein, and results were expressed as pg PGE_2/mg of protein. PLA_2 activity was determined as previously described [28]. Briefly, cells were homogenized in 50 mM Hepes (pH 7.5)/1 mM EDTA/1 mM EGTA/250 mM sucrose/20 μM pepstatin/20 μM leupeptin/100 μM PMSF/1000 units/ml aprotinin with a Dounce homogenizer. Soluble extracts were prepared by differential centrifugation as previously described and matched for protein concentration. In a reaction volume of 40 μl, extracts were incubated with 15 μM [^{14}C]arachidonoyl phosphatidylcholine (specific radioactivity approx. 57 mCi/mmol) in the presence of 4 mM $CaCl_2$ for 30 min at 37 °C. The reaction was quenched by the addition of 40 μl of unlabelled arachidonic acid (500 μg/ml) in ethanol/acetic acid (98:2, v/v), and 50 μl of each sample was spotted on Whatman LK5D silica gel TLC plates. The plates were developed in ethyl acetate/iso-octane/acetic acid/water (55:75:8:100, by vol.) from which the lower, aqueous, phase had been removed. Plates were stained with iodine vapour; arachidonic acid peaks and phosphatidylcholine peaks were scraped with a razor blade into vials and counted by liquid scintillation in the presence of 250 μl of water and 5 ml of Opti-Fluor. Results are expressed as pmol/min per mg of protein.

Statistical analysis

Results are expressed as means ± S.E.M. Differences between means were evaluated by Student's t test or ANOVA with the Newman–Keuls multiple comparisons procedure. A value of $P < 0.05$ was taken to be significant.

RESULTS

Transfection of VSMC with H-Ras

In rat aortic VSMC, both PDGF and epidermal growth factor stimulated Ras activity as defined by increased GTP loading

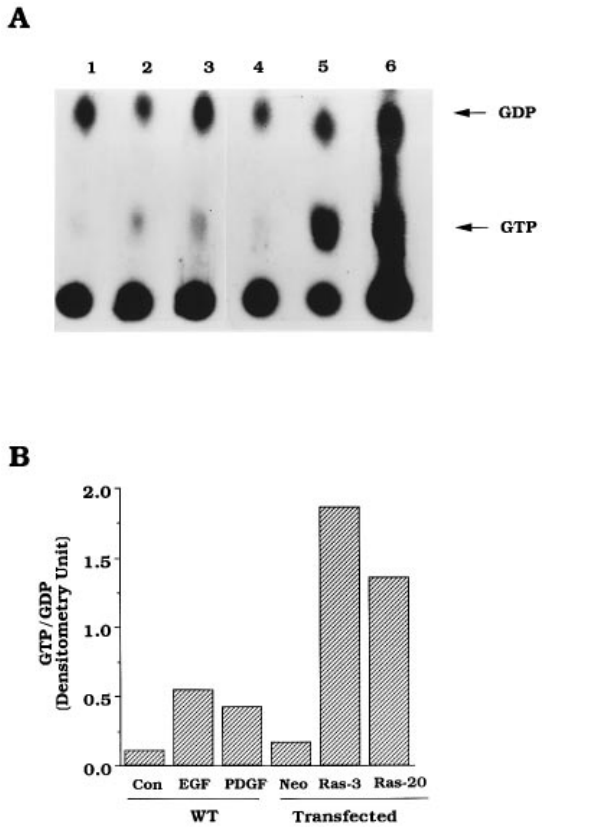

Figure 1 Ras activity in VSMC

(**A**) Cells grown on 100 mm dishes were serum-starved in phosphate-free medium and labelled with 30 μCi/ml [^{32}]P_i for 5 h. Cells were then stimulated for 5 min with PDGF or EGF. After this stimulation, cells were lysed in buffer containing 200 ng/ml Y13-259 anti-Ras antibody and Ras was immunoprecipitated as described in the Materials and methods section. Guanine nucleotides bound to Ras were resolved by poly(ethyleneimine)–cellulose TLC followed by autoradiography. GTP and GDP were detected by co-migration of unlabelled standards. (**B**) The ratio of ^{32}P-GTP/^{32}P-GDP was quantified by densitometry. Abbreviations: Con, control; EGF, epidermal growth factor.

