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### Research Article

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# The Mitogen-activated Protein Kinase Pathway Can Mediate Growth Inhibition and Proliferation in Smooth Muscle Cells

## Dependence on the Availability of Downstream Targets

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### Abstract

Activation of the classical mitogen-activated protein kinase (MAPK) pathway leads to proliferation of many cell types. Accordingly, an inhibitor of MAPK kinase, PD 098059, inhibits PDGF-induced proliferation of human arterial smooth muscle cells (SMCs) that do not secrete growth-inhibitory PGs such as PGE<sub>2</sub>. In striking contrast, in SMCs that express the inducible form of cyclooxygenase (COX-2), activation of MAPK serves as a negative regulator of proliferation. In these cells, PDGF-induced MAPK activation leads to cytosolic phospholipase A<sub>2</sub> activation, PGE<sub>2</sub> release, and subsequent activation of the cAMP-dependent protein kinase (PKA), which acts as a strong inhibitor of SMC proliferation. Inhibition of either MAPK kinase signaling or of COX-2 in these cells releases them from the influence of the growth-inhibitory PGs and results in the subsequent cell cycle traverse and proliferation. Thus, the MAPK pathway mediates either proliferation or growth inhibition in human arterial SMCs depending on the availability of specific downstream enzyme targets. (*J. Clin. Invest.* 1997. 100:875–885.)  
Key words: cyclooxygenase • human • phospholipase • platelet-derived growth factor • prostaglandin E

### Introduction

The classical mitogen-activated protein kinase (MAPK)<sup>1</sup> signaling pathway is a multistep phosphorylation cascade that transmits signals from various cell surface receptors to cytosolic

and nuclear targets in a number of different cell types (1). Activation of the MAPK pathway is initiated by ligand-binding to the cell surface receptor, activation of the receptor, and binding of adapter molecules (such as GRB<sub>2</sub>) to phosphotyrosine residues in the activated receptor or to proteins phosphorylated by the receptor, followed by activation of the small GTP-binding protein Ras by a guanine nucleotide exchange factor (e.g., SOS). Sequential phosphorylation leads to activation of the protein kinases Raf, MAP kinase kinase (MAPKK or MEK), and MAPK (also known as extracellular-signal regulated kinase, Erk). Two isoforms of MAPK, the p44 MAPK (Erk-1), and the p42 MAPK (Erk-2), are expressed in most cell types. The substrates of MAPK include nuclear transcription factors such as Ets proteins (2) and nonnuclear substrates such as the protein serine/threonine kinase p90<sup>orsk</sup>, cytoskeletal proteins, and cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) (3). cPLA<sub>2</sub> catalyzes the release of arachidonic acid from phospholipids in membranes and is one of the rate-limiting steps in the synthesis of PGs, thromboxanes, leukotrienes, and other arachidonic acid metabolites (4, 5).

Early on, the MAPK cascade was suggested to be a regulator of eukaryotic cell cycle progression. This concept was based on the fact that virtually all growth regulatory molecules activate MAPK, and on the striking homology of MAPK with two protein kinases, KSS-1 and FUS-3, which regulate cell cycle progression in yeast (6). An important role for MAPK in cell proliferation was later confirmed when dominant negative mutations in MAPKK, or overexpression of MAPK phosphatase-1, which inactivates MAPK, were shown to lead to reduced DNA synthesis and proliferation (7–9), whereas overexpression of a constitutively active MAPKK caused transformation-associated changes (7, 10). All of these studies are consistent with the concept that MAPKK and MAPK activities are directly correlated with proliferation. However, it is now clear that in addition to regulating proliferation, the MAPK cascade may be involved in a variety of biological effects, such as differentiation (11), cell attachment (12), smooth muscle contraction (13), and protein synthesis (14), depending on stimuli and cell type. For example, epidermal growth factor (EGF) activation of the MAPK pathway leads to proliferation, whereas nerve growth factor (NGF)-stimulated MAPK activation leads to differentiation in PC12 cells (11). However, it is not completely understood how activation of the same MAPK pathway can induce distinct biological responses.

To address this question, we took advantage of a synthetic, cell-permeable, noncompetitive inhibitor of MAPKK that had been identified by screening of a compound library (15). The inhibitor, PD 098059 ([2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one]), inhibits activation of MAPKK in intact cells (16) and has been shown to inhibit proliferation and to reverse the transformed phenotype induced by Ras in specific

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1. Abbreviations used in this paper: COX, cyclooxygenase; COX-2, the inducible isoform of cyclooxygenase; cPLA<sub>2</sub>, cytosolic phospholipase A<sub>2</sub>; EC<sub>50</sub>, concentration required for half-maximal stimulation; IC<sub>50</sub>, concentration required for half-maximal inhibition; MAPK (Erk), mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MBP, myelin basic protein; NGF, nerve growth factor; PDGF-BB, PDGF B-chain homodimer; PKA, cAMP-dependent protein kinase; PKI, protein kinase A inhibitor; SMC, smooth muscle cell.

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cell lines (15). This study was undertaken to elucidate the role of MAPK activation in normal human, diploid, arterial smooth muscle cells (SMCs). Proliferation of these cells is a key event in the formation and progression of atherosclerotic lesions and in restenosis after angioplasty (17). Thus, knowledge of the intracellular signals leading to SMC proliferation may be of clinical relevance. Using PD 098059, we show that MAPK activation induced by PDGF leads to proliferation of SMCs that do not secrete growth-inhibitory PGs. In contrast, in SMCs that secrete large amounts of inhibitory PGs, activation of the same pathway results in an attenuated mitogenic response due to a negative feedback mechanism. The inducible isoform of cyclooxygenase (COX-2), a downstream target regulating prostanoic acid release, is identified as the highly regulated molecule whose availability dramatically shifts the biological effects of MAPK activation in SMCs.

## Methods

**Cell cultures.** Human newborn (2 d–3 mo) arterial SMCs were obtained from the thoracic aortas of infants after accidental death, death from Sudden Infant Death Syndrome, or from congenital defects. Arterial SMCs from adult normal thoracic aortas were obtained from heart transplants. Arterial SMCs from atherosclerotic lesions were isolated from SMC-rich areas of well-characterized lesions of human adult carotid arteries. The cells were isolated by the explant method and cultured as described previously (18). Cells were used in passages 2–10, and were characterized as smooth muscle by morphologic criteria and by expression of smooth muscle  $\alpha$ -actin. In some experiments, the cells were allowed to become near-senescent and were used in passages 17–19. The cells were negative in mycoplasma assays and had a normal chromosome number. Before all experiments, the cells were kept in DME and 1% plasma-derived human serum for 48 h. Five different strains (donors) of newborn aortic SMC (strains 1–5), three different strains of adult aortic SMCs (strains 6–8) and three strains of lesion SMCs (strains 9–11) were used at different passages. Although many of the experiments were repeated in the different strains, the results shown are principally from strains 1 (newborn SMCs that secrete large amounts of growth-inhibitory PGs under basal conditions and express COX-2), 3 (newborn SMCs that do not secrete growth-inhibitory PGs and do not express COX-2), 6 (adult SMCs that do not secrete growth-inhibitory PGs and do not express COX-2), and 9 (lesion SMCs that do not secrete growth-inhibitory PGs and do not express COX-2), for easier comparison of the results.

**Growth factors, receptors, peptides, antibodies, and biochemicals.** Human recombinant PDGF-BB and PDGF-AA were kindly provided by Hoffman-La Roche Inc. (Basel, Switzerland). Determination of PDGF  $\alpha$ - and  $\beta$ -receptor numbers and affinities was performed as previously described (19, 20). SMCs express the two PDGF receptor subunits,  $\alpha$  and  $\beta$ . All strains expressed significant numbers of PDGF  $\alpha$ - and  $\beta$ -receptor subunits, and there were no consistent differences in receptor numbers or affinities between the different strains of SMC (data not shown). PDGF B-chain, which binds with high affinity to both  $\alpha$ - and  $\beta$ -subunits, was used in the study.

