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The MAP kinase inhibitors, PD098059, UO126 and SB203580, inhibit IL-1β-dependent PGE_2 release *via* mechanistically distinct processes

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1 In common with human bronchial epithelial cells, pulmonary A549 cells release prostaglandin (PG) E_2 in response to pro-inflammatory cytokines. We have therefore used these cells to examine the effect of the selective mitogen activated protein (MAP) kinase inhibitors; PD098059, a mitogen activated and extracellular regulated kinase kinase (MEK) 1 inhibitor, UO126, a dual MEK1 & MEK2 inhibitor, and SB203580, a p38 MAP kinase inhibitor in the IL-1β-dependent release of PGE_2.

2 Following IL-1β treatment the extracellular regulated kinases (ERKs) and the p38 MAP kinases were rapidly phosphorylated.

3 PD09059, UO126 and SB203580 prevented IL-1β-induced PGE_2 release at doses that correlated closely with published IC_{50} values. Small or partial effects at the relevant doses were observed on induction of cyclo-oxygenase (COX) activity or COX-2 protein suggesting that the primary effects were at the level of arachidonate availability.

4 Neither PD098059 nor SB203580 showed any effect on IL-1β-induced arachidonate release. We therefore speculate that the MEK1/ERK and p38 kinase cascades play a role in the functional coupling of arachidonate release to COX-2.

5 In contrast, UO126 was highly effective at inhibiting IL-1β-dependent arachidonate release, implicating MEK2 in the activation of the PLA_2 that is involved in IL-1β-dependent PGE_2 release.

6 We conclude that the MEK1, MEK2 and p38 MAP kinase inhibitors, PD098059, UO126 and SB203580, are highly potent in respect of inflammatory PG release. Finally, we conclude that these inhibitors act *via* mechanistically distinct processes, which may have anti-inflammatory benefits.

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Abbreviations: COX, cyclo-oxygenase; $cPLA_2$, cytosolic phospholipase A_2; DMSO, dimethyl sulphoxide; ERK, extrcellular regulated kinase; IL, interleukin; JNK, Jun N-terminal kinase; MAFP, methyl arachidonyl fluorophosphonate; MAP, mitogen activated protein; MAPK, MAP kinase; MAP2K, mitogen activated protein kinase kinase; MAP3K, mitogen activated protein kinase kinase kinase; MEK, mitogen activated protein kinase/extracellular regulated kinase kinase; PD098059, 2′-amino-3′methoxyflavone; PG, prostaglandin; PLA, phospholipase; RIA, radioimmuoassay; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)1H-imidazole; $sPLA_2$, secretory phospholipase A_2; UO126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene

Introduction

Prostaglandins (PGs) are lipid mediators that are involved in many normal physiological processes, and are implicated in many pathophysiological processes such as inflammation, edema, bronchoconstriction, platelet aggregation, fever and hyperalgesia (Mitchell *et al*., 1995). PG synthesis predominantly involves phospholipase A_2 (PLA_2)-catalysed release of arachidonic acid from the *sn*-2 position of membrane phospholipids and conversion by the two cyclo-oxygenase (COX) enzymes to PGH_2. Subsequently cell type-specific expression of downstream synthases is responsible for the production of biologically relevant PGs.

Numerous PLA_2 activities have been identified and many of the genes cloned (Balsinde & Dennis, 1997; Dennis, 1997). Of these, the group II secretory PLA_2 ($sPLA_2$) and the group IV Ca^{2+}-dependent cytosolic PLA_2 ($cPLA_2$) have long been thought to play the predominant roles in respect of inflammatory prostaglandin synthesis (Balsinde & Dennis, 1997; Dennis, 1997). Similarly, the two COX isoforms are encoded by distinct genes. COX-1 is a constitutively expressed housekeeping gene whilst COX-2 is an acute phase gene that is rapidly induced by inflammatory and mitogenic stimuli (Mitchell *et al*., 1995). As COX is a target for non-steroidal anti-inflammatory drugs (NSAIDs) this pathway is pharmacologically important (Mitchell *et al*., 1995). Furthermore the use of isoform selective COX inhibitors has revealed that many anti-inflammatory benefits of NSAIDs derive from COX-2 inhibition whilst many undesirable side effects result from COX-1 inhibition (DeWitt, 1999; Mitchell *et al*., 1995; Seibert *et al*., 1994). However, more recent evidence suggests that COX-2-dependent PG release may also play a role in the resolution of inflammation (Gilroy *et al*., 1999). Despite the clinical usefulness of NSAIDs, currently the most effective drugs in the treatment of chronic inflammatory diseases, such as asthma, are corticosteroids (Barnes, 1999). These down-regulate various inflammatory processes, including prostaglan-

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din synthesis, *via* repression of pro-inflammatory genes such as COX-2 (Barnes, 1999; Newton *et al*., 1997a).

Recently the development of small molecule inhibitors of the mitogen activated protein (MAP) kinase pathways has attracted much attention as potential therapeutic agents (Alessi *et al*., 1995; Cuenda *et al*., 1995; Dudley *et al*., 1995; Favata *et al*., 1998; Lee *et al*., 1994). Various cellular stresses, as well as pro-inflammatory cytokines such as IL-1β and TNFα, are known to activate multiple MAP kinase signalling pathways, which then play major effector roles in numerous cellular responses (Kyriakis & Avruch, 1996). These pathways comprise of kinase cascades in which small GTP-binding proteins, such as Ras, Rac or Rho, activate MAP kinase kinase kinases (MAP3K), such as Raf. These activate, by phosphorylation, MAP kinase kinases (MAP2K), which in turn activate the MAP kinases (MAPK). Finally, the MAP kinases phosphorylate and activate various effector molecules to elicit cellular responses. In particular, inhibitors of the MAP2K, mitogen activated protein kinase (MAPK)/extracellular regulated kinase (ERK) kinase (MEK), and the p38 MAPK are effective in preventing induction of pro-inflammatory genes (Alessi *et al*., 1995; Cuenda *et al*., 1995; Dudley *et al*., 1995; Lee *et al*., 1994; Scherle *et al*., 1998).