(Figure 1). Stimulation of cells with AVP, angiotensin II or PMA, which also stimulate ERK activity, did not result in a significant stimulation of Ras activity (results not shown). To examine the effects of Ras on VSMC phenotype and signalling pathways, cells were stably transfected with a constitutively active H-Ras G12V cDNA. Stable transfectants were selected by their ability to grow in G-418, and were screened by immunoblotting with anti-Ras antibodies. Cells expressing H-Ras grew more slowly than Neo controls or wild-type cells, but had normal morphology by phase contrast, and failed to form colonies in soft agar (X. Li and R. A. Nemenoff, unpublished work). Two clones, Ras-3 and Ras-20, were selected for further characterization. In both clones, Ras activity was constitutively increased to levels higher than achieved by stimulation of wild-type or Neo cells (Figure 1) by growth factor. Expression of constitutively active Ras resulted in an increased basal ERK activity (Figure 2A). In Ras-3 cells, activity was further increased by stimulation with either AVP or PDGF, whereas in Ras-20, ERK activity was maximally increased, and no further stimulation with agonists was observed.

Levels of steady-state SM-α-actin protein expression were examined by immunoblotting. Both Ras-3 and Ras-20 cells had low or undetectable steady-state levels of SM-α-actin compared

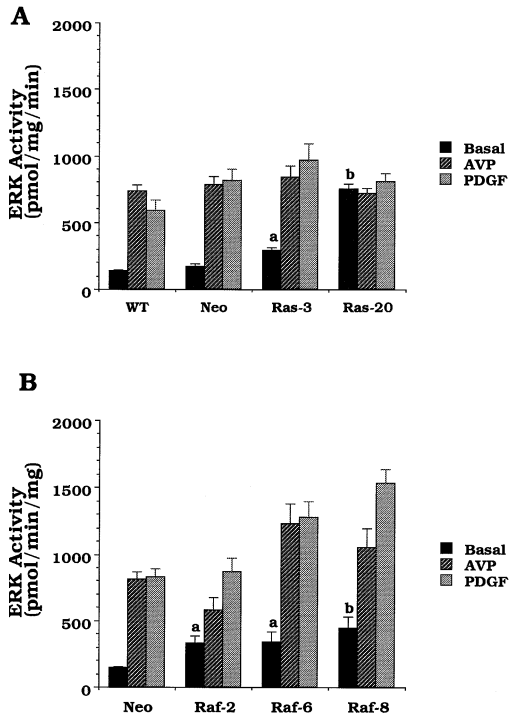

Figure 2 ERK activity in VSMC stably expressing H-Ras (A) and BxB-Raf (B)

Cells were placed in serum-free medium overnight and then incubated for 5 min with 100 nM PMA, 100 nM AVP or 20 ng/ml PDGF. Cell lysates were prepared and matched for protein. Extracts were assayed for ERK activity by using myelin basic protein. Activity measurements represent the means ± S.E.M. for four separate experiments. Statistical significance: a, $P < 0.05$ compared with wild type; b, $P < 0.01$ compared with wild type.

with Neo controls or untransfected VSMC (Figure 3A). To determine whether this effect of Ras was mediated through regulation of the SM-α-actin promoter, wild-type cells were transiently co-transfected with a 713 bp region of the SM-α-actin promoter ligated into a promoterless CAT vector [3], along with either a plasmid encoding H-Ras, or the same plasmid lacking an insert (Neo). Cells were then exposed to AVP for 72 h, which induces promoter activity [3], and CAT activity was determined. AVP induced promoter activity approx. 5-fold in cells co-