Protein kinase A inhibitor (PKI) peptide, a peptide inhibitor of cAMP-dependent protein kinase (PKA; TTYADFIASGRTGRRNA-IHD), was synthesized at the peptide synthesis facility, Howard Hughes Medical Institute (University of Washington, Seattle, WA). Recombinant rat Erk-2 was a gift from Dr. M. Cobb (Department of Pharmacology, University of Texas, Dallas, TX). Polyclonal antibodies against a carboxy-terminal Raf-1 peptide (CTLTSPRLPVF coupled to KLH) were generated in the laboratory of one of the authors (E.G. Krebs). MAPK mAbs for immunoprecipitation were obtained from Zymed Labs. Inc. (South San Francisco, CA). MAPKK-1 and -2 antibodies, as well as MAPKK-1 and -2 positive cell lysates, were obtained from Transduction Laboratories (Lexington, KY). COX-1 isolated

from ram seminal vesicles, COX-2 isolated from sheep placentas, and mAbs were obtained from Cayman Chem. Co. Inc. (Ann Arbor, MI).

The MAPKK inhibitor PD 098059 was generously given to us by Dr. Alan Saltiel and Dr. David Dudley (Research Division, Parke-Davis, Warner Lambert, Ann Arbor, MI), or was purchased from BIOMOL Research Labs., Inc. (Plymouth Meeting, PA). Of the COX inhibitors used, indomethacin (Sigma Chemical Co., St Louis, MO) inhibits both COX-1 and -2, whereas L-745,337 (5-methanesulfonamido-6-(2,4-difluorothio-phenyl)-1-indanone, kindly provided by Dr. A.W. Ford-Hutchinson (Merck Frosst Canada Inc., Dorval, Québec), NS-398, and nimesulide (Cayman Chem. Co.) are selective COX-2 inhibitors. The inhibitors were all dissolved at a concentration of 10 mM in dimethyl sulfoxide. Arachidonic acid (eicosa-5Z, 8Z, 11Z, 14Z-tetraenoic acid, 20:4, n-6), PGE<sub>2</sub>, and 8-bromoadenosine-3',5'-cyclic monophosphate (8-Br-cAMP) were obtained from BIOMOL Research Labs., Inc.

**Measurement of DNA synthesis, cell cycle distribution, and proliferation.** For estimation of DNA synthesis, cells were grown in 24-well trays, and were changed to DME/1% human plasma-derived serum for 48 h at  $\sim$ 80% confluence. After a 30-min preincubation with or without the indicated concentrations of MAPKK or COX inhibitors, PDGF-BB or vehicle (10 mM acetic acid/0.25% BSA) was added, and the cells were incubated for an additional 18 h and subsequently labeled with 2  $\mu$ Ci/ml [<sup>3</sup>H]thymidine (New England Nuclear, Boston, MA) for 2 h. For cell cycle distribution (analyzed by flow cytometry), the SMCs were incubated as for DNA synthesis assay, with and without inhibitors or PDGF-BB for 22 h, trypsinized, and stored at  $-70^{\circ}\text{C}$  in 10% DMSO. Before flow cytometric analysis, the cells ( $\sim$ 400,000) were pelleted (2,400 g for 5 min), resuspended in 250  $\mu$ l PBS containing 10% DMSO, and DNA was stained using 4,6-diamidino-2-phenylindole.

Proliferation was measured by determining cell number. Cells were treated as for DNA synthesis measurements, but the cells were incubated with inhibitors and PDGF-BB (1 nM) or vehicle for 96 h. The cells were trypsinized, fixed in Holley's fixative (3.7% formaldehyde, 86 mM NaCl, 106 mM Na<sub>2</sub>SO<sub>4</sub>), and counted with a cell counter (Coulter Corp., Hialeah, FL).

**Measurement of MAPKK, MAPK, Raf-1, and PKA activities.** Enzyme activities of MAPKK, MAPK, and PKA in cell extracts were measured as previously described (20, 21). Cells in 100-mm dishes (2–5 million cells) were preincubated for 30 min in the presence of the indicated concentrations of PD 098059 and then stimulated with PDGF-BB for 5 min (the time giving maximal activation). MAPK activities were measured as phosphorylation of myelin basic protein (MBP), and total MAPKK activities were measured in a double assay as activation of recombinant Erk-2 and subsequent phosphorylation of MBP. In some experiments, MAPK was immunoprecipitated before the activity assay. PKA activity was assayed by measuring phosphorylation of Kemptide (0.17 mM) in the presence or absence of PKI peptide (15  $\mu$ M) as described (21). PKA activity was calculated as the amount of Kemptide phosphorylated in the absence of PKI peptide per minute minus that phosphorylated in its presence. Protein concentrations in the supernatants were quantitated by either the BCA<sup>®</sup> protein assay (Pierce Chemical Co., Rockford, IL) or the Bio-Rad protein assay according to Bradford (Bio-Rad Laboratories, Hercules, CA).

Specific activities of MAPKK-1 and -2 were measured after immunoprecipitation of MAPKK-1 or -2. SMCs in 100-mm dishes were preincubated in the presence or absence of 30  $\mu$ M PD 098059 for 30 min, stimulated with 1 nM PDGF-BB, rinsed in ice-cold PBS, and then incubated for 5 min at 4 $^{\circ}\text{C}$  in 1 ml of MAPKK lysis buffer (10 mM Tris, pH 7.6, 140 mM NaCl, 1% NP-40, 5 mM EDTA, 50 mM NaF, 0.4 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, 1  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml leupeptin). Lysates were incubated for 10 min on ice with vortexing, and then centrifuged at 10,000 g for 10 min to collect supernatants. Samples were normalized for protein and then aliquoted for immunoprecipitation with MAPKK-1 or -2 antibodies. 500  $\mu$ g protein was incubated overnight at 4 $^{\circ}\text{C}$  with 2  $\mu$ g of antibody and then for an addi-

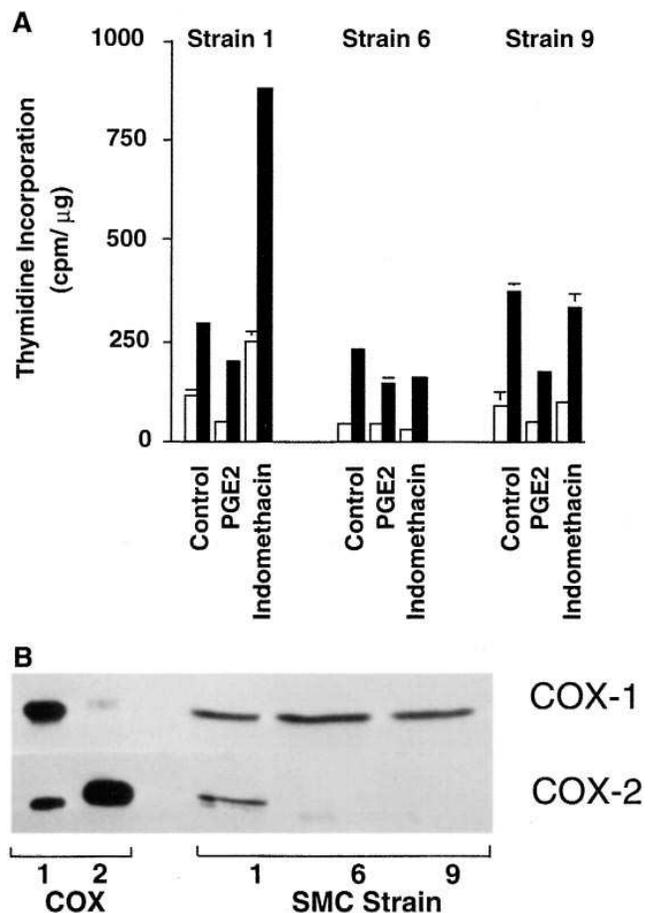
tional 3 h with 50  $\mu$ l protein A-Sepharose (1:1 [vol:vol] suspension; Sigma Chemical Co.). The beads were washed once with 0.5 M LiCl, 50 mM Tris (pH 7.5), 1 mM DTT, and 0.5 mM PMSF, once with 25 mM Hepes (pH 7.4), and once with 50 mM  $\beta$ -glycerophosphate, 1.5 mM EGTA, 0.1 mM  $\text{Na}_3\text{VO}_4$ , and 1 mM DTT. MAPKK-1 and -2 activities were determined by measuring phosphorylation of a purified recombinant kinase-dead MAPK (Erk-2 K52R mutant). The beads were incubated in assay buffer (25 mM  $\beta$ -glycerophosphate, 1.25 mM EGTA, 5 mM DTT, 0.15 mM  $\text{Na}_3\text{VO}_4$ , 10 mM  $\text{MgCl}_2$ , 0.1 mM [ $^{32}\text{P}$ ]ATP [3,000 cpm/pmol], 4  $\mu\text{M}$  PKI, 10  $\mu\text{M}$  calmidazolium) with 2  $\mu\text{g}$  of purified recombinant kinase-dead Erk-2 K52R for 30 min at 30°C. The reactions were terminated with SDS sample buffer and separated by SDS-PAGE. The gels were then dried and analyzed by autoradiography.

