Regulatory role of prostaglandin E_2 in induction of cyclo-oxygenase-2 by a thromboxane A_2 analogue (U46619) and basic fibroblast growth factor in porcine aortic smooth-muscle cells

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U46619, a thromboxane A_2 analogue, and basic fibroblast growth factor (FGF-2) both induced the expression of the inducible cyclo-oxygenase (Cox)-2 in porcine aortic smooth-muscle cells. This induction was dose-dependent (submaximal at 300 nM for U46619 and 1 ng/ml for FGF-2) and time-dependent, with similar intensity and maximal expression at 2 h. Under these conditions, both inducers stimulated rapid activation of extracellular signal-regulated kinase (ERK2) at 5–10 min, a transient and lower intensity being induced by U46619 whereas that induced by FGF-2 was sustained (> 1 h). PD98059, an inhibitor of the ERK pathway, inhibited the expression of Cox-2. In contrast, activation of Jun-N-terminal kinase (JNK1) was sustained with U46619 but poorly induced by FGF-2. Cox-2 expression induced by U46619 or FGF-2 was similarly reduced by prostaglandin (PGE_2), forskolin or dibutyryl-cAMP, suggesting a regulatory effect of adenylate cyclase on Cox-2 expression. However, activation of ERK2 by FGF-2 was not affected by PGE_2 whereas that of JNK1 by U46619 was inhibited, suggesting that inhibition of COX-2 expression by cAMP may be downstream of ERK2. The effects of PGE_2 and forskolin on Cox-2 and phosphorylation of JNK1 were reversed with the protein kinase A inhibitor H89. In addition, endogenous PGE_2 down-regulated the expression of Cox-2 by the two inducers, as stimulation of the cells in the presence of different Cox inhibitors increased the expression of the protein. Overall, these results suggest that exogenous and endogenous PGE_2 exert negative inhibitory effects on Cox-2 expression mediated by stimulation of protein kinase A.

INTRODUCTION

The synthesis of prostaglandins (PGs) in smooth-muscle cells (SMCs) involves the liberation of arachidonic acid and subsequent metabolism by cyclo-oxygenase (Cox). Although phospholipases have long been thought to play a major role in regulating the synthesis of PGs by controlling the amount of unesterified substrate necessary for subsequent metabolism, recent data have demonstrated the existence of two isoforms of Cox [1]. The first, Cox-1, is a product of the house-keeping genes and shows little variation on cell stimulation; however, the second, Cox-2, is the product of a primary response gene and is expressed rapidly in response to growth factors [2]. Induction of Cox-2 is accompanied by a major variation in the capacity of cells to generate PGs, suggesting that it may play an important role in controlling cellular events.

In addition, we have shown that induction of Cox-2 in SMCs after *in vivo* vascular injury extends over many days and may have a pathophysiological role in the early modulation of vascular responses to injury [3]. Thromboxane A_2 (TXA_2) is the main metabolite of arachidonic acid in platelets; it is a mediator in the amplification loop of platelet aggregation and a potent vasoconstrictor of SMCs [4]. These cells possess TX receptors associated with vasoconstriction but recent results suggest that this mediator acts on extracellular signal-regulated kinases (ERKs) and p85/S6 protein kinase [5]; it could thus have a role in proliferation [5–7]. Therefore the end effects of the G-protein-coupled seven-membrane-spanning TXA_2 receptor could be similar to those reported for growth factors with receptor tyrosine kinases. Recent results, however, show that activation of ERK2 by U46619, a stable analogue of TXA_2, is weak and transient [6–8]. These studies also showed a reversal of these effects by ciprostene, a stable analogue of prostacyclin, suggesting a down-regulation of this signalling cascade.

Since recent results have raised the possibility that the expression of Cox-2 is associated with cell growth and tumour cells [9], we investigated Cox-2 expression induced by U46619 and a growth factor, basic fibroblast growth factor (FGF-2), in SMCs from pig. Although little is known about the effect of U46619 and the expression of Cox-2, FGF-2 has been found to induce Cox-2 mRNA and protein in a mouse osteoblastic cell line [10]. Our data show that Cox-2 is expressed in a similar fashion in response to these two inducers; however, only FGF-2 induces strong activation of ERK2, which has been previously found associated with cell growth. In contrast, U46619 was more potent in activating another member of the mitogen-activated protein kinase (MAPK) family, the recently described Jun-N-terminal kinase (JNK1) [10, 11]. We also found that PGE_2, which is formed by these SMCs, down-regulated Cox-2 expression induced by U46619 or FGF-2 but had mixed effects on MAPKs. These negative effects appear to be mediated by stimulation of protein kinase A.

MATERIALS AND METHODS

Materials

BSA (fraction V), leupeptin, myelin basic protein (MBP), pepstatin A, arachidonic acid, monoclonal anti-β-actin, 2-mercaptoethanol and 3-isobutyl-1-methylxanthine were from Sigma (St.

Abbreviations used: TX, thromboxane; Cox, cyclo-oxygenase; PG, prostaglandin; FGF-2, basic fibroblast growth factor; SMC, smooth-muscle cell; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK1, Jun-N-terminal kinase; MBP, myelin basic protein; GST, glutathione S-transferase.