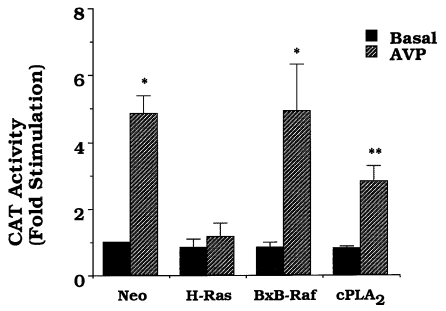

Figure 4 Regulation of SM-α-actin promoter activity

Wild-type VSMC were co-transfected with pCAT-α-actin construct pCATACT(−713/52) together with plasmids encoding H-Ras, BxB-Raf or $cPLA_2$. Control transfections were performed with pcDNA-3 lacking an insert (Neo). Cells were then exposed for 3 days to 0.2% (v/v) FCS alone (Basal) or 0.2% FCS/AVP. CAT activity, normalized to β-galactosidase activity, was determined. The results represent the means ± S.E.M. for three independent experiments. $^{*}P < 0.05$ compared with basal; $^{**}P < 0.05$ compared with Neo + AVP or BxB-Raf + AVP.

transfected with control vector, but failed to stimulate promoter activity cells transfected with H-Ras (Figure 4). Thus the expression of constitutively active Ras in VSMC mimicked the ability of PDGF to suppress SM-α-actin expression.

Transfection of VSMC with BxB-Raf

To establish whether the effects of Ras on SM-α-actin expression were mediated through the activation of Raf, VSMC were stably transfected with a constitutively active form of Raf-1 (BxB-Raf). G-418-resistant clones were screened by immunoblotting with anti-Raf antibodies. Similarly to Ras-transfected cells, cells expressing BxB-Raf grew more slowly than Neo controls and were not transformed, as assessed by their inability to form colonies in soft agar (X. Li and R. A. Nemenoff, unpublished work). Three clones (Raf-2, Raf-6 and Raf-8) were selected for further characterization. All three clones had elevated levels of basal ERK kinase activity, comparable to that seen with one of the Ras clones (Figure 2B). ERK activity could be further stimulated by the addition of either AVP or PDGF. The stimulated activity was greater than that obtained in Neo controls treated with the same agents. In contrast with cells expressing H-

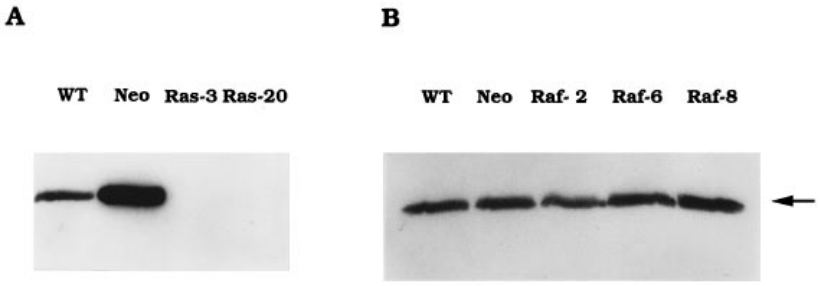

Figure 3 Regulation of SM-α-actin protein in VSMC expressing H-Ras (A) and BxB-Raf (B)

The indicated cell lines were grown in full medium containing 10% (v/v) FCS. Lysates were prepared, matched for protein and immunoblotted with an antibody against SM-α-actin. The arrow indicates the migration position of SM-α-actin. (**A**) Cells expressing H-Ras; no detectable SM-α-actin was detected in either of the Ras clones despite extreme overexposure of the autoradiograms. (**B**) Cells expressing BxB-Raf; all clones had comparable levels of SM-α-actin as determined by densitometry.

Table 1 Effect of PD90859 and wortmannin on regulation of SM-α-actin promoter in WT-VSMC

For studies with PD90859, wild-type VSMC were transiently co-transfected with the full-length pCAT-α-actin construct pCATACT(−713/52) together with a cytomegalovirus (CMV)-β-galactosidase vector. After attachment in full medium, cells were incubated with medium containing 0.2% (v/v) FCS alone (basal), medium containing 0.2% (v/v) FCS and AVP, or PDGF for 72 h in the absence (DMSO alone) or presence of 50 μM PD09859. Extracts were assayed for CAT activity and normalized to β-galactosidase activity in the same sample. For studies with wortmannin, separate dishes of cells were transfected under identical conditions and exposed to agonists in the absence (DMSO alone) or presence of 1 μM wortmannin. Extracts were assayed for CAT activity as with PD90859. Results, expressed as pmol of chloramphenicol acetylated/h per m-unit of β-galactosidase, are means ± S.E.M. for three or four experiments for PD90859, and for five or six experiments for wortmannin. $^*P < 0.05$ compared with basal or PDGF by using a paired t test.