For measurement of Raf-1 activity, cells were treated as for MAPK analysis and lysed in 0.5 ml lysis buffer (25 mM Hepes, pH 7.5, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 150 mM NaCl, 100 mM sodium pyrophosphate, 50 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM benzamide, 10  $\mu\text{g}/\text{ml}$  leupeptin, 2  $\mu\text{g}/\text{ml}$  pepstatin A, and 0.5 mM AEBBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; ICN Pharmaceuticals, Inc., Costa Mesa, CA] for 20 min by rocking at 4°C. The lysate was scraped and briefly sonicated for 4 s using a sonicator (Braun-Sonic 2000; B. Braun Biotech Inc., Allentown, PA) at 50% output. The samples were centrifuged for 10 min at 10,000  $g$  in a microfuge. To each sample (400  $\mu\text{g}$ –2 mg protein, depending on SMC strain), 4  $\mu\text{l}$  of an anti-carboxy-terminal Raf-1 peptide rabbit antiserum was added, and the volume was equalized to 0.5 ml with lysis buffer. After incubation overnight at 4°C, protein A-Sepharose was added, and the incubation was continued for 2 h. Each sample was washed sequentially with 1.0 ml lysis buffer, 2  $\times$  1 ml TTBS (20 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 0.5 M NaCl, 0.1%  $\beta$ -mercaptoethanol), 1 ml 20 mM Tris-HCl, pH 7.5, and 0.5 M LiCl, 1 ml assay buffer (50 mM Hepes, 0.1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM DTT, 1 mM EDTA). Raf-1 activity was assayed using a coupled assay in which inactive MAPKK is activated by the immunoprecipitated Raf which, in turn, phosphorylates Erk-2. Inactive MAPKK, prepared as described below, was added to each immunoprecipitate along with kinase-dead Erk-2 K52R, and the assay was initiated by the addition of assay mix containing [ $\gamma$ - $^{32}\text{P}$ ]ATP. The final concentrations in the assay were 60 mM Hepes, pH 7.3, 10 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$  ATP (specific activity 3,000 cpm/pmol), 1 mM DTT, 1 mM EDTA, 0.01% Triton X-100, 1.25  $\mu\text{g}$  Erk-2 (25  $\mu\text{g}/\text{ml}$ ), and 5  $\mu\text{g}$  partially purified MAPKK. After 30 min at 30°C with shaking, the reactions were subjected to SDS-PAGE and autoradiography, and the phosphorylated Erk-2 bands were excised and counted. Inactive MAPKK was partially purified from serum-starved NIH 3T3 cells (using eight 150-mm confluent plates) by binding the flow-through from a DE-52 column to Heparin agarose (1.5 ml; Sigma Chemical Co.) similar to the purification procedure used by Seger et al. (22). Inactive MAPKK was eluted by an NaCl step-gradient, and the fractions were tested for their ability to be stimulated by immunoprecipitated Raf-1 and phosphorylate kinase-inactive Erk-2. Fractions containing activatable MAPKK (protein concentration 0.19 mg/ml) were pooled and stored at -20°C.

**PGE<sub>2</sub> release, and immunoblotting of cPLA<sub>2</sub>, COX, and MAPKK isoforms.** PGE<sub>2</sub> is one of the principal growth-inhibitory prostaglandins secreted by human SMCs (23). PGE<sub>2</sub> secretion into the culture medium was determined as previously described (24). For immunoblotting experiments, lysates from cells incubated with PDGF-BB for 5 min with or without a 30-min preincubation with 10–30  $\mu\text{M}$  PD 098059 or 10  $\mu\text{M}$  indomethacin were used for immunoblotting and detection of cPLA<sub>2</sub> band shift due to phosphorylation as described (24). Immunodetection of MAPKK-1 and COX-1 and -2 was carried out in cell extracts, using standards provided by the manufacturer.

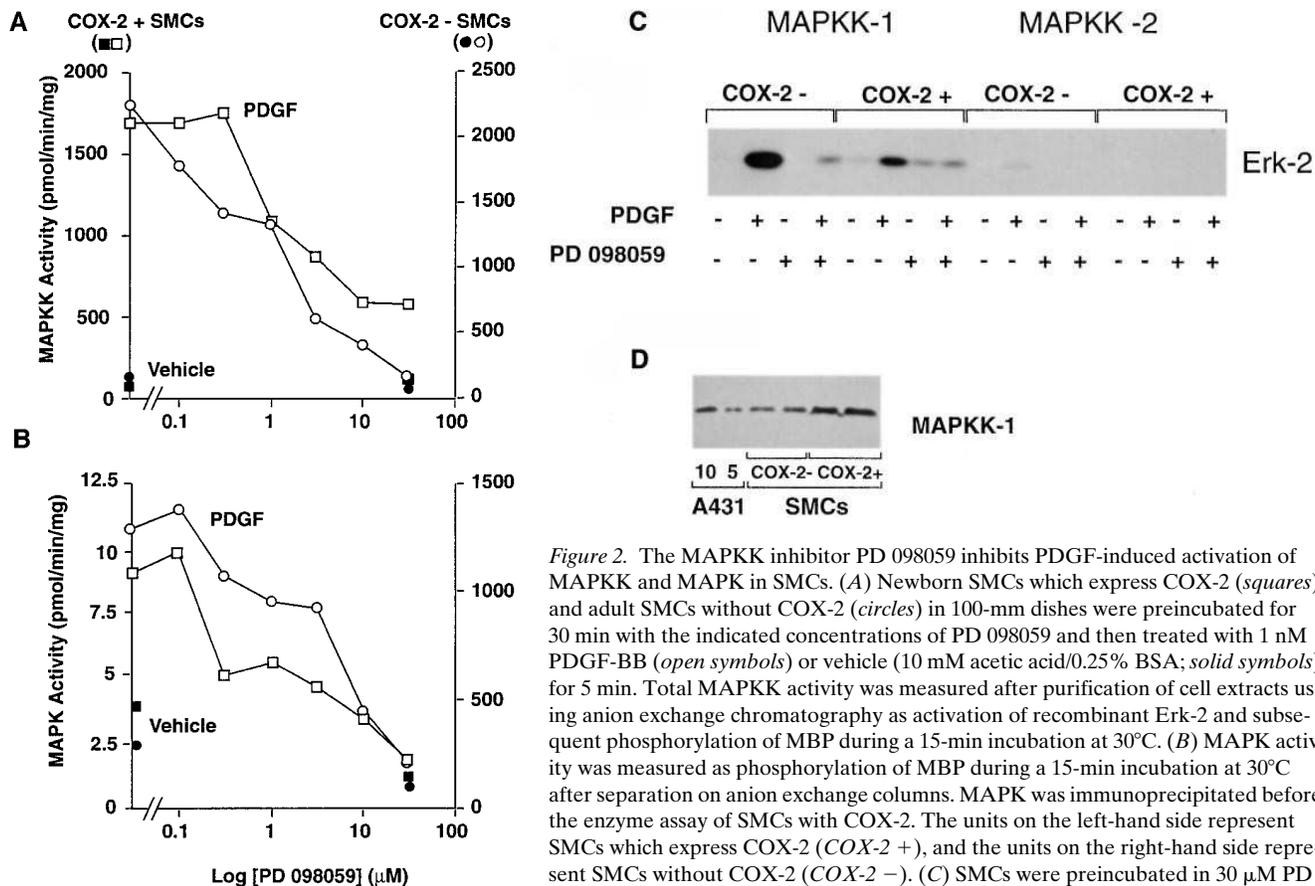
## Results

*Expression of COX-2 is associated with secretion of large amounts of growth-inhibitory PGs in human SMCs.* It is known