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Louis, MO, U.S.A.). Arachidonic acid was purified by silicic acid column chromatography and stored at −70 °C. U46619 was from Cayman Chemical, Ann Arbor, MI, U.S.A. PD98059 was obtained from Biomol (Plymouth Meeting, PA, U.S.A.). Recombinant FGF-2 was from Biotech Trade Service (St. Leon-Rot, Germany). Fetal calf serum, minimum essential medium with Earle's salts, Albumax I, L-glutamine, penicillin and streptomycin were purchased from Gibco (Grand Island, NY, U.S.A.). [γ-^{32}P]ATP (111 Tbq/mmol) was from ICN Biomedicals (Costa Mesa, CA, U.S.A.). [*methyl*-^{3}H]Thymidine, (74 Gbq/mmol) and enhanced chemiluminescence substrates were obtained from Amersham (Les Ulis, France). Monoclonal antibodies raised against Cox-1 and against the C-terminal extremity of Cox-2 and specific for these isoenzymes have been described previously [12]. Protein G–Sepharose, anti-(rat MAPK) (C-14), a rabbit polyclonal antibody raised against the C-terminal peptide of ERK2 and a rabbit (C-17) polyclonal antibody raised against the C-terminal peptide of JNK1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Glutathione S-transferase (GST)–Jun fusion peptide was from UBI (Lake Placid, NY, U.S.A.). Protein donkey polyclonal anti-mouse IgG coupled to peroxidase was from Jackson (West Grove, PA, U.S.A.). Electrophoresis reagents were from Bio-Rad (Richmond, CA, U.S.A.), and chemical products were from Prolabo (Paris, France).

Cell culture

SMCs were isolated from pig thoracic aorta and cultured as previously described [13]. Cultures at subconfluence were rendered quiescent by incubation for 5 days in serum-free medium containing 0.2 % Albumax I. All experiments were performed with cell cultures of less than seven passages.

Cell incubations and measurement of PGs and cAMP

Quiescent SMCs were activated with the inducers at different times as indicated in the Figure legends. In some experiments, after activation, cells were washed in Hanks buffer, pH 7.4, containing 1 mg/ml BSA and incubated for 30 min in the same buffer with 10 μM arachidonic acid to evaluate Cox activity. The supernatants were collected and the stable metabolites of prostacyclin, 6-oxo-$PGF_{1\alpha}$, $PGF_{2\alpha}$ or PGE_2 were determined by enzyme immunoassay with acetylcholinesterase-labelled 6-oxo-$PGF_{1\alpha}$, $PGF_{2\alpha}$ or PGE_2 as tracers [14]. Results are expressed in ng/ml and the cell concentration is indicated in the Figure legends. Verification of cell counts revealed no major variation in number for a given setting throughout the experimental period. For cAMP determination, cells were incubated in the presence of PGE_2 or forskolin in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (300 μM). Ice-cold ethanol (2 vol.) was added, and after 2 h at 20 °C the supernatant was centrifuged and evaporated under vacuum. The dry extract was dissolved in enzyme immunoassay buffer and the assay performed as previously established using acetylcholinesterase label [15]. Cells were lysed for Western-blot analysis (see below).

Assay of ERK2 and JNK1 activity

Quiescent SMCs in six-well plates (5×10^5 cells/well) were stimulated with FGF-2 (0.1 ng/ml) or U46619 (300 nM) for 10 min at 37 °C before lysis in Triton X-100 lysis buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 0.2 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μM pepstatin A and 1 % Triton X-100). Cell lysates were then immunoprecipitated overnight at 4 °C with 1 μg of polyclonal antibody against ERK2. The Protein G–Sepharose beads were added for 1 h at 4 °C. Immunocomplexes were collected by centrifugation at 12000 ***g*** and washed three times in lysis buffer and once with kinase buffer (20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol and 10 mM *p*-nitrophenyl phosphate) and then resuspended in 40 μl of kinase buffer containing 10 μg of MBP, 50 μM unlabelled ATP and 3 μCi of [γ-^{32}P]ATP (5000 c.p.m./pmol) per sample [16]. After 10 min at 30 °C the reaction was stopped by adding 40 μl of 2 × Laemmli's sample buffer and samples were subjected to SDS–PAGE on a 12 % polyacrylamide gel. The JNK immunocomplex kinase assay was performed as described [16]. Briefly, quiescent SMCs in six-well plates were stimulated with FGF-2 (0.1 ng/ml) or U46619 (300 nM) for 20 min at 37 °C. Cell lysates were incubated overnight with 1 μg of polyclonal antibody recognizing $p46^{JNK1}$. The same procedure was used for washing immunocomplexes adsorbed to Protein G–Sepharose before being resuspended in 40 μl of kinase buffer containing 2 μg of GSH–Jun, 50 μM unlabelled ATP and 5 μCi of [γ-^{32}P]ATP (5000 c.p.m./pmol) per sample and allowed to react for 30 min at 30 °C [17]. The samples were processed as above.