Epithelial cells have an active role in inflammation by producing multiple mediators and airway epithelial cells respond to pro-inflammatory cytokines, such as IL-1β, by induction of COX-2 and PGE_2 release (Mitchell *et al*., 1994). As this response is also observed in human A549 cells (Mitchell *et al*., 1994; Newton *et al*., 1997a), we have used these cells to examine the effect of selective MAP kinase pathway inhibitors on the induction of COX-2 and PGE_2 release by IL-1β.

Methods

Cell culture

A549 cells were grown to confluency as previously described (Newton *et al*., 1997a). Cells were incubated over-night in serum-free medium before changing to fresh medium containing 1 ng ml^{-1} IL-1β (2×10^5 u μg^{-1}) (Genzyme, MA, U.S.A.), 3 μM ionomycin (Sigma, Poole, U.K.), drugs or vehicle. PD098059 (2′-amino-3′methoxyflavone) (Alexis, Bingham, U.K.), UO126 (1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio)butadiene) (Calbiochem, Nottingham, U.K., SB203580 (4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)1H-imidazole) (Alexis) and methyl arachidonyl fluorophosphonate (MAFP) (Calbiochem) were dissolved in DMSO and diluted at least 1 : 1000 in culture media prior to use. DMSO at such concentrations had no effect on cell viability, PGE_2 release or COX activity (data not shown).

PGE_2 release and COX activity determination

Culture medium was removed and released PGE_2 measured by radioimmunoassay (RIA) using an anti-PGE_2 antibody (Sigma) essentially according to the manufacturers instructions (Mitchell *et al*., 1994). After rinsing cells twice, COX activity assays were performed as described (Mitchell *et al*., 1994). Briefly, fresh medium containing 30 μM arachidonic acid (Sigma) was added and the plates incubated for 10 min at 37°C. PGE_2 produced was measured by RIA and taken as an index of COX activity.

Immunoblot analysis

Cells were harvested and lysed as previously described (Newton *et al*., 1997c). Total cellular protein was separated by 10% SDS–PAGE (or as indicated) and electroblotted to hybond-ECL membranes (Amersham, Buckinghamshire, U.K.). Membranes were probed with rabbit anti-human antibodies directed to either phosphorylated or total ERK and p38 MAP kinases according to the manufacturers specification (New England Biolabs, Hitchin, U.K.). After washing, membranes were incubated with horseradish peroxidase-linked anti-rabbit immunoglobulin (Dakko) and immune complexes detected by enhanced chemiluminescence (ECL) (Amersham). For COX-2 immunodetection, goat anti-rabbit primary (Santa Cruz) and rabbit-anti-goat secondary (Dakko) antibodies were used with ECL.

Release of [^{3}H]-arachidonate

Confluent cells, in 24-well plates, were incubated over-night in 0.5 ml serum free media supplemented with 0.125 μCi [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid (Amersham). Cells were washed three times with media and treated in media containing 2 mg ml^{-1} bovine serum albumin (Sigma) to absorb arachidonate metabolites. Following stimulation, supernatants were collected as indicated for liquid scintillation counting. In all cases, [^{3}H]-arachidonate incorporation into cells was measured by harvesting cells in 1% SDS and liquid scintillation counting. Using this methodology [^{3}H]-arachidonate incorporation into cells was typically 75–80%. In each case, release of [^{3}H]-arachidonate and its metabolites was expressed as a percentage of the total incorporated into cells.

Statistical analysis

Data were expressed as means ± s.e.mean and statistical analysis performed by ANOVA using a Bonferoni correction for multiple comparisions. Significance is indicated where: $*P<0.05$, $**P<0.01$, $***P<0.001$.

Results

IL-1β induces ERK and p38 MAP kinase phosphorylation

In A549 cells, IL-1β has previously been shown to activate the p54 and p46 Jun N-terminal kinases (JNK) (Newton *et al*., 1997c). The effect of IL-1β was therefore examined on the ERK and p38 MAP kinase pathways. As threonine/tyrosine phosphorylation of residues 202 and 204 in ERK1 (p44) or the equivalent residues in ERK2 (p42) and residues 180 and 182 in p38 result in enzymatic activation, we used Western blot analysis to monitor phosphorylation of these sites as an index of kinase activation (Boulton *et al*., 1991; Payne *et al*., 1991; Raingeaud *et al*., 1995). In resting cells, basal ERK phosphorylation was detected whereas no basal level of p38 phosphorylation was observed. Both ERK, p44 and p42, and p38 MAP kinases were rapidly (<5 min) phosphorylated following IL-1β treatment (Figure 1). In each case this was maximal at around 30 min and was declining by 1 h after stimulation.

Effect of PD098059 and UO126 on IL-1β-induced PGE_2 release

As previously observed, 24 h IL-1β resulted in a substantial increase in PGE_2 release, COX activity and COX-2 immunoreactivity (Figure 2) (Mitchell *et al*., 1994; Newton *et al*., 1997a, 1998). Since COX-1 message is 100 fold less abundant than COX-2 and COX-1 protein is undetectable in both cytokine stimulated and untreated cells, the increase in COX activity and PGE_2 release can be attributed to *de novo* COX-2 synthesis (Mitchell *et al*., 1994; Newton *et al*., 1997a). The increase in PGE_2 release was dose-dependently inhibited by PD098059 (EC_{50} 3.0 μM) (Figure 2) and corresponds well with the published values for MEK1 inhibition (IC_{50} 2–10 μM) (Alessi *et al*., 1995; Dudley *et al*., 1995). However, 10 μM PD098059 showed little effect on induction of COX activity and protein, indicating that the inhibition of PGE_2 release must occur upstream of COX-2, presumably at the level of PLA_2 and arachidonic acid release. The partial inhibition of 50 μM PD098059 on COX activity and COX-2 protein suggest an effect on COX-2 synthesis (Figure 2A,C). However, this effect failed to reach statistical significance.

Likewise, the inhibition of IL-1β-dependent PGE_2 release by UO126 (EC_{50} 0.8 μM) is also consistent with doses previously reported to inhibit MEK1 activity and ERK phosphorylation (Favata *et al*., 1998). Similarly, whilst a partial inhibition was observed on COX activity (EC_{50} 1.0 μM) and COX-2 (EC_{50} 2.0 μM), these effects were insufficient to account for the total loss of PGE_2 release. Thus these data indicate that UO126 inhibits IL-1β-dependent PGE_2 release primarily by inhibiting a step (or steps) upstream of COX-2.