**Figure 1.** Differential expression of COX-1 and -2 in SMCs correlates with effects of inhibition of COX on proliferation. (A) Different strains of human arterial SMCs were preincubated for 30 min with 10  $\mu\text{M}$  indomethacin or PGE<sub>2</sub> and then stimulated for 18 h with PDGF-BB (solid bars) or vehicle (open bars), and pulse labeled with 2  $\mu\text{Ci}/\text{ml}$  [ $^3\text{H}$ ]thymidine for 2 h. DNA synthesis was measured as TCA-insoluble radioactivity. The strains examined are newborn SMCs which secrete growth-inhibitory PGs (Strain 1), adult normal SMCs, and adult lesion SMCs, both of which do not secrete growth-inhibitory PGs (Strains 6 and 9, respectively). The values are presented as mean  $\pm$  SD of triplicate samples of representative experiments ( $n = 4$ ). The inhibitory effects of PGE<sub>2</sub> on proliferation are due to activation of PKA. The cAMP analog 8-bromo-cAMP inhibits PDGF-induced DNA synthesis by 35  $\pm$  7% at 100  $\mu\text{M}$  and 64  $\pm$  1% at 1 mM ( $n = 3$ ). (B) Cell lysates from the SMC strains in A were prepared as described in Methods. The samples were separated on 10% SDS gels, transferred to membranes, and incubated with COX-1- or -2-specific antibodies for Western blot analysis. Sheep COX-1 (lane 1) and -2 (lane 2) were used as standards.

that SMCs can secrete large amounts of growth-inhibitory PGs under different conditions (23, 24), and that the secretion of PGE<sub>2</sub> is inhibited by pharmacologic inhibitors of the COX isoforms, such as indomethacin (24). Here we show that indomethacin markedly stimulates basal and PDGF-stimulated DNA synthesis in some strains of human diploid SMCs (Fig. 1 A). Significant amounts of PGE<sub>2</sub> are secreted into the culture medium by the strains in which indomethacin stimulates DNA synthesis (the amount of PGE<sub>2</sub> secreted under basal conditions is on the order of 1 ng PGE<sub>2</sub>/min/million cells, and after PDGF



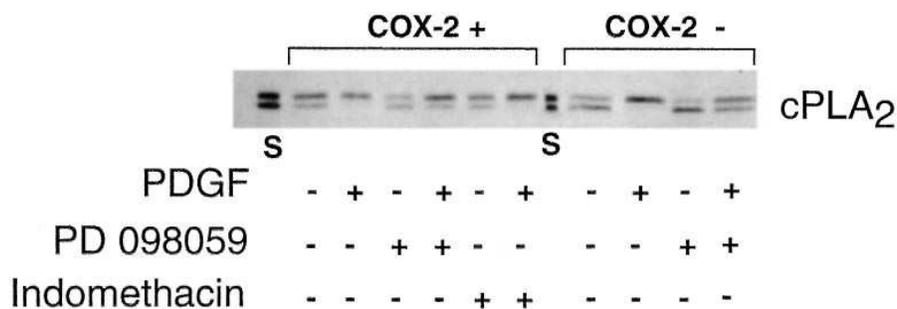
**Figure 2.** The MAPKK inhibitor PD 098059 inhibits PDGF-induced activation of MAPKK and MAPK in SMCs. (A) Newborn SMCs which express COX-2 (squares) and adult SMCs without COX-2 (circles) in 100-mm dishes were preincubated for 30 min with the indicated concentrations of PD 098059 and then treated with 1 nM PDGF-BB (open symbols) or vehicle (10 mM acetic acid/0.25% BSA; solid symbols) for 5 min. Total MAPKK activity was measured after purification of cell extracts using anion exchange chromatography as activation of recombinant Erk-2 and subsequent phosphorylation of MBP during a 15-min incubation at 30°C. (B) MAPK activity was measured as phosphorylation of MBP during a 15-min incubation at 30°C after separation on anion exchange columns. MAPK was immunoprecipitated before the enzyme assay of SMCs with COX-2. The units on the left-hand side represent SMCs which express COX-2 (COX-2 +), and the units on the right-hand side represent SMCs without COX-2 (COX-2 -). (C) SMCs were preincubated in 30 μM PD 098059 or vehicle (DMSO) in the presence or absence of 1 nM PDGF or vehicle

(10 mM acetic acid/0.25% BSA). MAPKK-1 and -2 were immunoprecipitated, and the activity in the MAPKK-1 and -2 immunoprecipitates was measured as phosphorylation of recombinant kinase-dead Erk-2 K52R during a 30-min incubation at 30°C. The samples were subsequently separated by SDS-PAGE, and the radioactivity in the Erk-2 band was estimated by autoradiography. The apparent induction of MAPKK-1 by PD 098059 in the basal state in COX-2 + cells is not a consistent finding. (D) MAPKK-1 expression in the SMCs was estimated by Western blot analysis. Total cell extracts (30 μg/lane) were separated on a 10% SDS gel by SDS-PAGE, and MAPKK-1 was detected using an MAPKK-1-specific antibody (Transduction Laboratories). Control cell lysates from A431 cells were used as positive controls (10 μg; 5 μg). The results in A and B are expressed as the mean of duplicate samples from a representative experiment ( $n = 2$ ). The experiments shown in C and D were repeated three times with similar results.

stimulation is increased to 6 ng PGE<sub>2</sub>/min/million cells; reference 24), whereas PGE<sub>2</sub> is not detectable in strains in which indomethacin does not stimulate DNA synthesis (see below). Exogenously added PGE<sub>2</sub>, on the other hand, inhibits basal and PDGF-induced DNA synthesis in all SMC strains examined (Fig. 1 A), and also markedly inhibits the stimulatory effects of indomethacin in strains where indomethacin enhances DNA synthesis (data not shown). PGs are synthesized by two isoforms of COX, the constitutive COX-1 and the inducible COX-2. The different strains of SMCs all express the constitutive COX-1 (Fig. 1 B). However, SMC strains in which indomethacin stimulates DNA synthesis also express the inducible COX-2 under basal conditions (Fig. 1 B). In striking contrast, levels of COX-2 are undetectable in SMCs that do not secrete growth-inhibitory PGs, which include strains of newborn SMCs, normal adult SMCs, and SMCs derived from atherosclerotic lesions (Fig. 1, and data not shown). Taken together, these results suggest that strains of human SMCs that express COX-2 secrete high levels of growth-inhibitory PGs, and that the PGs constitute a negative feedback loop that inhibits proliferation.

*The MAPKK inhibitor, PD 098059, has similar effects on MAPKK in the strains with and without COX-2.* To investigate the role of PDGF-induced activation of MAPKK and MAPK in different strains of SMCs, the effects of PD 098059, a synthetic, cell-permeable, noncompetitive inhibitor of MAPKK, were studied. PDGF-BB stimulates MAPKK and MAPK activities in both SMC strains which do not secrete growth-inhibitory PGs (COX-2-) and in SMC strains which secrete growth-inhibitory PGs (COX-2 +), as shown in Fig. 2, A-C. The concentration of PD 098059 required for half-maximal inhibition (IC<sub>50</sub>) of MAPKK is estimated to 2-3 μM in the different SMC strains (Fig. 2 A). The inhibition of MAPKK by PD 098059 is also transferred to MAPK (Fig. 2 B), and 30 μM PD 098059 blocks PDGF-induced MAPK (Erk-1 and -2) activation almost completely in the different strains of SMCs (Fig. 2 B).