Western-blot analysis

After incubation, monolayers of SMCs in six-well plates were washed twice in PBS and lysed for 30 min in ice-cold lysis buffer (see above) for Cox analysis. Cells were scraped with a rubber 'policeman' and centrifuged at 4 °C for 10 min at 12000 ***g***. Protein content was determined by a micro-bicinchoninic acid assay (Pierce) with BSA as standard. SDS–PAGE analysis was performed as described previously [18]. Monoclonal antibodies were used for Cox-1 and Cox-2 (Cox-1 no. 10 and Cox-2 no. 29) [12]. Monoclonal antibodies against β-actin were used as internal standard for control of protein load. Blots were further incubated for 1 h at room temperature with sheep anti-mouse IgG conjugated with horseradish peroxidase. Enhanced chemiluminescence substrates were used to reveal positive bands according to the manufacturer's instructions, and bands were visualized after exposure to Hyperfilm™ ECL (Amersham). Protein bands were quantified on the films by densitometry using an LKB Ultrascan XL laser densitometer (Pharmacia).

For ERK2 and JNK1, 30 and 80 μl of Triton X-100 cell lysate respectively, corresponding to 20–50 μg of protein, were mixed with 5 × Laemmli's buffer and heated for 5 min at 95 °C. Soluble cell lysates were separated by SDS–PAGE (12 % gel), transferred by electroblot to nitrocellulose filters and probed with polyclonal antibodies raised against $p42^{ERK2}$ and $p46^{JNK1}$. The primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG. The blots were visualized as above.

RESULTS

Dose- and time-dependence effects of U46619 and FGF-2 on Cox-2 expression

When SMCs were stimulated with various concentrations of U46619 or FGF-2 for 2 h, Western-blot analysis showed a concentration-dependent increase in Cox-2 using monoclonal antibodies specific for this protein (Figure 1A). Expression of Cox-2 was already detectable at concentrations as low as 1.6 nM U46619 and 0.1 ng/ml FGF-2 compared with control unstimulated cells. A sub-maximal increase in protein expression was obtained at 300 nM and 1 ng/ml of U46619 and FGF-2 respectively. Stimulation of cells by U46619 in the presence of SQ29548, a thromboxane receptor antagonist, prevented the induction of Cox-2 (not shown). There were no synergistic effects

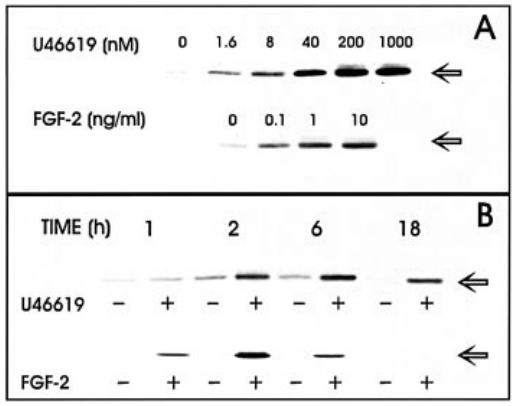

Figure 1 Effect of U46619 and FGF-2 on the expression of Cox-2 in SMCs

(**A**) Concentration-dependent expression of Cox-2; (**B**) time-dependent effect. Cells were incubated for 2 h in the presence of the two inducers for (**A**) and using 300 nM U46619 and 0.1 ng/ml FGF-2 in (**B**). All treatments were carried out as described in the Materials and methods section. These data are representative of at least five separate experiments.

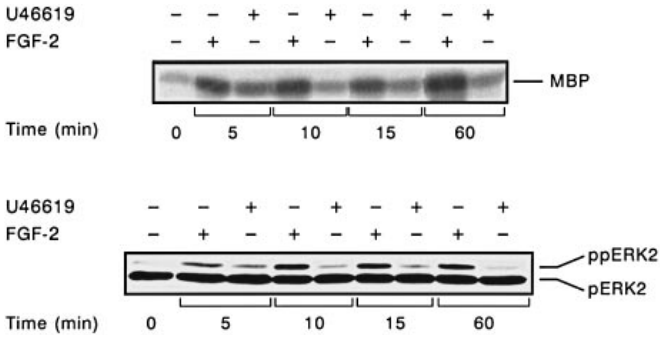

Figure 2 Time-dependent effect of U46619 and FGF-2 on the activation of ERK2

Top, U46619- or FGF-2 induced ERK2 activity; bottom, mobility shift of U46619- or FGF-2-induced ERK2 kinase. After 48 h of serum deprivation, SMCs were treated for different lengths of time with FGF-2 (0.1 ng/ml) or U46619 (300 nM). Cells were lysed and lysates were either immunoprecipitated with the anti-ERK2 antibody and the ERK2 activity was measured as described in the Materials and methods section or they were reduced and subjected to SDS/PAGE (12% gel), and Western-blot analysis was performed using anti-ERK2. These results are representative of a total of three similar experiments.

between the two inducers, even when used at threshold concentrations (not shown). Time-course induction of Cox-2 after stimulation by the inducers was also evaluated. The protein was detected as early as 1 h after stimulation, reaching a maximum between 2 and 6 h for both inducers (Figure 1B). After 18 h, Cox-2 expression induced by U46619 was only slightly decreased whereas it had disappeared in the presence of FGF-2. Neither maximal concentrations of inducers nor early-time appearance of expression revealed divergences between the two inducers in their ability to induce more intense expression of Cox-2. In subsequent experiments, 300 nM U46619 and 0.1 ng/ml FGF-2 and 2 h stimulation were used, as these conditions provided similar, albeit submaximal, amounts of Cox-2 for both inducers. In the presence of these inducers, Cox-1 remain constant (not shown).