To confirm the effects of PD098059 and UO126 on MEK activation, the phosphorylation status of the immediate downstream kinases, ERK1 and 2, was examined (Figure 3). As in Figure 1 above, IL-1β markedly induced phosphorylation of both the p44 and p42 ERK. Pre-incubation with either PD098059 or UO126 totally inhibited this response with

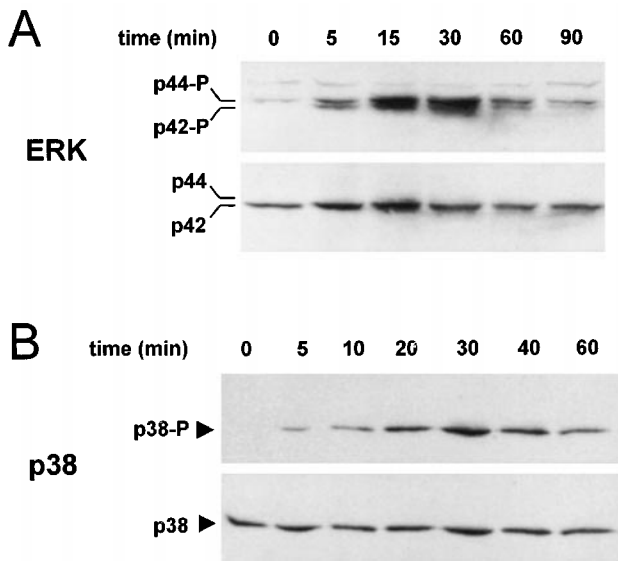

Figure 1 Activation of ERK and p38 MAP kinases by IL-1β. Cells were treated with IL-1β (1 ng/ml) for the times indicated and harvested for Western blotting. Immunodetection was performed with antibodies directed to; (A) phosphorylated ERK (upper panel) and pan ERK (lower panel) or, (B) phosphorylated p38 (upper panel) or pan p38 (lower panel). Data presented are representative of at least three similar experiments.

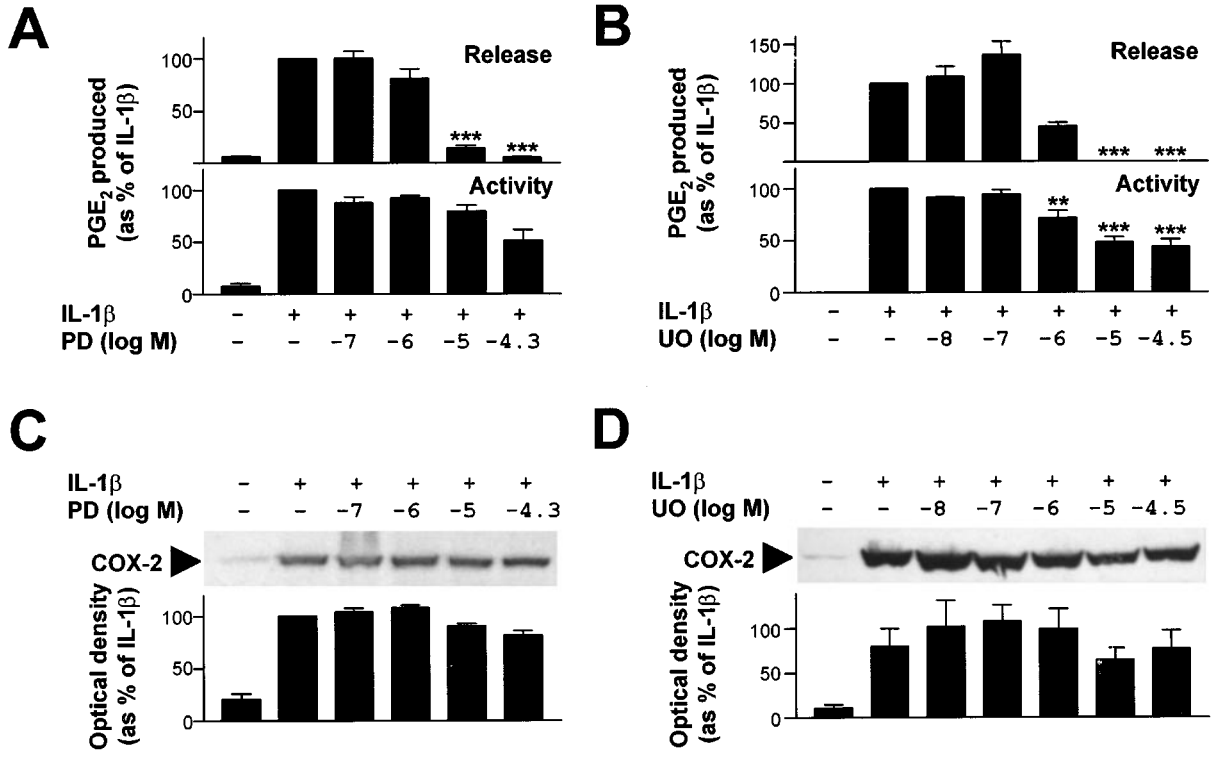

Figure 2 Effect of PD098059 and UO126 on PGE_2 release, COX activity and COX-2 protein expression. Cells were treated with various concentrations of PD098059 (PD) or UO126 (UO) as indicated and then stimulated with IL-1β (1 ng ml^{-1}) for 24 h. (A, B) Supernatants were collected for PGE_2 release measurement (upper panels) and COX activity (lower panels) determination performed. Data ($n=7$ and 6 for PD098059 and UO126 respectively) are expressed as a percentage of IL-1β treated as means$\pm$s.e.mean. (C, D) In addition cells were harvested for Western blot analysis of COX-2 protein. Representative blots are shown (upper panels). After densitometric analysis, data from four or five (PD098059 and UO126 respectively) experiments were expressed as a percentage of IL-1β treated and are shown as means$\pm$s.e.mean (lower panels).