Since PD 098059 has been reported to inhibit principally MAPKK-1 (16), the relative activities and inhibitions of the two isoforms were estimated. MAPKK-1 and -2 were immunoprecipitated from cell extracts from SMCs with and without COX-2. MAPKK-1 is significantly activated by PDGF-BB in both strains of SMCs, whereas activation of MAPKK-2 is low



**Figure 3.** PD 098059 inhibits phosphorylation of cPLA<sub>2</sub> in SMCs. Lysates (50 μg) from newborn SMCs with or adult SMCs without COX-2, treated with or without 10 μM PD 098059 or indomethacin for 30 min and 1 nM PDGF-BB for 5 min, were immunoblotted using an antibody to cPLA<sub>2</sub> as described in Methods. The standard (S; 10 ng of partially phosphorylated human cPLA<sub>2</sub>) was purified from baculovirus-infected Sf9 cells. The upper band represents the phosphorylated form of cPLA<sub>2</sub>. The experiment was repeated twice with similar results.

or undetectable (Fig. 2 C). PD 098059 (30 μM) inhibits PDGF-stimulated MAPKK-1 activation in both SMCs with and without COX-2. Thus, the effect of PD 098059 appears to be due principally to inhibition of MAPKK-1, which is expressed at significant levels in both strains (Fig. 2 D).

To address whether inhibition of MAPKK by PD 098059 affects the activity of its upstream kinase, Raf-1, SMCs were incubated with PD 098059 and then stimulated with PDGF-BB for 0, 2, 4, 8, 10, and 15 min. In SMCs without COX-2, PDGF induces a marked activation of Raf-1, whereas the response of SMCs with COX-2 is weaker (data not shown). Maximal activation of Raf-1 is observed 4 min after stimulation in both strains of SMCs (data not shown), and PD 098059 does not inhibit Raf-1 activity at any time point examined. Instead, PD 098059 slightly increases Raf-1 activity in SMCs with COX-2 and tends to prolong the time course of PDGF-stimulated Raf-1 activation in both strains of SMCs (data not shown). The stimulatory effect of PD 098059 on Raf activity has been described in other cell types, and has been reported to be due to release of Raf from a negative regulation by MAPK (16).

cPLA<sub>2</sub>, a rate-limiting step in release of arachidonic acid used for PG synthesis, is activated by MAPK (4). To examine the role of the MAPK cascade in cPLA<sub>2</sub> phosphorylation and activation, the effects of PD 098059 on cPLA<sub>2</sub> were investigated. cPLA<sub>2</sub> is expressed at similar levels in both SMCs with and without COX-2 (Fig. 3). Phosphorylation and activation of cPLA<sub>2</sub> can be measured as a band-shift after separation of the different forms using SDS-PAGE, where the phosphorylated and active form is the slower migrating form. Approximately 50% of cPLA<sub>2</sub> in both strains of SMCs is phosphorylated in the basal state. As demonstrated in Fig. 3, PDGF stimulates a near complete phosphorylation of cPLA<sub>2</sub> within 5 min after addition to both SMC strains. 10 μM PD 098059 inhibits PDGF-induced phosphorylation of cPLA<sub>2</sub> without affecting the basal level of cPLA<sub>2</sub> phosphorylation, and results are identical using 30 μM PD 098059 (data not shown). Indomethacin (10 μM) does not alter the basal phosphorylation state of cPLA<sub>2</sub>, nor does it inhibit PDGF-induced phosphorylation of cPLA<sub>2</sub> (Fig. 3).

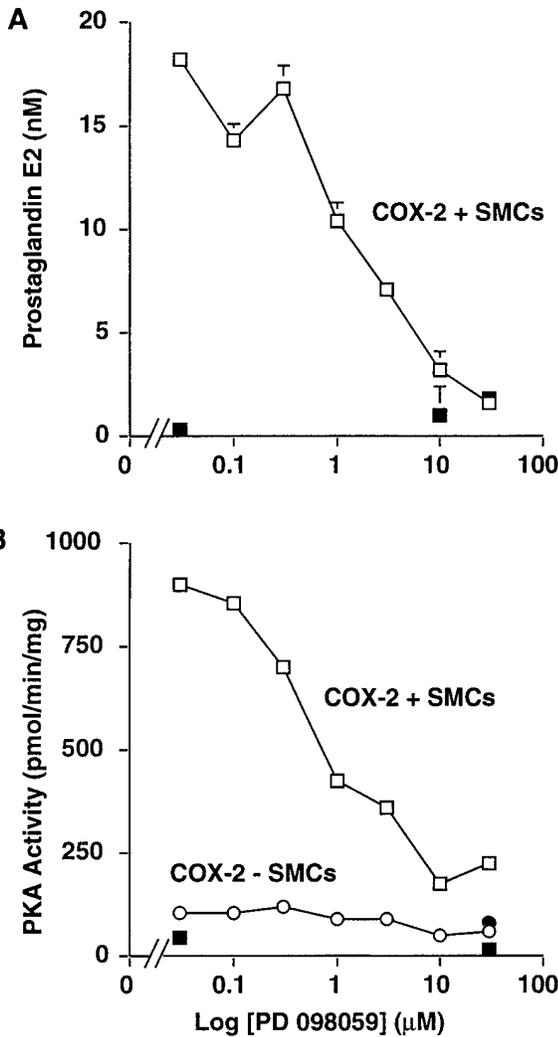
*The MAPK cascade mediates PGE<sub>2</sub> secretion and PKA activation in human SMCs that express COX-2.* Since PDGF stimulates MAPK, cPLA<sub>2</sub>, and PGE<sub>2</sub> release (24), it is possible that the MAPK cascade regulates PGE<sub>2</sub> secretion in SMCs and, thus, the negative feedback loop that can inhibit proliferation in strains expressing COX-2. Although protein levels and activation of cPLA<sub>2</sub> are similar in the different strains of

SMCs, activation of cPLA<sub>2</sub> leads to synthesis of PGE<sub>2</sub> only in the strains of SMCs which express COX-2 and in which proliferation is enhanced by indomethacin (e.g., *Strain 1* in Fig. 1 A). In fact, within 5 min after stimulation of these SMCs with 1 nM PDGF-BB, levels of PGE<sub>2</sub> secreted into the culture medium are stimulated 18-fold (Fig. 4 A), whereas levels of PGE<sub>2</sub> released by SMC strains which do not express COX-2 (e.g., *Strain 6* in Fig. 1 A) are below the detection limit of the assay (45 pM) after stimulation with PDGF-BB. Thus, the levels of PGE<sub>2</sub> released into the medium after PDGF stimulation are > 400-fold lower in SMCs that do not express COX-2 compared to COX-2-expressing SMCs. PD 098059 inhibits PDGF-induced PGE<sub>2</sub> synthesis with an IC<sub>50</sub> value of 3 μM in these SMCs (Fig. 4 A).

Since stimulation of PGE<sub>2</sub> receptors activates adenylate cyclase and leads to a subsequent increase in the levels of cAMP and activation of PKA, we studied the effects of PD 098059 on PKA activities. Fig. 4 B demonstrates that PKA is activated by PDGF in SMCs that express COX-2 and secrete growth-inhibitory PGs, but not in the SMCs that do not. The PKA activation is inhibited by PD 098059, with an IC<sub>50</sub> value of ~ 1 μM. In addition, both PDGF-induced release of PGE<sub>2</sub> and the subsequent activation of PKA are completely inhibited by 10 μM indomethacin (24). Indomethacin (10 μM) does not inhibit PKA activation induced by PGE<sub>2</sub>, showing that the effects of indomethacin are mediated by inhibition of COX (data not shown).

The lack of effect of PDGF on PKA activities in SMCs that do not express COX-2 is not due to a lack of PGE<sub>2</sub> receptors or their inability to activate PKA, since 10 μM PGE<sub>2</sub> gives a 24-fold activation of PKA within 5 min in these SMCs (PKA activity increased from 87.9 pmol/min/mg to 2,113.6 pmol/min/mg). Further, quantitative Western blot analysis of the expression of PKA catalytic subunits shows that SMCs without COX-2 have a twofold higher expression of PKA catalytic subunit compared to those with COX-2 (400 ng PKA catalytic subunit/mg protein compared to 200 ng/mg protein in newborn SMC). Taken together, these results show that the MAPK cascade stimulates activation of cPLA<sub>2</sub>, secretion of PGE<sub>2</sub>, and activation of PKA in human arterial SMCs which express COX-2.