In SMCs stimulated with various concentrations of U46619 or FGF-2, Cox-2 expression was also accompanied by a change in

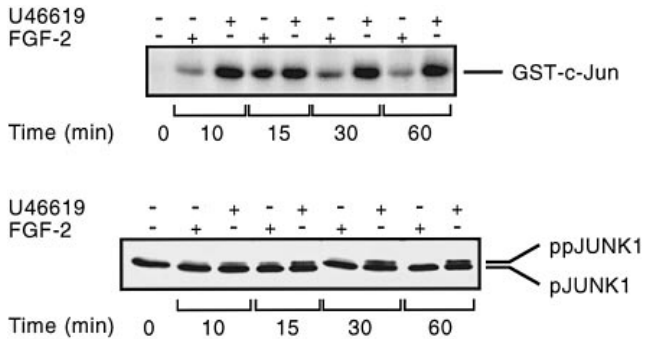

Figure 3 Time-dependent effect of U46619 and FGF-2 on the activation of JNK1

Top, U46619- or FGF-2-induced JNK1 activity; bottom, mobility shift of U46619- or FGF-2-induced JNK1 kinase. Incubation was similar to that in Figure 2 and cell lysates were also treated similarly using an anti-JNK1 antibody to perform the immunoprecipitation and Western-blot analysis. These results are representative of a total of three independent experiments.

PGs production evaluated by the measurement of 6-oxo-$PGF_{1\alpha}$ and PGE_2. After 2 h of cell activation with U46619 and FGF-2, cells released 4–5 times and 3 times respectively the amount of 6-oxo-$PGF_{1\alpha}$ present in the supernatant of control cells (mean $\pm$ S.E.M.: control, 0.2 ± 0.04 ng/ml; U46619, 1.0 ± 0.3 ng/ml; FGF-2, 0.7 ± 0.2 ng/ml). Similar results were obtained with PGE_2 (mean $\pm$ S.E.M.: control, 0.9 ± 0.2 ng/ml; U46619, 5.4 ± 1.1 ng/ml; FGF-2, 3.8 ± 0.5 ng/ml). Cox activity in the same cells after addition of exogenous arachidonic acid for a brief period of time (i.e. 30 min) showed a 2-fold increase in the production of 6-oxo-$PGF_{1\alpha}$ (mean $\pm$ S.E.M.: control, 2.8 ± 0.3 ng/ml; U46619, 5.6 ± 0.3 ng/ml; FGF-2, 5.2 ± 0.4 ng/ml). The production of PGE_2 was comparable after stimulation by either inducer (mean $\pm$ S.E.M.: control, 14 ± 1.8 ng/ml; U46619, 26 ± 3.3 ng/ml; FGF-2, 30 ± 4 ng/ml). These results are consistent with the up-regulation of Cox-2 shown in Figures 1 and 2. In all cases, the amount of PGE_2 was higher than that of 6-oxo-$PGF_{1\alpha}$.

Time-dependent effect of U46619 and FGF-2 on MAPK activation

Stimulation of ERK by a TX receptor agonist has been reported in cultured guinea-pig coronary SMCs [5]. We determined the time-dependent effect of U46619 (300 nM) and FGF-2 (0.1 ng/ml) on ERK2 activation at concentrations inducing [^{3}H]thymidine incorporation. Using a standard immunocomplex kinase assay, a low and transient (i.e. maximal at 5 min) level of ERK2 activation was found in the presence of U46619 whereas a higher and sustained kinase activity was observed with FGF-2 (Figure 2, top). These results were confirmed by analysing the state of ERK2 phosphorylation, which is directly correlated with a reduction in the electrophoretic mobility. In quiescent control cells, there was no change in electrophoretic mobility, whereas treatment of cells with U46619 induced a low and transient (5–10 min) ERK2 phosphorylation, which returned to baseline after 15 min (Figure 2, bottom). U46619 at 1 μM induced a maximal, though significant, 3-fold increase in maximal incorporation of [^{3}H]thymidine over control cells (not shown). These results are in sharp contrast with those for FGF-2, which also induced a rapid increase in phosphorylation at 5 min with a maximum at 10 min but this was followed by a long and progressive decline at later times (i.e. beyond 1–2 h). Under these conditions, incorporation of [^{3}H]thymidine was 10-fold higher

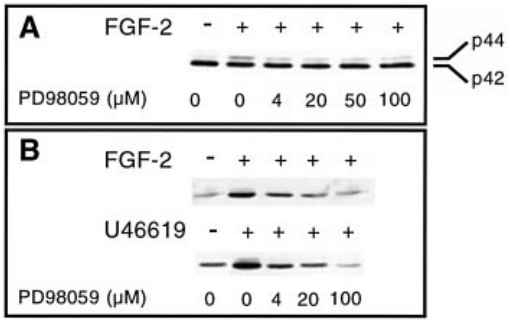

Figure 4. Dose–response effect of PD98059 on the activation of ERK2 (A) and induction of Cox-2 (B)