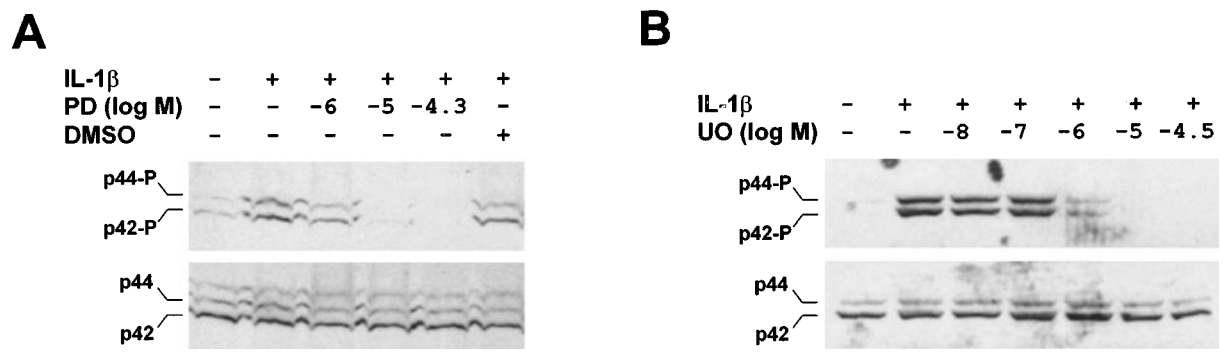

Figure 3 Effect of PD098059 and UO126 on ERK phosphorylation. Cells were treated with IL-1β (1 ng ml^{-1}) and the indicated concentrations of (A) PD098059 (PD) or (B) UO126 (UO). After 30 min cells were harvested for Western blot analysis using antibodies to phosphorylated ERK (upper panel) or pan ERK (lower panel). Blots representative of three such experiments are shown.

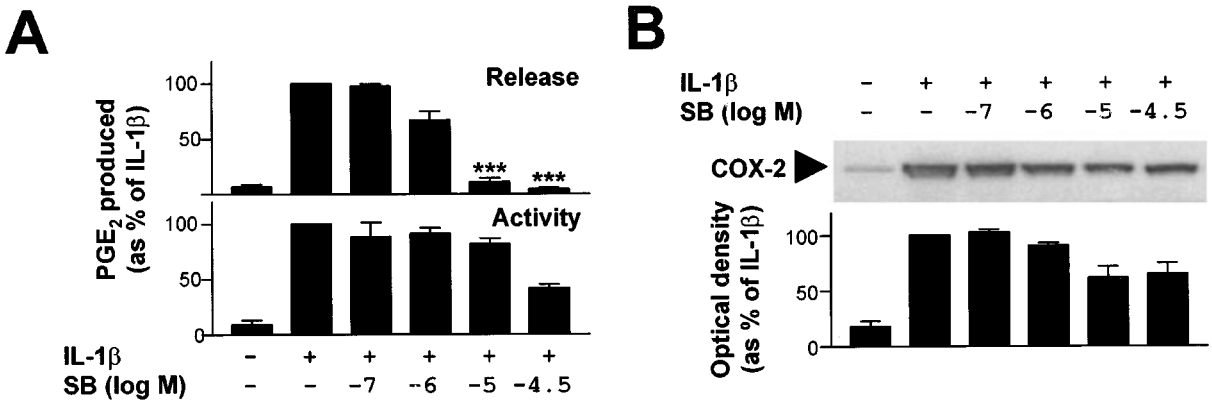

Figure 4 Effect of SB203850 on PGE_2 release, COX activity and COX-2 protein expression. Cells were treated with various concentrations of SB203850 (SB) as indicated and then stimulated with IL-1β (1 ng ml^{-1}) for 24 h. (A) Supernatants were collected for PGE_2 release measurement (upper panel) and COX activity (lower panel) determination performed. Data ($n=5$) are expressed as a percentage of IL-1β treated as means $\pm$ s.e.mean. (B) In addition cells were harvested for Western blot analysis of COX-2 protein. A representative blot is shown (upper panel). After densitometric analysis, data from four such experiments were expressed as a percentage of IL-1β treated and are shown as means $\pm$ s.e.mean (lower panel).

similar dose-response characteristics to that for the inhibition of PGE_2 release.

Effect of SB203580 on IL-1β-induced PGE_2 release

Analysis of the p38 MAP kinase inhibitor, SB203580, again revealed a dose-dependent inhibition of IL-1β-induced PGE_2 release (Figure 4). The EC_{50} for this was 0.18 μM and is consistent with published values for p38 inhibition (IC_{50} 0.6 μM) (Cuenda *et al*., 1995; Kramer *et al*., 1996). As little inhibition of COX activity and COX-2 protein was observed at these doses, these data again imply an inhibitory step upstream of COX-2.

Effect of PD098059, UO126 and SB203580 on arachidonate release

Following 1 h of IL-1β treatment only modest increases in [^{3}H]-arachidonate metabolite release into the supernatant were observed (untreated 0.90% $\pm$ 0.09, IL-1β 1.60% $\pm$ 0.11, $n=16$, $P>0.05$). Because of this low level of inducibility and release, cells were treated for 6 h with or with out IL-1β and the effects of the PD098059, UO126 and SB203580 examined (Figure 5). At this time point IL-1β caused a 1.5–2 fold increase over basal levels of [^{3}H]-arachidonate release (untreated 3.34% $\pm$ 0.18, IL-1β 5.97% $\pm$ 0.32, $n=14$, $P<0.001$). Pretreatment with either PD098059 or SB203580 had little or no effect on [^{3}H]-arachidonate release at or below 10 μM and at higher concentrations a partial inhibition was observed. In marked contrast, UO126 dose-dependently inhibited IL-1β-dependent release of [^{3}H]-arachidonate (EC_{50} 0.3 μM). To confirm that PD098059 and SB203580 were indeed functioning on PGE_2 release at this time, measurements of PGE_2 release, COX activity and COX-2 protein were carried out as in Figure 2 except that samples were harvested after 6 h. Essentially identical data was obtained to that shown in Figure 2 (data not shown).

A role for $cPLA_2$ in arachidonate release?

To further examine the mechanisms of arachidonate release in this system, we tested the effect of the highly selective group IV $cPLA_2$ inhibitor, MAFP (Balsinde & Dennis, 1996; Huang *et al*., 1994). To our surprise there was no inhibitory effect on IL-1β-dependent PGE_2 release and at higher doses a significant enhancement of PGE_2 release was observed (Figure 6). Little or no effect was observed on COX activity or COX-2 protein expression. Consistent with the elevated levels of PGE_2, a dose-dependent increase in arachidonate release was observed, which failed to reach statistical significance.