	CAT activity (pmol/h per m-unit of β-galactosidase)			
Incubation	−PD90859	+PD90859	−Wortmannin	+Wortmannin
Basal	3.25 ± 0.4	5.53 ± 1.6	8.2 ± 2.5	8.3 ± 0.5
AVP	23.5 ± 1.8*	21.7 ± 1.8*	42.8 ± 5.5*	36.6 ± 5.1*
PDGF	0.69 ± 0.1	1.4 ± 1.0	1.3 ± 0.5	2.4 ± 1.0

RasG12V, cells expressing BxB-Raf had steady-state levels of SM-α-actin protein comparable to those observed in Neo controls or wild-type cells (Figure 3B). In contrast with results obtained with H-Ras, transient co-transfection with BxB-Raf did not impair the ability of AVP to stimulate SM-α-actin promoter activity (Figure 4).

The lack of involvement of the Raf/MEK/ERK pathway in mediating the suppression of SM-α-actin expression was further confirmed with a specific MEK inhibitor, PD90859 [29,30]. Pretreatment of wild-type VSMC with 50 μM PD90859 for 30 min completely blocked the ability of either AVP or PDGF to stimulate ERK activity (results not shown), consistent with what has been observed in VSMC by other workers [31]. Exposure for 3 days to this concentration of inhibitor did not impair either the ability of AVP to stimulate SM-α-actin promoter activity or of PDGF to suppress promoter activity (Table 1).

Other Ras effectors

Because the constitutive activation of Ras but not Raf led to the suppression of SM-α-actin, other potential effectors of Ras were examined. A number of studies have indicated that Ras activation can lead to the activation of another branch of the MAP kinase family: JNK/SAPK [32]. Stable expression of constitutively active Ras resulted in a modest increase in basal JNK/SAPK activity (Ras-3, 2.10 ± 0.82-fold; Ras-20, 3.98 ± 1.35-fold compared with Neo). Expression of BxB-Raf did not increase JNK/SAPK activity above basal levels seen in Neo or wild-type cells (results not shown). Activation of Ras has also been implicated in the activation of phosphatidylinositol 3-kinase [33]. However, wortmannin, an inhibitor of phosphatidylinositol 3-kinase [34], did not affect either the ability of AVP to stimulate SM-α-actin promoter activity or the ability of PDGF to suppress activity (Table 1).

Prostaglandin production

VSMC are vigorous producers of eicosanoids, which may have a role in the regulation of growth in these cells. PDGF stimulates PGE_2 production in VMSC, resulting in the activation of protein kinase A [35]. We therefore examined PGE_2 production in VSMC expressing constitutively active Ras. In both clones