Incubations were performed using 0.1 ng/ml FGF-2 and 300 nM U46619. The mobility shift of FGF-2-induced ERK2 kinase was tested at 10 min in the absence or presence of increasing concentrations of PD98059 whereas Cox-2 expression was analysed after 2 h of incubation

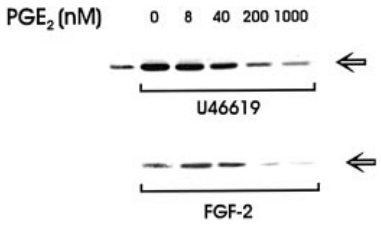

Figure 5 Dose–response effect of PGE_2 on the induction of Cox-2 by U46619 or FGF-2

Incubation was similar to that in Figure 1 using 300 nM U46619 or 0.1 ng/ml FGF-2 for 2 h. This Figure represents one experiment of a total of three.

than in controls but went up to 80-fold when 5 ng/ml FGF-2 was used (not shown). These results suggest that U46619 and FGF-2 induced different levels of ERK2 activation, consistent with the low mitogenic effect of U46619 compared with that of FGF-2.

We therefore investigated the effect of U46619 and FGF-2 on the activation of JNK1, a novel member of the MAPK cascade. Incubation with U46619 caused a marked and sustained activity of JNK1 with a plateau after 10 min still visible at 1 h (Figure 3, top). A transient and modest activation of JNK1 was obtained with FGF-2, and the phosphorylation pattern of JNK1 induced by FGF-2 was virtually undetectable at all times of induction (Figure 3, top and bottom). These results contrast with the high phosphorylation of JNK1 obtained with U46619, which was maximal at 30 min with a progressive decline after 2 h (Figure 3, bottom). Finally, the effect of the specific ERK2 inhibitor PD98059 was tested on the expression of Cox-2. There was a dose-dependent effect of PD98059 on the inhibition of ERK2 phosphorylation (Figure 4A) parallel to a reduction in Cox-2 expression (Figure 4B, top). A similar reduction was observed for U46619 (Figure 4B, bottom), although the phosphorylation of JNK1 was not modified (not shown).

Effect of PGE_2 on the expression of Cox-2 and MAPK activation induced by U46619 and FGF-2

Various reports have described a regulatory effect of prostacyclin on the stimulation of various cells [19]. We initially examined the effects of carbacyclin, a prostacyclin analogue, on the expression of Cox-2 induced by U46619 or FGF-2. This compound attenuated the induction of Cox-2 but its effect was moderate (not shown). Because PGE_2 is the main metabolite of these cells we also evaluated its effects; it inhibited Cox-2 expression dose-dependently from 40 nM up to 1000 nM (Figure 5). The possible relationship between Cox-2 expression and MAPK pathways was investigated by analysing the effect of PGE_2 at concentrations that inhibit Cox-2 expression (i.e. 200 nM). Concomitant addition of PGE_2 with U46619 for FGF-2 had no effect. However, pretreatment of cells with PGE_2 or 15 min completely inhibited the phosphorylation and activity of ERK2 induced by U46619. Inhibition was less pronounced with FGF-2 and most of the activity was still present 30 min after PGE_2 treatment (Figure 6). Similar results were obtained when higher concentrations (i.e. up to 1 μM) of U46619 were used to obtain a more pronounced phosphorylation of ERK2, although at this concentration PGE_2 was still effective in suppressing Cox-2 expression (not shown). In contrast, PGE_2 completely inhibited JNK1 phosphorylation and activity with both inducers. Although an effect was observed in the absence of preincubation, pretreatment of cells with PGE_2

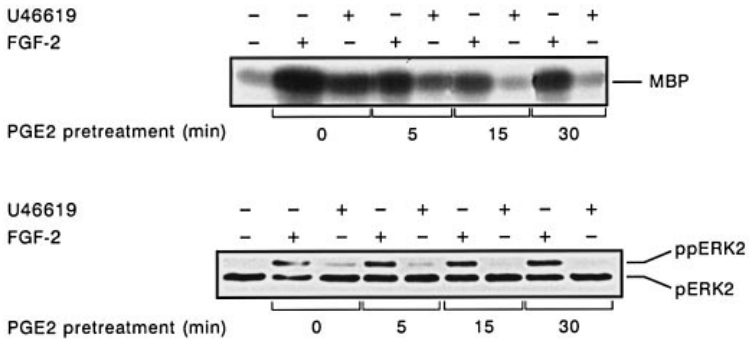

Figure 6 Time-dependent effect of PGE_2 pretreatment on ERK2 activity and mobility shift induced by U46619 or by FGF-2

Top, ERK activity; bottom, mobility shift of ERK2 kinase on activation by U46619 or FGF-2. SMCs were pretreated for different times (0–30 min) with PGE_2 (200 nM) and incubated in the presence of U46619 (300 nM) or FGF-2 (0.1 ng/ml). After 10 min of stimulation, cells were lysed and lysates were treated as described in the legend to Figure 2. These results are representative of at least three independent experiments.