As a number of studies have implicated $cPLA_2$ in the IL-1β-dependent release of PGE_2 and/or arachidonate, we decided to further investigate this possibility. The small increases in [^{3}H]-arachidonate release may be explained by the fact that whilst

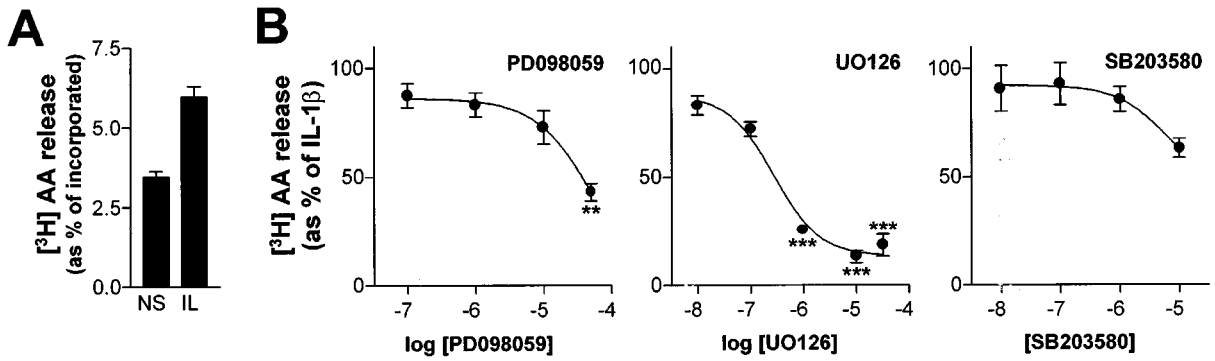

Figure 5 Effect of IL-1β, PD098059, UO126 and SB203580 on arachidonate release. Cellular lipids were loaded with [^{3}H]-arachidonate and then treated with various concentrations of PD098059, UO126 or SB203580, as indicated, prior to stimulation with IL-1β (1 ng ml^{-1}). After 6 h supernatants and cells were harvested for liquid scintillation counting. (A) Data ($n=14$) are expressed as arachidonate release as a percentage of the total incorporated. (B) The effects of PD098059 ($n=7$), UO126 ($n=4$) and SB203580 ($n=5$) each performed in duplicate are shown as arachidonate release expressed as a percentage of IL-1β treated as means$\pm$s.e.mean.

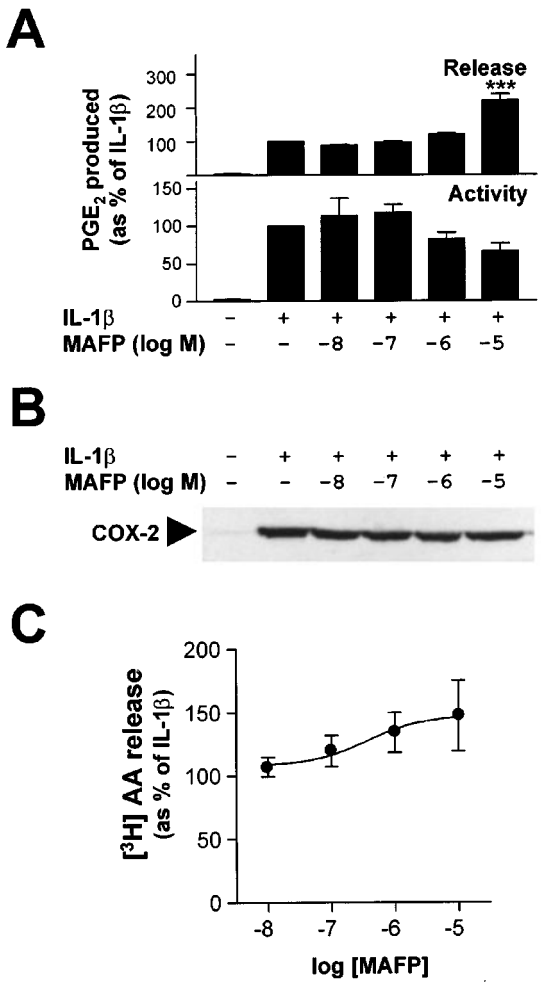

Figure 6 Effect of MAFP on PGE_2 release, COX activity, COX-2 protein expression and arachidonate release. Cells were treated with various concentrations of MAFP as indicated and then stimulated with IL-1β (1 ng ml^{-1}). (A) After 24 h, supernatants were collected for PGE_2 release measurement (upper panel) and COX activity (lower panel) determination performed. Data ($n=6$) are expressed as a percentage of IL-1β treated as means$\pm$s.e.mean. In addition cells were harvested for Western blot analysis of COX-2 protein (B). A blot representative of two such experiments is shown. (C) Cellular lipids were loaded with [^{3}H]-arachidonate and then treated with various concentrations of MAFP, as indicated, prior to stimulation with IL-1β (1 ng ml^{-1}). After 6 h supernatants were removed and cells harvested for liquid scintillation counting. Data ($n=4$) are shown as arachidonate release expressed as a percentage of IL-1β treated as means$\pm$s.e.mean.

IL-1β is a good inducer of COX-2 synthesis, it is a poor inducer of the PLA_2, possibly $cPLA_2$, required for inflammatory PGE_2 release (Schalkwijk *et al*., 1996; Tokumoto *et al*., 1994). Treatment of cells with Ca^{2+} mobilizing agents produces substantial increases in $cPLA_2$-dependent arachidonate release (Sa *et al*., 1995; Schalkwijk *et al*., 1996). A similar effect is also reported in A549 cells using ionomycin induced Ca^{2+} mobilisation (Tokumoto *et al*., 1994). In this case induction of arachidonate release was shown to be Ca^{2+}-dependent as ionomycin had no effect in Ca^{2+}-free media or media containing the Ca^{2+} chelator, EGTA. We therefore treated cells with combinations of IL-1β and ionomycin (Figure 7). Ionomycin alone produced a significant increase in arachidonate release. In the presence of ionmycin, IL-1β resulted in a consistent doubling of released arachidonate, which was significantly reduced by pre-treatment with 10 and 50 μM PD098059. Consistent with the 6 h data, SB203580, failed to inhibit IL-1β+ionomycin induced arachidonate and at higher concentrations actually enhanced arachidonate release. When PD098059 and SB203580 were tested on IL-1β-induced release of arachidonate at 1 h, again no effect was observed, which is consistent with the data in Figure 5 (data not shown). In contrast to IL-1β stimulation alone, MAFP produced a significant decrease in IL-1β+ionomycin stimulated arachidonate release.