*PD 098059 stimulates proliferation in SMCs that secrete growth-inhibitory PGs and express COX-2.* PD 098059 inhibits DNA synthesis in SMCs that do not express COX-2. The IC<sub>50</sub> value is ~ 10 μM (Fig. 5, A and B). The decrease by PD 098059 in DNA synthesis in these SMCs is associated with a



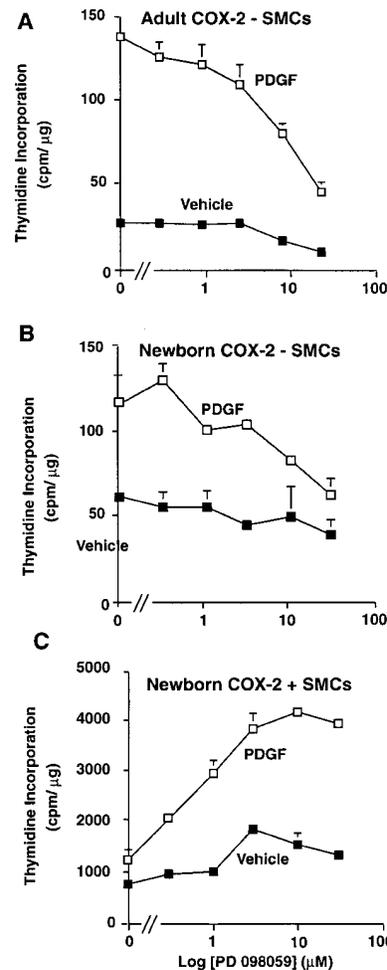
**Figure 4.** PGE<sub>2</sub> synthesis and PKA activation in SMCs with COX-2 are inhibited by PD 098059. Newborn SMCs with (squares) and adult SMCs without COX-2 (circles) were cultured in 100-mm dishes and were washed in fresh DME without serum before the experiments. The cells were incubated with the indicated concentrations of PD 098059 for 30 min, and then stimulated with 1 nM PDGF-BB (open symbols) or vehicle (solid symbols) for 5 min (a time which gives maximal stimulation of PGE<sub>2</sub> or PKA activity). (A) Levels of PGE<sub>2</sub> in the cell culture medium were measured using a PGE<sub>2</sub> enzyme-immunoassay (Amersham Corp., Arlington Heights, IL). (B) PKA activity was measured as phosphorylation of Kemptide in the presence or absence of the PKA inhibitor PKI. The experiment was repeated twice with similar results.

decreased fraction of cells that enter the S-phase of the cell cycle, as judged from flow cytometric analysis, and this leads to a reduced number of cells 96 h after stimulation with PDGF in the presence of 10–30 μM PD 098059 (Table I). A marked decrease of PDGF-induced DNA synthesis by 10–30 μM PD 098059 was also seen in aortic SMCs isolated from adult monkey (*Macaca nemestrina*) and adult and newborn rat (data not shown). Neither of these SMCs secrete significant amounts of growth-inhibitory PGs in the basal state (data not shown). The inhibitory effects of PD 098059 were not due to cytotoxicity measured by release of lactate dehydrogenase into the me-

dium, even after a 4-d incubation with 30 μM PD 098059 (data not shown).

In contrast to human SMCs without COX-2, inhibition of MAPKK by PD 098059 results in a dose-dependent increase of PDGF-stimulated DNA synthesis in strains of SMCs that express COX-2 (Fig. 5 C), with a half-maximal effective concentration (EC<sub>50</sub>) value of ~1 μM. Basal levels of DNA synthesis were also increased by PD 098059, although to a lesser extent than those induced by PDGF. To verify that the stimulation of DNA synthesis leads to increased cell cycle traverse, the fraction of cells in the different stages of the cycle were measured using flow cytometric analysis. Table I shows the cell cycle distribution of SMCs that express COX-2 and secrete growth-inhibitory PGs. An increased number of cells are in the S-phase at 22 h after stimulation with PD 098059 in either the absence or presence of PDGF. The increase in DNA synthesis and cell cycle traverse is reflected by an increased number of SMCs 96 h after addition of 10–30 μM PD 098059 (Table I).

The stimulatory effects of PD 098059 on DNA synthesis and cell cycle traverse of SMCs that secrete growth-inhibitory PGs and express COX-2 are mimicked by 10 μM indomethacin (Fig. 1 A and Table I). The proliferative effects of indomethacin are not due to inhibition of MAPKK or MAPK activities (data not shown). Furthermore, indomethacin-mediated enhancement of DNA synthesis and proliferation is limited to SMCs that express COX-2 and proliferate in response



**Figure 5.** Inhibition of MAPKK inhibits DNA synthesis in SMCs without COX-2, but stimulates DNA synthesis in SMCs with COX-2. (A) Adult normal SMCs without COX-2, (B) newborn SMCs without COX-2, and (C) newborn SMCs with COX-2 were preincubated for 30 min with the indicated concentrations of PD 098059 and then stimulated for 18 h with PDGF-BB (open squares) or vehicle (solid squares), and pulse labeled with 2 μCi/ml [<sup>3</sup>H]thymidine for 2 h. DNA synthesis was measured as TCA-insoluble radioactivity. The values are presented as mean ± SD of triplicate samples of representative experiments (n = 4).

**Table I. PD 098059 and Indomethacin Increase Cell Cycle Traverse and Cell Number in SMCs Which Express COX-2, but not in SMCs without COX-2**

	S phase (%) (22 h)	Cell number (× 1,000) (96 h)
<b>COX-2 + SMCs</b>		
Vehicle	9.3±1.5	222.3±10.1
10 μM PD 098059	14.6±1.8	245.5±9.7
30 μM PD 098059	13.3±0.3	238.1±8.7
Indomethacin	13.8±0.5	294.2±6.8
PDGF	14.5±1.4	278.4±10.7
PDGF + 10 μM PD 098059	19.2±1.5	345.0±5.1
PDGF + 30 μM PD 098059	19.6±0.0	310.0±14.8
PDGF + indomethacin	19.8±1.1	413.2±19.8
<b>COX-2 – SMCs</b>		
Vehicle	4.5±0.5	112.1±2.7
10 μM PD 098059	4.5±0.8	119.1±3.9
30 μM PD 098059	3.3±0.6	101.9±5.6
Indomethacin	4.8±0.7	102.4±2.9
PDGF	10.3±2.2	268.5±8.6
PDGF + 10 μM PD 098059	8.3±1.9	222.3±9.9
PDGF + 30 μM PD 098059	4.0±1.3	176.1±10.0
PDGF + indomethacin	9.9±0.3	232.0±10.0

Newborn (COX-2 +) or adult SMCs (COX -2 -) in 24-well trays were preincubated for 30 min with the indicated concentrations of PD 098059 or 10 μM indomethacin and then stimulated for 22h (flow cytometric analysis) or 96 h (cell number) with 1 nM PDGF-BB or vehicle. Cell cycle distribution was determined by estimating the amount of DNA per cell using flow cytometry. The results are expressed as mean±SD of triplicate (flow cytometric analysis) or quadruplicate (cell number) samples.

to PD 098059, and is not observed in other SMC strains (Fig. 1 A, Table I, and data not shown).