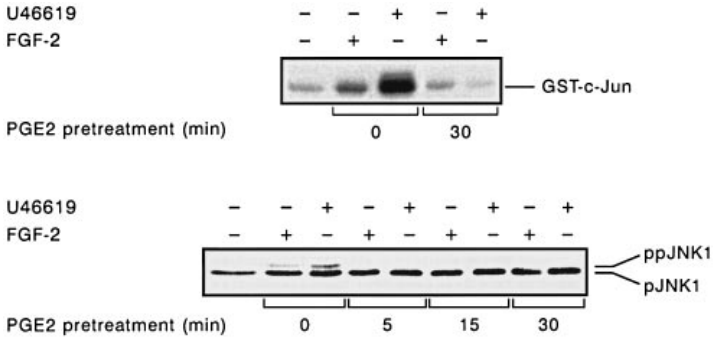

Figure 7 Time-dependent effect of PGE_2 pretreatment on JNK1 activity (top) and mobility shift induced by U46619 or by FGF-2

SMCs were pretreated for different times (0–30 min) with PGE_2 (200 nM) and incubated in the presence of U46619 (300 nM) and FGF-2 (0.1 ng/ml). After 20 min of stimulation, cells were lysed and lysates were treated as described in the legend to Figure 3. These results are representative of a total of three independent experiments.

Table 1 Production of cAMP in SMCs stimulated by PGE_2 or forskolin

SMCs were incubated for various times (0–30 min as indicated) with PGE_2 (200 nM) or forskolin (10 μM) in the presence of 300 μM) in the presence of 300 μM isobutylmethylxanthine. The extraction and assay were performed as described in the Materials and methods section. The values are means $\pm$ S.D. for three to five different experiments for PGE_2 and three experiments for forskolin. n.d., not done.

	cAMP (pmol/mg of protein)					
Time (min) ...	0	2	5	10	20	30
PGE_2	143 $\pm$ 44	491 $\pm$ 220	665 $\pm$ 210	722 $\pm$ 242	1029 $\pm$ 371	995 $\pm$ 174
Forskolin	n.d.	n.d.	n.d.	2190 $\pm$ 308	n.d.	n.d.

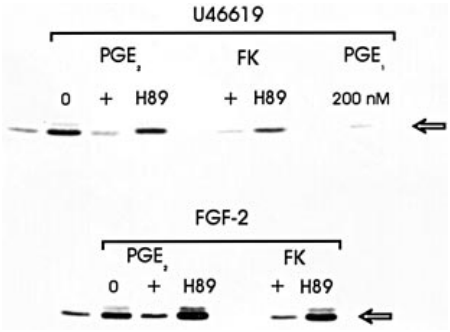

Figure 8 Reversal by a protein kinase A inhibitor of the inhibitory effect of PGE_2 on Cox-2 induction

Cells were incubated for 2 h with U46619 (300 nM) or FGF-2 (0.1 ng/ml), as in Figure 4 using PGE_2, forskolin (FK) or PGE_1 (200 nM, 5 μM or 200 nM respectively). H89 (10 μM) was added concomitantly with the different compounds. The results are representative of three incubations from different experiments.

showed a time-dependent inhibition of phosphorylation after 5 min of preincubation (Figure 7). These results suggest that there is no correlation between the capacity of PGE_2 to inhibit Cox-2 expression induced by FGF-2 and its ability to modify ERK2 activity. In contrast, the effect of PGE_2 on the induction of Cox-2 appears to vary concomitantly with JNK1 activity.

Regulation of Cox-2 by PGE_2 is mediated by cAMP

The mode of action of PGE_2 on Cox-2 expression was investigated. We initially measured cAMP levels in SMCs in response to PGE_2, as its biological effects, when coupled to the EP2 receptors, are exerted by elevation of cAMP [20]. Dose–response experiments indicated a significant increase in cAMP at the lowest concentration of PGE_2 able to inhibit Cox-2 expression, i.e. 8 nM (266 $\pm$ 65 compared with 129 $\pm$ 19 pmol/mg of protein; mean $\pm$ S.D.; $n = 3$). Maximal response was obtained at 200 nM PGE_2 (972 $\pm$ 44 pmol/mg of protein). This compound induced a significant increase in cAMP after 2 min (491 $\pm$ 220 pmol/mg of protein) reaching a plateau after 5 min (Table 1). In the next set of experiments, we investigated whether elevation of cAMP concentration could inhibit Co-2 expression via activation of protein kinase A. We used H89, a protein kinase A inhibitor [21], in cells stimulated by U46619 or FGF-2; this compound reversed the inhibition of Cox-2 expression induced by PGE_2 (Figure 8). Under the same conditions, H89 reversed the inhibitory effect of PGE_2 on the phosphorylation of JNK1 (not shown).