Time-dependence of MAP kinase inhibitors on PGE_2 release

Confluent cells that had been treated with IL-1β for 20 h (in 12-well plates) were found to produce detectable levels of PGE_2 in a 10 min period (0.625 ± 0.04 ng ml^{-1}, $n=4$), whereas in unstimulated cells any PGE_2 release was below the level of detection (Figure 8). This response was totally prevented by both PD098059 and UO126, whereas SB203580 had no effect. Therefore the steps that are inhibited by PD098059 and UO126 are continually required for PGE_2 release whereas inhibition by SB203580 is time-dependent.

Immediate effects of PD098058, UO126 and SB203580 on COX activity

It has been suggested that PD098059 and SB203580 may be capable of acting as direct inhibitors of COX activity (Borsch-Haubold *et al*., 1998). To examine this possibility, cells were treated with IL-1β for 20 h to induce COX-2. Cells were then pre-treated with each drug before the addition of exogenous

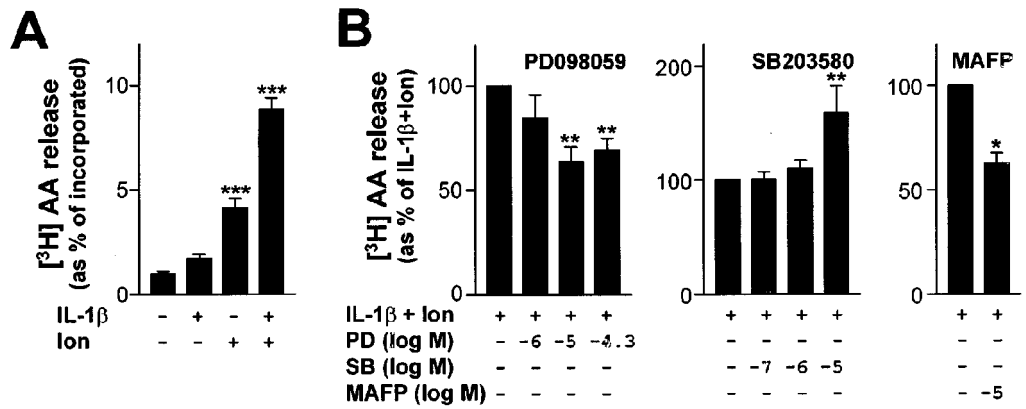

Figure 7 Effect of PD098059, SB203580 and MAFP on IL-1β and ionomycin stimulated arachidonate release. (A) Cells were loaded with [^{3}H]-arachidonate and then treated with various combinations of IL-1β (1 ng ml^{-1}) and ionomycin (3 μM) (as indicated). After 1 h supernatants were removed and cells harvested for liquid scintillation counting. Data ($n=6$) are expressed as arachidonate release as a percentage of the total incorporated. (B) Cells were loaded with [^{3}H]-arachidonate and then treated with various concentrations of PD098059 (PD), SB203580 (SB) or MAFP, as indicated, prior to stimulation with IL-1β (1 ng ml^{-1}) and ionomycin (3 μM) (Ion). After 1 h, supernatants were removed and cells harvested for liquid scintillation counting. Data ($n=4$) are shown as arachidonate release expressed as a percentage IL-1β + Ionomycin treated as means ± s.e.mean.

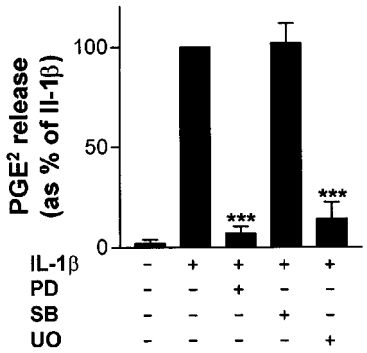

Figure 8 Acute effect of PD098059, UO126 and SB203580 on PGE_2 release. Cells, in 12-well plates, were treated with IL-1β (1 ng ml^{-1}) for 20 h. Then serum free medium containing PD098059 (50 μM), SB203580 (10 μM) or UO126 (30 μM) was added for 10 min. After this time the media was changed to fresh serum free medium (also containing PD098059, SB203580 or UO126, but not IL-1β). After a further 10 min the medium was harvested for PGE_2 release measurements. Data from four experiments are shown as a percentage of IL-1β treated as means ± s.e.mean.

arachidonic acid at concentrations of either 1 or 100 μM. After 10 min, PGE_2 release was assessed and taken as an index of COX activity (Figure 9). In the absence of inhibitors these doses of arachidonic acid gave rise to 3.07 ± 0.27 and 28.73 ± 3.16 ng ml^{-1} 10 min^{-1} well^{-1} of PGE_2 respectively. At 100 μM arachidonic acid none of the three inhibitors showed any effect. At 1 μM arachidonic acid, PD098059 and to a lesser extent UO126 showed some degree of inhibition whilst no effect was observed for SB203580.