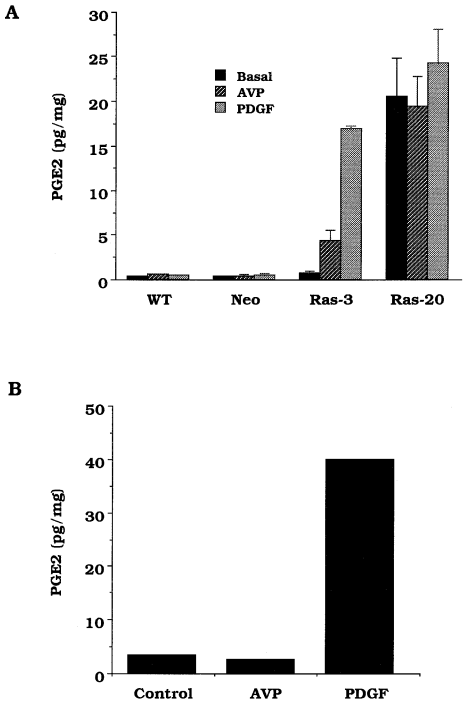

Figure 5 PGE_2 production in VSMC

(**A**) PGE_2 production in cells expressing H-Ras. Cells expressing H-Ras, or Neo controls, were incubated overnight in serum-free medium. Cells were then acutely stimulated for 20 min with AVP (100 nM) or PDGF (20 ng/ml), or received no further addition. Medium was collected at the end of the incubation and assayed for PGE_2 production with a radioimmunoassay kit. (**B**) Effect of chronic exposure to PDGF on PGE_2 production. Wild-type VSMC were incubated for 3 days in the presence of medium containing 0.2% (v/v) FCS or medium containing AVP (100 nM) or PDGF (20 ng/ml). Cells were then stimulated for 20 min with ionomycin (100 nM) and PMA (100 nM) for 20 min. Medium was collected and PGE_2 production determined.

expressing H-Ras, PGE_2 production was markedly elevated compared with Neo controls (Figure 5A). Exposure of untransfected cells to PDGF for 3 days caused a similar increase in PGE_2 production, whereas AVP had no effect (Figure 5B). Release of arachidonic acid, through the action of phospholipase A_2, represents the rate-limiting step in eicosanoid production in most cells [36]. We and others have described an arachidonic-selective isoform of PLA_2 ($cPLA_2$) whose activity is regulated by growth factors [25,28,37–39]. To determine whether increases in $cPLA_2$ activity were associated with the enhanced PGE_2 production observed in Ras-transfected VSMC, extracts were prepared and assayed for $cPLA_2$ activity. Both clones expressing H-Ras had increased $cPLA_2$ activity compared with either wild-type or Neo cells (Figure 6A). Increased $cPLA_2$ activity was also detected in pools of Ras-transfected cells. In contrast, $cPLA_2$ activity in three independent Raf clones was not significantly different from those in Neo or wild-type cells. Exposure to PDGF also constitutively induced steady-state $cPLA_2$ activity (Figure 6B). Immunoblotting of extracts with anti-$cPLA_2$ antibodies demonstrated that steady-state levels of $cPLA_2$ were increased 3–5-fold in the Ras clones (Figure 6C). To establish directly a role for $cPLA_2$ in mediating the suppression of SM-α-actin expression, wild-type VSMC were transiently co-tranfected with plasmids encoding $cPLA_2$ and the SM-α-actin promoter/CAT. Expression of $cPLA_2$ decreased AVP stimulation of promoter activity by approx. 50% (Figure 4).