In order to confirm that these results were not attributable to secondary effects resulting from the interaction of PGE_2 with its receptor(s), we used forskolin which directly stimulates adenylate cyclase or we added dibutyryl-cAMP. Forskolin raised the level of cAMP (from 143 $\pm$ 44 to 2190 $\pm$ 308 pmol/mg or protein; Table 1) and suppressed the expression of Cox-2 induced by both inducers; this effect was reversed by H89, similarly to PGE_2 (Figure 8). A similar effect on the inhibition of Cox-2 expression by U46619 or FGF-2 was obtained using dibutyryl-cAMP (1 mM) (not shown). There was no induction of Cox-2 when PGE_2, forskolin or dibutyryl-cAMP were incubated alone with the cells (not shown). In the light of these results it is likely that inhibition of Cox-2 expression by PGE_2 is the consequence of a rise in cAMP followed by activation of protein kinase A. The ability of PGE_2 to stimulate cAMP in SMCs suggests the presence on these cell membranes of receptors coupled to a G_s-protein stimulating adenylate cyclase activity. Because the isoform(s) of PGE_2 receptors have not yet been characterized in porcine SMCs, we used PGE_1, which has been reported also to act on the EP2 receptor isoform coupled to adenylate cyclase. The expression of Cox-2 induced by U46619 was totally suppressed by PGE_1 at the same concentration as PGE_2 (Figure 8). These results support the concept that the inhibitory effect of PGE_2 on Cox-2 expression may be caused by activation of EP2 receptors coupled to adenylate cyclase. When U46619 is the inducer,

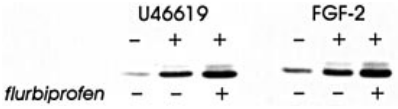

Figure 9 Effect of Cox inhibition on the induction of Cox-2 by U46619 or FGF-2

Activation of cells with 300 nM U46619 or 0.1 ng/ml FGF-2 was carried out for 2 h in the absence or presence of 5 μM flurbiprofen. The Figure is representative of three separate experiments.

inhibition of Cox-2 is parallel to that of JNK and appears to be dependent on subsequent activation of protein kinase A.

Autocrine regulation of Cox-2 by PGE_2

Finally, because PGE_2 is found in appreciable amounts in the supernatant of these cells, we investigated the contribution of endogenous PGE_2 to the regulation of Cox-2 expression induced by U46619 or FGF-2. Stimulation of cells in the presence of flurbiprofen, a Cox inhibitor, resulted in profound suppression ($> 95\%$) of PGs released into the supernatant (not shown). Inhibition of PG synthesis was accompanied by a substantial increase in the expression of Cox-2 after stimulation with both inducers (Figure 9). Similar results were obtained using low concentrations of other structurally unrelated Cox inhibitors, such as aspirin (100 μM) and indomethacin (2.5 μM) (not shown). None of these molecules incubated alone induced a significant induction of Cox-2. Overall these results suggest that PGE_2 can regulate the expression of Cox-2 in SMCs via an autocrine loop.

DISCUSSION

TXA_2 and other eicosanoids have profound effects on SMC contractility. Various reports suggest a possible role for these mediators in cell growth, and recent publications have shown that TX mimetics such as U46619 activate the ERK pathway [6–8] and stimulate p85/S6 kinase [5]. However, recent studies [8,22] failed to show a significant effect of U46619 on cell growth in spite of an increased activity of ERK2 and other upstream signalling molecules such as Shc–GRB2 complexes [8]. Induction of Cox-2 has been found to be associated with cell transformation and metastatic cell potential [9], and we have shown in a previous report that various growth promoters induce the expression of Cox-2 in rat vascular SMCs [3]. These data support the view that, in spite of similarities in biochemical responses such as induction of Cox-2, differences between the effects of growth factors and G-protein-coupled receptors exist on the signalling pathways and growth effects on cell. However, the relation between these issues was not addressed in the previous study.

The expression of Cox-2 was increased by the two inducers with similar rapid kinetics (i.e. 1–2 h) and intensity. When both inducers were used at concentrations that produced a similar induction of Cox-2, the ability of U46619 to elicit ERK2 phosphorylation and activation was very low compared with that of FGF-2. In addition, as reported by Morinelli et al. [5], U46619 failed to induce a biphasic and sustained activation of ERK2 comparable with that of FGF-2. A recent study reports that $PGF_{2\alpha}$ and 8-epi-$PGF_{2\alpha}$, presumably acting on TX receptors, induce a sustained increase in MAPK activity (up to 2 h) in porcine carotid artery SMCs [23]. This result is at odds with previous ones [8], but this discrepancy could be due to molecular differences between the inducers. Sustained activation of ERK2 has been shown to be necessary to initiate the signalling pathways involved in cell proliferation [24]. The low ability of TX to act as an inducer of proliferation compared with FGF-2 is consistent with these results, and recent studies on rat aortic SMCs showed a low mitogenic effect of U46619 with a 2-fold increase over control, similar to our results [23]. In contrast, the effects of U46619 on JNK were quite distinct. ERKs and JNKs are the main members of the growing family of MAPKs and define parallel cascades that lead to the phosphorylation of both distinct and overlapping sets of transcription factors [25,26]. There was a marked difference from the previous results, as U46619 was more potent in stimulating JNK1 phosphorylation and activity than FGF-2. However, the short-lived, less intense effect of FGF-2 on this kinase compared with that of U46619 was consistent in nearly all experiments. The inhibitory effect of PD98059 on the expression of Cox-2 suggests that activation of ERK2 may still control the expression of Cox-2 by both inducers, irrespective of the difference in mitogenesis.