Discussion

In these studies, PD098059, a selective MEK1 inhibitor (Alessi *et al*., 1995; Dudley *et al*., 1995), UO126, a selective MEK1 & MEK2 inhibitor (Favata *et al*., 1998), and SB203850, a p38 MAP kinase inhibitor (Cuenda *et al*., 1995), were highly potent inhibitors of IL-1β-induced PGE_2 release. In all three cases the inhibitory effect occurred at the published IC_{50} values obtained from *in vitro* kinase assays, indicating that these compounds are readily taken up by the cells and that access to the relevant target enzyme was unhindered. At the concentrations required for almost total inhibition of PGE_2 release, 10 μM for PD0998058 and 1 μM for SB203580, little or no effect was observed on IL-1β-induced COX activity or COX-2 protein. These data indicate that neither, activation of MEK1 and the downstream kinases ERK1 and 2, nor the p38 MAP kinase play a major role in the induction of COX-2 by IL-1β. By contrast, inhibition of PG synthesis in zymosan or lipopolysaccharide treated monocytes correlated with inhibition of COX-2 by SB203580 and suggests a major role for p38 kinase in COX-2 synthesis in this system (Dean *et al*., 1999; Pouliot *et al*., 1997). Furthermore, a related p38 inhibitor, SC68376, also inhibited PG synthesis seemingly *via* inhibition of COX-2 mRNA and protein induction in IL-1β-treated rat mesangial cells (Guan *et al*., 1997). However, our results are consistent with the fact that the NF-κB, C/EBP and JNK signalling pathways, which do not involve p38 MAPK, are primarily thought to mediate COX-2 transcriptional induction (Inoue *et al*., 1995; Newton *et al*., 1997b; Xie & Herschman, 1995; Yamamoto *et al*., 1995). Nevertheless, as higher doses of both PD098059 and SB203850 appeared to inhibit induction of COX activity and COX-2 protein, it is possible that one of these other pathways also becomes partially inhibited. In the case of PD098059 the inhibitory effect on COX activity may be explained by MEK2 inhibition, which occurs with an IC_{50} of around 50 μM (Alessi *et al*., 1995). This proposal is supported by the fact that UO126, which is equipotent on both MEK1 and MEK2 (Favata *et al*., 1998), also results in a partial inhibition of COX-2 protein and COX activity (EC50 1.1 μM) (Figure 2B). Furthermore, a similar inhibitory effect of UO126 was reported at the level of COX-2 mRNA (Scherle *et al*., 1998). However, whilst providing support for a role of MEKs in the induction of COX-2, this study failed to distinguish between MEK1 and MEK2 inhibition by UO126.

The relative lack of effect of PD098059, UO126 and SB203850 on induction of COX activity and COX-2 protein points to arachidonate release or availability as the primary target of inhibition. However, neither PD098059 nor SB203580 showed any significant effect on IL-1β stimulated arachidonate release at concentrations at or below 10 μM. At 50 μM, PD098059 produced a greater than 50% inhibition of arachidonate release which correlates more closely with inhibition of MEK2 rather than MEK1 (Alessi *et al*., 1995).

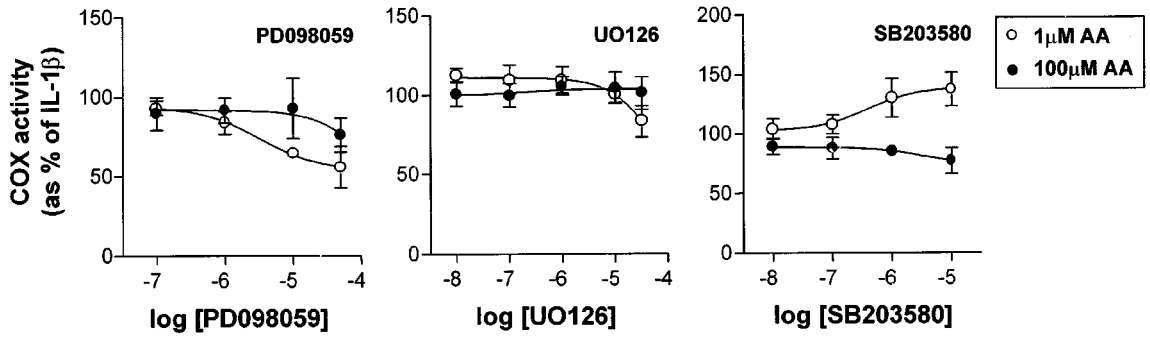

Figure 9 Effect of PD098059, UO126 and SB203580 on arachidonic acid induced PGE_2 release (COX activity). Cells, in 12-well plates, were treated with IL-1β (1 ng ml^{-1}) for 20 h. Then serum free medium containing PD098059 (50 μM), SB203580 (10 μM) or UO126 (30 μM) was added for 10 min. After this time the media was changed to fresh serum free medium supplemented with either 100 or 1 μM arachidonic acid (also containing PD098059, SB203580 or UO126). After a further 10 min the medium was harvested and PGE_2 release taken as an index of COX activity. Data from four experiments are shown as a percentage of IL-1β treated (i.e. no drug) as means$\pm$s.e.mean.

This hypothesis is supported by the data using UO126, which dose-dependently inhibits IL-1β-stimulated arachidonate release (Figure 5). The fact that ERK phosphorylation was inhibited over this same range of concentrations shows that the UO126 was gaining access to the cells and was inhibiting MEK1, which is upstream of ERK1 and 2. However, the lack of effect of PD098059 over doses that clearly inhibit MEK1, as measured by ERK1 and 2 phosphorylation, indicate that it is inhibition of MEK2 by UO126 which is responsible for the inhibition of arachidonate release and subsequent PGE_2 release.

Previous mRNA and biochemical data point to involvement of group IV $cPLA_2$ and not group II $sPLA_2$ in cytokine mediated PGE_2 release in A549 cells (Neagos *et al*., 1993; Newton *et al*., 1997a; Tokumoto *et al*., 1994). However, in the present study the highly selective $cPLA_2$ inhibitor, MAFP, was ineffective at inhibiting IL-1β-dependent PGE_2 release suggesting that the group IV $cPLA_2$ may not be involved (Figure 6). As a means of validating this effect, we tested the effect of PD098059 and MAFP in cells that had been co-stimulated with the Ca^{2+} mobilizing agent, ionomycin, and IL-1β. It is well established that Ca^{2+} flux and phosphorylation of Ser 505 are both required for full $cPLA_2$ activity (Clark *et al*., 1991; Lin *et al*., 1993; Sa *et al*., 1995; Schalkwijk *et al*., 1996). The fact that Ca^{2+} mobilization in the presence of IL-1β gave rise to synergistically increased arachidonate release supports the existence of a role for $cPLA_2$ in A549 cells and this is further substantiated by the inhibitory effect of MAFP (Figure 7). In addition, the inhibition by PD098059 of the synergistic effect of IL-1β on Ca^{2+}-dependent arachidonate release is consistent with previous studies, which indicate that $cPLA_2$ is a target for ERK MAP kinases (Sa *et al*., 1995; Schalkwijk *et al*., 1996). Therefore these data indicate that whilst ERK activation of $cPLA_2$ may play a role in IL-1β+Ca^{2+} stimulated release of arachidonate, the IL-1β-dependent release of PGE_2 does not appear to involve $cPLA_2$ and inhibition by PD098059 is not mediated *via* an effect on arachidonate release. Similarly, the p38 inhibitor, SB203580, resulted in total suppression of IL-1β-dependent release of PGE_2 at doses that correlated closely with published IC_{50} values (Cuenda *et al*., 1995; Kramer *et al*., 1996) . However, as no effect of SB203580 was observed on release of arachidonate metabolites at either 1 or 6 h, these data show that p38 MAP kinase is also not directly involved in PLA_2 activation *per se*. This ability of SB203580 to inhibit PGE_2 release is time-dependent as cells that have been IL-1β stimulated for 20 h and then treated with SB203580 do not show any inhibition of PGE_2 release (Figure 8). Thus either the downstream substrate is fully activated and p38 MAP kinase is no longer required or synthesis of a p38 MAP kinase-dependent protein that is required for PGE_2 release has occurred and can no longer be blocked by SB203580.