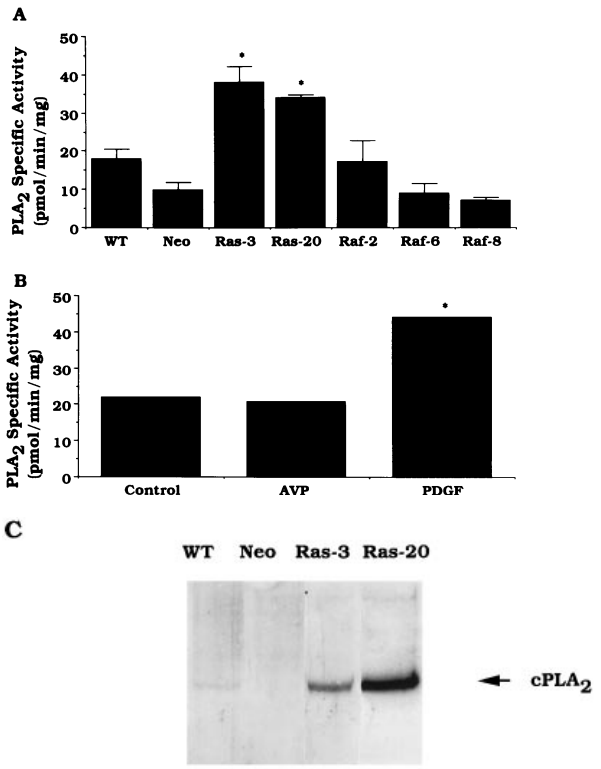

Figure 6 $cPLA_2$ activity in VSMC expressing H-Ras and BxB-Raf

(**A**) PLA_2 activity in transfected cells. Cells were placed in serum-free medium overnight and then homogenized in PLA_2 homogenization buffer. Cytosolic fractions were prepared by centrifugation. Soluble extracts were matched for protein and assayed for PLA_2 activity with [^{14}C]arachidonoyl phosphatidylcholine as substrate in the presence of 5 mM Ca^{2+} as described in the Materials and methods section. Results are expressed as pmol/min per mg of protein and represent the means of two experiments with triplicate determinations in each experiment. $^*P < 0.05$ compared with wild-type or Neo cells. (**B**) Effect of PDGF on $cPLA_2$ activity. Wild-type VSMC were placed for 3 days in medium containing 0.2% (v/v) FCS alone (control) or medium containing 100 nM AVP (AVP) or medium containing 20 ng/ml PDGF (PDGF). After 3 days cell lysates were prepared as in (**A**), matched for protein and assayed for $cPLA_2$ activity. $^*P < 0.05$ compared with control or AVP. (**C**) Immunoblotting. Extracts from wild-type, Neo and H-Ras-expressing cells were prepared, then separated by SDS/PAGE and immunoblotted with a polyclonal antibody against $cPLA_2$.

DISCUSSION

PDGF engages multiple signalling pathways through the activation of the PDGF receptor tyrosine kinase [40–42]. These include the activation of phospholipase Cγ, phosphatidylinositol 3-kinase, c-Src and Ras. In this study we sought to delineate the pathways mediating the PDGF-induced suppression of SM-α-actin expression in VSMC. Decreased expression of SM-α-actin and other muscle-specific genes precedes the onset of cell proliferation and migration to the intima in atherosclerotic VSMC [43,44]. Furthermore vasoconstrictors that are comitogens for VSMC increase the expression of these genes, suggesting that the suppression of SM-α-actin expression is not secondary to a growth response. It was therefore reasonable to hypothesize that PDGF suppression of SM-α-actin expression might be mediated by a signalling pathway distinct from those promoting cell proliferation.

Our results suggest that the activation of Ras is sufficient to suppress SM-α-actin expression. Cells stably expressing constitutively active H-Ras showed undetectable levels of SM-α-actin compared with Neo controls. Furthermore the AVP-induced stimulation of SM-α-actin promoter activity was blocked in cells transiently expressing H-Ras. The failure of AVP to increase SM-α-actin expression was not due to the absence of functional AVP receptors, because AVP stimulated ERK activity in the Ras-3 clone. It should be noted that AVP stimulation of ERK activity in VSMC is PKC-dependent and does not involve the significant activation of Ras or Raf [8]. Some degree of heterogeneity was observed in these Ras clones, with Ras-20 cells having a much larger increase in basal ERK activity than Ras-3 cells, in spite of comparable levels of Ras activity. Morphologically these two cell lines were indistinguishable and had comparable growth rates. We examined several other clones expressing H-Ras; although all had increased basal ERK activity, there was heterogeneity in this response. We would propose that this heterogeneity is a result of a differential activation of compensatory mechanisms. Ras transformation has also been shown to suppress SM-α-actin expression in fibroblasts [45]. In these cells the expression of serum response factor restored SM-α-actin expression and reversed the transformed phenotype induced by Ras.

The effects of Ras on the suppression of SM-α-actin do not seem to be mediated by the Raf/ERK pathway. The stable expression of BxB-Raf, which resulted in an activation of basal ERK activity similar to that with H-Ras, had no effect on steady-state levels of SM-α-actin protein. Unlike Ras, transient expression of BxB-Raf did not impair the ability of AVP to induce promoter activity. The inability of BxB-Raf to suppress SM-α-actin expression was not due to differential activation of ERKs or to differences in the growth properties of the two cell types. Consistent with these results, a specific MEK inhibitor that blocked ERK activation completely had no effect on the regulation of SM-α-actin expression by either AVP or PDGF.