Earlier results had shown that ciprostene, a prostacyclin analogue, interferes with ERK2 activity [8]. We found that carbacyclin, a PGI_2 mimetic, and PGE_2 dose-dependently reduced the expression of Cox-2 induced by U46619 or FGF-2. However, PGE_2 had no effect on the activation of ERK2 induced by FGF-2, and preincubation of the cells with PGE_2 for up to 30 min did not modify this result, although Jones et al. [8] reported that this step was critical to inhibit MAPK activation in rat aortic SMCs by ciprostene. Although results for kinase activation and mobility shift are mostly parallel, some discrepancies are observed because of different thresholds of detection of the kinase activity and mobility shift. The inhibitory effect of PGE_2 on ERK2 activation induced by U46619 could be due to a weaker activation of this kinase compared with that induced by FGF-2. Alternatively, a species difference (rat compared with pig) may account for this effect as distinct TX receptors with a unique C-terminus have been identified in the rat which may be associated with transducing mechanisms [27]. Our results are in agreement with a recent study on airway SMCs showing that seven-transmembrane-induced activation of ERK1 was inhibited by an increase in cAMP level, whereas the effect of platelet-derived growth factor was unaltered [28]. In contrast, JNK1 phosphorylation and activity were suppressed for both inducers even after a short (5 min) preincubation with PGE_2. This time is compatible with the results of the time-course study of cAMP increase (Table 1), which reached a maximum within 5 min of the addition of PGE_2. Inhibition of JNK1 by cAMP with no effect on ERK has been reported in T-lymphocytes [29]. The mechanism of Cox-2 regulation by PGE_2 in SMCs stimulated with U46619 or FGF-2 appears to be related to the variation in cAMP levels, since addition of dibutyryl-cAMP inhibited the expression of Cox-2. Furthermore the reversal of the inhibitory effects of PGE_2 or forskolin on Cox-2 and phosphorylation of JNK brought about by the protein kinase A inhibitor, H89, is compatible with a mechanism involving activation of adenylate cyclase after binding of PGE_2 to its receptor(s). The ensuing increase in cAMP leading to activation of protein kinase A is followed by inhibition of JNK1 activity with parallel inhibition of Cox-2 expression. Inhibition of JNK activation has also been reported in airway SMCs after stimulation of protein kinase A by forskolin [28]. The possibility that this effect could be mediated by EP2 receptors coupled to adenylate cyclase is suggested by the inhibitory effect of PGE_1, which shares the same effector response as PGE_2 on this receptor [20]. It has been shown that activation of adenylate cyclase by forskolin in 3T3 cells induced an increased expression of Cox-2, and the human Cox-2 gene has been shown to contain a cAMP response element [30–32]; the down-regulation observed

here may be explained by different effector systems coupled to protein kinase A [33]. Consistent with our data, neither dibutyryl-cAMP nor forskolin added alone were able to induce the expression of Cox-2. A similar negative effect of PGE_2 on the expression of Cox-2 in uterine stromal cells has recently been reported [34]. In addition to the role of exogenous PGE_2 as a negative regulator of Cox-2 expression in U46619-or FGF-2-activated SMCs, our study indicates that endogenous PGE_2 (and presumably PGI_2) generated by the vascular cells can exert a moderate but reproducible negative effect on the induction of Cox-2. Since flurbiprofen, aspirin and indomethacin are structurally unrelated, their comparable ability to up-regulate Cox-2 is likely to be due to inhibition of endogenous prostaglandins (PGE_2 but probably also PGI_2) rather than to a direct effect on Cox-2 expression. Apparently contradictory results have been obtained on the effects of non-steroidal anti-inflammatory molecules on Cox-2 expression. Whereas aspirin, by inhibiting Nfκb [35], could interfere with Cox-2 expression, a variety of related drugs including flurbiprofen have been reported to induce the expression of this enzyme [36]. However, effective concentrations for induction of these effects were 10–500-fold higher than those used here. Such results support an autocrine role for endogenous PGs in limiting the expression of Cox-2 in SMCs.

Overall, our results show that TX or FGF-2 can induce the expression of Cox-2 in a similar fashion in spite of substantial discrepancies in the magnitude of MAPK activation. These results suggest that ERK2 activation, cell proliferation and induction of Cox-2 are differentially regulated and that several signalling pathways are involved in these separate events. Involvement of MAPK in the induction of Cox-2 has recently been suggested, indicating a convergence of JNK signal-transduction pathways activating c-Jun and ERK pathways in the activation of the Cox-2 gene via a cAMP response element in the murine Cox-2 promoter [17]. Since PD98059 inhibits the expression of Cox-2, our results suggest that cAMP act downstream of ERK2. Recent results show that inhibition of inducible Cox-2 correlates with that of MAPK in rat macrophages stimulated by lipopolysaccharide [37]. In addition, since PGE_2 mediates parallel inhibition of Cox-2 expression and JNK activity, our results also suggest the involvement of this MAPK in the induction of Cox-2.

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