In A549 cells, epidermal growth factor is reported to cause $cPLA_2$ phosphorylation detectable by mobility shift on SDS–PAGE (Croxtall *et al*., 1996). However, in our studies we have been unable to reproducibly demonstrate an IL-1β-dependent shift in $cPLA_2$ mobility on SDS–PAGE, and likewise have been unable to show changes in mobility as a result of PD098059 or SB203580 treatment (data not shown). Due to the inherent variability of this technique, these data do not exclude $cPLA_2$ as targets of ERK and/or p38 MAP kinase phosphorylation. However, the lack of inhibition by MAFP suggest that $cPLA_2$ may not actually be involved in the IL-1β-dependent response. Furthermore, a p38 MAP kinase inhibitor inhibited phosphorylation of $cPLA_2$ in platelets, yet no effect on arachidonate release was observed, suggesting that phosphorylation of $cPLA_2$ is not necessarily linked to arachidonate release (Kramer *et al*., 1996). In addition, numerous new PLA_2 activities and genes have been identified (Balsinde & Dennis, 1997). In addition, a 1-O- acylceramide synthase that shows PLA_2 activity (Abe & Shayman, 1998), the $cPLA_2$ homologues, $cPLA_2\beta$ and $cPLA_2\gamma$ (Pickard *et al*., 1999; Underwood *et al*., 1998), a novel $cPLA_2$ splice variant (Gordon *et al*., 1996) and a number of novel group II secretory PLA_2 (IID, IIE and IIF) have all been cloned (Ishizaki *et al*., 1999; Valentin *et al*., 1999). These discoveries raise the possibility that another PLA_2 is in fact responsible for IL-1β-dependent PGE_2 release in these cells and that this PLA_2 activity is downstream of the MEK2-dependent kinase cascade that was inhibited by UO126.

One explanation for the effect of PD098059 and SB203580 is that these compounds may directly inhibit COX-2 activity (Borsch-Haubold *et al*., 1998). In the case of SB203580, this possibility was excluded as incubation of SB203580 with cells in which COX-2 had been induced, in the presence of odifferent exogenous arachidonic acid concentrations, showed no inhibition of COX activity (Figure 9). Furthermore, high dose SB203580 had no acute effect on release of PGE_2 from IL-1β-treated cells, again indicating that there is no direct effect on COX-2 enzyme (Figure 8). In the case of PD098059, a possible competitive inhibitory effect on COX (or a downstream enzyme) was apparent (Figure 9). However, as the inhibition of PGE_2 release occurred at the same concentrations as that for inhibition of ERK phosphorylation, it is unlikely that this effect was the result of direct COX-2 inhibition.

Another explanation for the effect of PD098059 and SB203580 is that the phospholipid pool, which is labelled with [^{3}H]-arachidonate, is not the same pool that gives rise to IL-1β-induced PGE_2. However, this hypothesis again seems unlikely as UO126 inhibited both release of PGE_2 and [^{3}H]-arachidonate release.

The question therefore remains as to the roles of the MEK1/ERK and p38 MAP kinases in IL-1β-dependent PGE_2 release. Whilst MEK1/ERK and p38 inhibition resulted in no change in arachidonate release, our data indicate a functional role for both these MAP kinase pathways in PGE_2 generation. Recently the group IV $cPLA_2$ and the group IIA and V $sPLA_2$'s, but not the group VI Ca^{2+}-independent PLA_2 (iPLA2), have been shown to be functionally linked to COX-2 and PG generation (Murakami *et al*., 1998). Thus simple release of arachidonic acid in the presence of COX-2 is not sufficient for PG production suggesting that a specific relationship between PLA_2 and COX-2 is required (Murakami *et al*., 1998). It is therefore possible that phosphorylation of the relevant PLA_2, by ERK or p38 has no effect on activity, but is important in coupling to or interaction with COX-2. In this context, it has become clear that adaptor or scaffold proteins, which provide a structural support for the various components of signalling pathways, also play an important function in the signal transduction process (Pawson & Scott, 1997). Such proteins may confer a specific spatial arrangement to the various pathway components thus enhancing specificity as well as ensuring that kinase and substrate domains are in close proximity to allow rapid kinetics. Furthermore, it is known that prostaglandin synthetic enzymes are co-localized to the endoplasmic reticulum and the nuclear envelope following cell stimulation (Morita *et al*., 1995; Schievella *et al*., 1995). Therefore it is possible that phosphorylation by ERKs or p38 MAP kinase provide an accessory function, which is important in the interaction of the IL-1β-dependent PLA_2 with specific anchoring proteins, or even COX-2, such that the correct spatial arrangement can be adopted for transfer of arachidonic acid to COX-2.

In conclusion, this study identifies the MEK1/ERK and MEK2 as well as the p38 MAP kinase pathways as major regulators of IL-1β-dependent PGE_2 release in human airway epithelial-like A549 cells. At the relevant doses, inhibitors of these pathways had little or partial effects on induction of COX activity or COX-2 protein yet totally inhibited IL-1β-dependent PGE_2 release indicating a primary role upstream of COX-2. Repression of PGE_2 release by UO126 was attributed to inhibition of MEK2, which lead to reduced PLA_2 activity of a non-$cPLA_2$ phospholipase. Inhibition of MEK1/ERK by PD098059 and of p38 MAP kinase by SB203580 prevented PGE_2 release but had no effect on arachidonate release suggesting that these pathways play roles in linking the relevant PLA_2 activity to COX-2. In each case, we suggest that inhibitors of the MEK1/ERK, MEK2 and p38 MAP kinase pathways are potent and effective inhibitors of PG production from epithelial cells and may therefore provide useful therapeutic tools.

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