*The mitogenic effects of PD 098059 are due to inhibition of growth-inhibitory PGs synthesized by COX-2.* The similar effects of PD 098059 and indomethacin in SMCs which express COX-2 and secrete growth-inhibitory PGs suggest that the effects of PD 098059 are due to inhibition of PG synthesis. To determine whether the stimulatory effects of PD 098059 and indomethacin in these SMCs are due to regulation of molecules released from the cells (such as inhibitory prostanoids) or to stimulation of intracellular signaling, strains of SMCs that secrete growth-inhibitory PGs were studied at a density of 200,000 cells/well in 24-well plates with different volumes of media conditioned by the cells in the presence or absence of 10 μM PD 098059 or indomethacin. When the volume of conditioned medium was increased 5-fold in the absence of inhibitors, PDGF-induced DNA synthesis increased 3.5-fold (from 12,238±55 to 42,753±2,476 cpm). This suggests that DNA synthesis is suppressed by molecules released into the medium. In contrast, the stimulating activity of PD 098059 on PDGF-induced DNA synthesis was reduced from 3.3-fold to 1.2-fold when the volume of the culture medium was increased 5-fold (from 400,000 to 80,000 cells/ml). Accordingly, the effects of indomethacin decreased from a 4.7-fold stimulation to a 1.4-fold stimulation (data not shown). These results suggest that the major mitogenic effects of both PD 098059 and indomethacin are due to suppression of an inhibitory molecule secreted

**Table II. Stimulation of DNA Synthesis by COX-2 Inhibitors in SMCs That Secrete Growth-inhibitory PGs**

Cox inhibitor	EC <sub>50</sub> DNA repl.	IC <sub>50</sub> COX-1	IC <sub>50</sub> COX-2	Reference
Indomethacin	10 nM	5 nM	10 nM	61
L-745,337	10 nM	> 10 μM	20 nM	61
NS-398	300 nM	> 100 μM	4 μM	62
Nimesulide	30 nM	> 100 μM	70 nM	63

Newborn SMCs which express COX-1 and -2 and secrete inhibitory PGs were preincubated for 30 min with COX inhibitors and then stimulated for 18 h with PDGF-BB or vehicle (10 mM acetic acid/0.25% BSA), and pulse labeled with 2 μCi/ml [<sup>3</sup>H]thymidine for 2 h. DNA replication was measured as TCA-insoluble radioactivity. The EC<sub>50</sub> values are compared with IC<sub>50</sub> values previously reported in the references provided in the table. The EC<sub>50</sub> values are expressed as mean of two independent experiments.

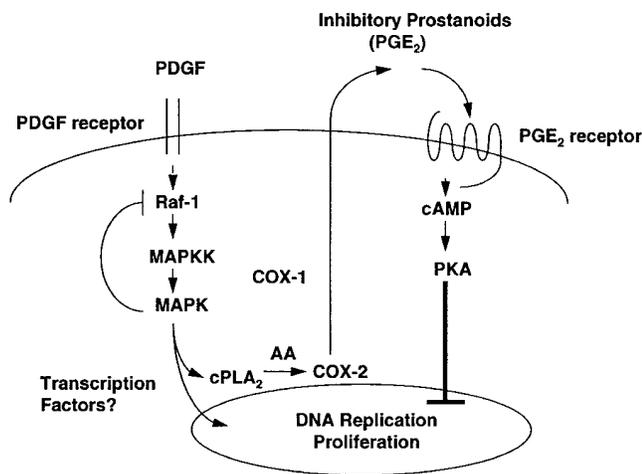
into the medium. In agreement with this possibility is the fact that neither indomethacin nor PD 098059 is able to stimulate SMC proliferation if the number of cells per volume of medium is lower than 30,000 cells/ml (data not shown).

The significant synthesis of PGE<sub>2</sub> and other growth-inhibitory prostanoids (PGE<sub>1</sub> and PGI<sub>2</sub>) that lead to PKA activation in SMCs is associated with an increased expression of the inducible COX-2 (Fig. 1 B). By using the selective COX-2 inhibitors, L-745,337, NS-398, and Nimesulide, a role of COX-2 in inhibiting SMC proliferation was verified (Table II). All of the COX-2 inhibitors result in enhanced DNA replication in SMCs strains which express COX-2 to an extent similar to that of the nonselective COX inhibitor indomethacin. Further, in each case the EC<sub>50</sub> of stimulation of DNA synthesis is in agreement with the IC<sub>50</sub> of inhibition of COX-2 (Table II and references therein), and COX-2 inhibitors do not enhance DNA replication in SMCs that do not secrete growth-inhibitory PGs (data not shown).

To further investigate the role of MAPKK and COX in inhibiting proliferation in SMC strains that secrete growth-inhibitory PGs and express COX-2, arachidonic acid was added to the culture medium, and DNA synthesis was measured as described above. In a representative experiment (out of three different experiments), basal DNA synthesis was 41±3 cpm/μg, and PDGF-induced DNA synthesis was 67±11 cpm/μg. In the presence of 10 μM indomethacin, L-745,337, or PD 098059, PDGF-induced DNA synthesis increased to 146±3, 149±7, and 147±8 cpm/μg, respectively. Arachidonic acid (10 μM) added to the cells reversed the mitogenic effects of PD 098059 but not of the COX inhibitors. The PDGF-stimulated DNA synthesis in the presence of arachidonic acid, in arachidonic acid plus indomethacin, arachidonic acid plus L-745,337, or arachidonic acid plus PD 098059 was 27±0, 178±3, 136±4, and 59±1 cpm/μg, respectively. Together, these data suggest that the stimulatory effect of PD 098059 is due to inhibition of growth-inhibitory arachidonic acid metabolites (PGs).

## Discussion

*Activation of the MAPK cascade can serve as a negative feedback on proliferation through release of PGs that activate PKA.* Fig. 6 represents the summation of the results presented



**Figure 6.** Model of MAPK-regulated proliferation in human arterial SMCs. Activation of MAPK stimulates cPLA<sub>2</sub>, and in SMCs that express COX-2, this results in generation of arachidonic acid (AA), and synthesis and release of PGE<sub>2</sub> and other inhibitory PGs. cPLA<sub>2</sub> and COX-2 colocalize in the nuclear membrane, and the secretion of PGE<sub>2</sub> leads to activation of cAMP-coupled prostaglandin receptors and the subsequent activation of PKA in an autocrine or paracrine manner. PKA inhibits cell cycle traverse. Although MAPK activates cPLA<sub>2</sub> in all SMCs, this does not result in release of growth-inhibitory PGs in SMCs that do not express significant levels of COX-2. Instead, in SMCs without COX-2, activation of the MAPK pathway by PDGF mediates cell cycle traverse and proliferation, most likely through activation of transcription factors. The biological outcome of MAPK signaling can thus be modulated by the presence or absence of downstream targets, in this case COX-2 that regulates the endogenous secretion of growth-inhibitory prostanoids in human SMCs.

in this study. The results show that in the arterial SMCs investigated in this study, the classical MAPK cascade can mediate either proliferation or growth inhibition, depending on regulation by the particular cell strain of the PGs secreted into the extracellular environment. Although a signaling loop from MAPK activation to release of prostanoids is present in many cell types (3), the extent to which this can occur in some SMC strains is exceptional. In fact, in these cells the main effect of MAPK activation on proliferation is inhibitory by a negative feedback loop resulting from PDGF stimulation. This is most likely explained by preferential secretion of inhibitory PGs (PGE<sub>1</sub>, PGE<sub>2</sub>, and PGI<sub>2</sub>) from human SMCs (23), in which PKA activated by these PGs is a powerful growth inhibitor.

The fact that human SMC proliferation can be markedly inhibited by endogenously produced PGs has been known for some time (23, 25, 26). Significant inhibition of human SMC proliferation by endogenous prostanoids has been observed in human SMC isolates from different vascular beds. We have shown recently that PDGF-induced MAPK activation is associated with phosphorylation, and activation of cPLA<sub>2</sub> with the subsequent release of PGE<sub>2</sub> (and most likely other inhibitory prostaglandins, such as PGE<sub>1</sub> and PGI<sub>2</sub>) in some strains of arterial SMC (24). PGE<sub>2</sub> is a strong activator of PKA, a well-known inhibitor of SMC proliferation, through binding to its adenylate cyclase-coupled receptors (26–30). The results presented here show that the effects of PDGF on cPLA<sub>2</sub>, PGE<sub>2</sub> (and other PKA-activating PGs) release, and PKA activation can be blocked by the MAPKK inhibitor PD 098059, verifying

that these events are mediated by the MAPK cascade in SMCs (see Fig. 6).