We therefore propose that the effects of PDGF on SM-α-actin expression are mediated through the Ras-induced activation of a pathway distinct from Raf/MEK/ERK. We have attempted to confirm this finding by examining the ability of a dominant-negative Ras construct (N17Ras) to block this effect of PDGF. However, transfection efficiency in the presence of N17Ras was extremely low compared with controls. We concluded from these studies that the expression of N17Ras was cytotoxic to VSMC and we therefore could not use this approach to examine the regulation of SM-α-actin expression. A number of other effectors of Ras have been described [46,47]. VSMC expressing H-Ras had moderately increased basal JNK/SAPK activity, which was not detected in cells expressing BxB-Raf (results not shown). This is consistent with other studies in which expression of H-Ras activated JNKs modestly compared with the expression of members of the Rho family [48,49]. We have recently demonstrated that AVP and angiotensin II, which increase SM-α-actin expression, stimulated JNK activity 5–8-fold in VSMC (X. Li and R. A. Nemenoff, unpublished work), making it unlikely that Ras-induced activation of JNKs mediates the suppression of SM-α-actin expression. The inability of wortmannin to affect the regulation of SM-α-actin expression also suggests that phosphatidylinositol 3-kinase, another Ras effector [33], does not have a major role in this process.

Expression of H-Ras, but not BxB-Raf, did, however, increase PGE_2 production and increased expression of $cPLA_2$. Analogously, exposure of wild-type-cells to PDGF increased constitutive $cPLA_2$ activity and PGE_2 production, whereas vasoconstrictors had no similar effect. Although we have previously shown that acute regulation of $cPLA_2$ activity involves phosphorylation of the enzyme by ERKs [50], the constitutive increase in activity induced by Ras seems to be a consequence of the increased expression of $cPLA_2$ protein. The ability of transient

expression of $cPLA_2$ to block the stimulation of SM-α-actin promoter activity by AVP suggests that this induction of $cPLA_2$ has a role in the suppressive effect of PDGF on SM-α-actin expression. Although PGE_2 is the major eicosanoid produced by VSMC, the available results do not support a role for this molecule in the suppression of SM-α-actin expression. PGE_2 increases cAMP levels [35], which has antimitogenic effects in VSMC cells [51]. Although chronic elevation of cAMP has also been reported to regulate mRNA levels negatively for both SM-α-actin and tropomyosin, this effect is mediated through increases in mRNA degradation and not inhibition of transcription [52]. We have found no direct effect of cAMP on SM-α-actin promoter activity [3]. Exposure of cells to exogenous PGE_2 or to the cyclo-oxygenase inhibitor indomethacin failed to affect vasoconstrictor-induced increases in promoter activity (results not shown). Because VSMC have been shown to produce a variety of eicosanoids whose biological function in these cells remains to be determined [53], we propose that additional eicosanoids, perhaps produced by lipoxygenase pathways, might mediate the effects of PDGF on SM-α-actin expression.

Enhanced release of arachidonic acid has been associated with the transformation of fibroblasts by H-Ras [54]. Several lines of non-small-cell lung cancer cells, which have a high incidence of Ras mutations, have been shown to produce prostaglandins [55,56]. Our own preliminary studies in non-small-cell lung cancer reveal that cell lines bearing activating mutations in Ki-Ras also exhibit strong induction of $cPLA_2$ and high basal PGE_2 production (L. E. Heasley, B. Price and R. A. Nemenoff, unpublished work). Thus Ras-dependent increases in $cPLA_2$ expression might represent a more widespread signalling pathway regulating growth and differentiation. The mechanism whereby Ras induces $cPLA_2$ expression remains to be defined, although activation of the $cPLA_2$ promoter through a pathway independent of Raf/MEK/ERK is attractive. Multiple potential regulatory elements have been identified in the $cPLA_2$ promoter [57–59]. Recent studies suggest that the low-molecular-mass G-protein Rac might be implicated in this process [60].

In summary, we have demonstrated that PDGF-activation of Ras mediates two distinct signalling pathways in VSMC. One of these, through the activation of Raf, stimulates ERK activity and is likely to contribute to the proliferative response initiated by PDGF. A second pathway, involving the induction of $cPLA_2$, suppresses SM-α-expression and promotes conversion to the proliferative phenotype. The ability of PDGF, as distinct from vasoconstrictors, to promote conversion to the proliferative phenotype might underlie the specific growth responses seen with these agents: proliferation compared with hypertrophy.

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