The following observations support the concept that the growth-inhibitory effects of MAPK signaling are mediated by release of growth-inhibitory PGs. First, PD 098059 inhibits PGE<sub>2</sub> synthesis and PKA activation at concentrations that stimulate cell cycle traverse and proliferation. Second, the mitogenic effects of the MAPKK inhibitor PD 098059 are mimicked by COX inhibitors. This is seen at the level of PKA activation, cell cycle traverse (22 h), and long-term (4 d) proliferation. In no case can a mitogenic effect by PD 098059 be found without a concurrent stimulation by COX inhibitors. Neither PD 098059 nor COX inhibitors are capable of enhancing DNA synthesis in SMCs that do not express COX-2 or secrete growth-inhibitory PGs under basal conditions, in agreement with results showing lack of PGE<sub>2</sub> release and PKA activation by PDGF in these SMCs. Third, the mitogenic effects of PD 098059 but not of COX inhibitors can be counteracted by arachidonic acid. The mitogenic effects of both PD 098059 and COX inhibitors can be counteracted by exogenously added PGE<sub>2</sub>; the maximal stimulatory effect of PD 098059 on DNA synthesis can be inhibited by 85% with one addition of 10 μM PGE<sub>2</sub> (data not shown). Fourth, the effects on DNA synthesis of PD 098059 and indomethacin are not additive, suggesting that these compounds act through a common mechanism (data not shown). Fifth, the mitogenic effects of PD 098059 and of COX inhibitors can both be reversed by dilution of the culture medium, indicating that these inhibitors probably target inhibitory molecules (i.e., prostaglandins) secreted into the medium, and are not likely to result from altered intracellular signaling.

Activation of PKA inhibits multiple signaling events in G1, including the MAPK cascade (21, 31–33), p70 S6 kinase (34, 35), and the eukaryotic initiation factor 4E (34). Since MAPK signaling is inhibited in the presence of PD 098059, the MAPK pathway does not appear to be the target of PKA-mediated inhibition of the cell cycle in this study. PKA has been reported to inhibit events later in the G1 phase of the cell cycle, such as cyclin D1 expression, cdk-4 activity, cyclin E expression, and cyclin E-associated kinase activity (36, 37). Our results show that cyclin E-associated kinase activity is significantly inhibited by exogenously added PGE<sub>2</sub> (data not shown). This is in agreement with a PKA-mediated inhibition of G1 cyclins, which may explain the mitogenic effects of PD 098059 and COX inhibitors.

*PD 098059 as a tool for studying the biological effects of MAPK signaling in arterial SMCs.* Although the MAPK pathway in normal arterial smooth muscle can be stimulated by a variety of conditions, such as growth factor administration (20), angiotensin II (38), cell adhesion, fluid shear stress (39), mechanical loading (13), and acute hypertension (40), elucidation of the physiological relevance of this signaling has been carried out in just a few cases. Pharmacological tools to inhibit the MAPK pathway have only recently been available (41), and transfection studies using dominant negative mutants of the MAPK cascade have been performed primarily in established cell lines, such as 3T3 cells. The biological outcome of intracellular signaling in such immortalized, nondiploid cells may not properly reflect the complex signaling in normal diploid human arterial SMCs. In this study, we used the selective MAPKK inhibitor PD 098059 to examine normal human SMC proliferation.

We cannot exclude that PD 098059 inhibits enzymes other

than MAPKK-1, and to a lesser extent -2. However, a number of observations indicate that this is unlikely. First, PD 098059 does not inhibit activation of Raf, the MAPKK and MAPK homologues SEK, JNK, p38 (16, 42), the p70 S6 kinase (14), or the PHAS-1 kinase (43) at concentrations that completely inhibit MAPKK in intact cells. In vitro studies show that PD 098059 (at concentrations up to 50  $\mu$ M) does not inhibit a variety of protein Ser/Thr kinases (16). Second, PD 098059 does not inhibit migration of SMCs (data not shown). These observations support previous results that MAPK signaling is not required for SMC migration and chemotaxis (20), and show that 30  $\mu$ M PD 098059 does not interfere nonspecifically with kinases in signal transduction pathways involved in cytoskeletal reorganization, adhesion, and migration in SMCs. PD 098059 also does not alter any other MBP-phosphorylating kinases as evaluated by an in-gel kinase assay (data not shown). The selectivity of PD 098059 may be explained by the fact that it does not appear to bind to the active site of MAPKK, and it does not compete with the ATP-binding site of the kinase, as do many other, less specific kinase inhibitors (16). The notion that it may be possible to use inhibitors of specific signaling pathways in the treatment of disease is appealing (44); however, our studies point out that the biological effect of a signaling pathway may differ in the same cell type, and show how this may occur.

*The effects of MAPK signaling on SMCs depend on their ability to express COX-2.* In this study, we demonstrate that the synthesis of the growth-inhibitory prostanoids is dependent on expression of COX<sub>2</sub>. Two different forms of COX (also known as prostaglandin endoperoxide H synthase) have been identified, COX-1 and -2 (45). COX-1 is constitutively expressed in most tissues and is thought to mediate "housekeeping" functions. COX-2 is inducible and is generally considered to be a mediator of inflammation. Its expression is stimulated by cytokines, tumor promoters, or growth factors (46). Induction of COX-2 has been regarded as a mechanism by which cells increase their capacity to synthesize prostanoids in excess of that provided by COX-1 (47, 48). Recent studies of COX-1- and -2-deficient mice suggest that there may be functional differences between COX-1 and -2 (49–51). Further, COX-1 and -2 may use different subcellular pools of arachidonic acid. Interestingly, large amounts of both COX-2 and cPLA<sub>2</sub> are localized in the nuclear envelope, whereas COX-1 is also found in the endoplasmic reticulum (52). Thus, MAPK-mediated activation of cPLA<sub>2</sub> in the nuclear envelope may lead to a preferential generation of prostanoids from COX-2.

Expression of COX-2 is highly regulated in SMCs. COX-2 mRNA is increased 50-fold after balloon injury of the rat aorta, with no significant induction of COX-1 (53, 54). Further, COX-2 expression is increased in rat aortic SMCs stimulated to proliferate by serum or growth factors, including PDGF. This increase is associated with a 2.5–3.0-fold-increased rate of arachidonic acid conversion to PGE<sub>2</sub> (52–54). In this study, expression of COX-2 is associated with a > 400-fold increase in secretion of PGE<sub>2</sub> and a marked inhibition of proliferation. By using several selective COX-2 inhibitors, we observed that the negative feedback on proliferation in SMCs is mainly due to COX-2 activity (Table II). This is consistent with recent results showing that PDGF-stimulated PGE<sub>2</sub> release in primary osteoblasts is mediated primarily by COX-2 (55).

The mechanism behind the increased expression of COX-2 in selected strains of newborn SMCs is not clear, but it is possible that increased secretion of endogenous growth factors by

newborn SMCs (56) induces expression of COX-2. Preliminary studies show that near-senescent SMCs (passages 17–19) lose their ability to secrete both growth-inhibitory PGs (data not shown) and PDGF (57). It is likely that COX-2 expression and the negative feedback induced by MAPK signaling can be regulated in SMCs, depending on the extracellular milieu.

*Availability of downstream targets and substrates can significantly shift the biological effects of the MAPK pathway.* During recent years it has become clear that the MAPK pathway regulates a variety of different physiological responses in different cell types. In some cases, activation of MAPK by distinct ligands in the same cell type can lead to different biological effects. Such a mechanism has been suggested in PC12 cells in which EGF-stimulated MAPK activation may mediate mitogenesis, whereas NGF-stimulated MAPK has been implicated in growth arrest and differentiation (11). Indeed, the MAPKK inhibitor PD 098059 has recently been shown to reverse NGF-mediated growth inhibition (58). Experiments to define the mechanism by which one signaling pathway regulates such distinct biological effects in the same cell type have suggested that differences in the strength and/or duration of MAPK activation (11, 59) or restricted access of signaling molecules to potential substrates (60) may be involved.

This study demonstrates that the biological outcome and specificity of activation of the MAPK cascade is highly dependent on downstream enzymes expressed by the cell. This is the first example of distinct biological effects mediated by the MAPK cascade in a normal, diploid human cell. In these human SMCs, activation of the MAPK cascade by one ligand (PDGF-BB) can lead to either proliferation or to growth inhibition, depending on the extent of generation of growth-inhibitory PGs. It is likely that in cell types that secrete mainly growth-stimulatory prostaglandins, the effect of the MAPK cascade on PG release may contribute to its mitogenic effects. Significant synthesis of PGE<sub>2</sub> is dependent on expression of COX-2. Interestingly, the nuclear localization of the MAPK substrate cPLA<sub>2</sub> and of COX-2 may be important for the observed generation of growth-inhibitory PGs. Thus, our studies have identified a highly regulated but localized downstream target of MAPK, the presence or absence of which can dramatically shift the biological effects of MAPK activation.

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