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Investigation into the involvement of phospholipases A_2 and MAP kinases in modulation of AA release and cell growth in A549 cells

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1 We have investigated the contribution of specific PLA_2s to eicosanoid release from A549 cells by using specific inhibitors of secretory PLA_2 (ONO-RS-82 and oleyloxyethylphosphocholine), cytosolic PLA_2 ($AACOCF_3$ and MAFP) and calcium-independent PLA_2 (HELSS, MAFP and $PACOCF_3$). Similarly, by using specific inhibitors of p38 MAPK (SB 203580), ERK1/2 MAPK (Apigenin) and MEK1/2 (PD 98059) we have further evaluated potential pathways of AA release in this cell line.

2 ONO-RS-82 and oleyloxyethylphosphocholine had no significant effect on EGF or IL-1β stimulated ^{3}H-AA or PGE_2 release or cell proliferation. $AACOCF_3$, HELSS, MAFP and $PACOCF_3$ significantly inhibited both EGF and IL-1β stimulated ^{3}H-AA and PGE_2 release as well as cell proliferation. Apigenin and PD 98509 significantly inhibited both EGF and IL-1β stimulated ^{3}H-AA and PGE_2 release and cell proliferation whereas, SB 203580 had no significant effect on EGF or IL-1β stimulated ^{3}H-AA release, or cell proliferation but significantly suppressed EGF or IL-1β stimulated PGE_2 release.

3 These results confirm that the liberation of AA release, generation of PGE_2 and cell proliferation is mediated largely through the actions of $cPLA_2$ whereas, $sPLA_2$ plays no significant role. We now also report a hitherto unsuspected contribution of $iPLA_2$ to this process and demonstrate that the stimulating action of EGF and IL-1β in AA release and cell proliferation is mediated in part *via* a MEK and ERK-dependent pathway (but not through p38MAPK). We therefore propose that selective inhibitors of MEK and MAPK pathways may be useful in controlling AA release, eicosanoid production and cell proliferation.

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Abbreviations: AA, arachidonic acid; $AACOF_3$, arachidonyl triflouromethyl ketone; AP or apigenin, 4′,5,7-trihydroxyflavone; COX, cyclo-oxygenase; $cPLA_2$, cytosolic phospholipase A_2; DMEM, Dulbecco's modified Eagle medium; EGF, epidermal growth factor; FCS, foetal calf serum; HELSS, E-6-(bromomethylene)tetrahydro-3(1-napthalenyl)-2H-pyran-2-one haloenol lactone; IL-1β, interleukin-1β; $iPLA_2$, calcium independent phospholipase A_2; MAPK, mitogen acitivated protein kinase; ONO-RS-82, 2-(p-amylacinnamoyl)-amino-p-chlorobenzoic acid; PD 98059′ 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one; PGE_2, prostaglandin E_2; PS, phoshoserine; PT phosphotreonine; PY, phosphotyrosine; SB 203580, 4-(4-flourophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)imidazole; $sPLA_2$, secretory phospholipase A_2.

Introduction

Prostaglandins (PGs) act as local hormones to regulate both physiological and pathological responses within the host. They have been implicated as causative factors in the pathogenesis of many diseases, including, asthma, atherosclerosis and cancer (Vane, 1978; Cuthbert, 1973; Borg *et al*., 1994; Earnest *et al*., 1992). Additionally, PGs can also act as autocrine modulators of cell proliferation in several cell lines (Karmali *et al*., 1979; Qiao *et al*., 1995). We have identified PGE_2 as an autocrine mediator of cell growth in the human lung adenocarcinoma A549 cell line (Croxtall & Flower, 1992). PGE_2 production is regulated both by the release of AA from cellular phospholipids by PLA_2 enzymes (Mukherjee *et al*., 1994) and by the conversion of AA to PGH_2, the common substrate for all PGs, by the enzyme cyclo-oxygenase (COX) (Smith & Marnett, 1991).

AA, the precursor for PGs biosynthesis, does not exist in free form but is released from cellular phospholipids by the rate-limiting PLA_2 enzymes in response to a variety of extracellular stimuli. PLA_2 enzymes are generally characterized according to their cellular localization, calcium dependency and molecular weight. The two main forms are the secretory PLA_2 ($sPLA_2$) and the cytosolic PLA_2 ($cPLA_2$) (reviewed by Leslie, 1997; Kramer & Sharp, 1997; Dennis, 1997). However, another calcium independent form of PLA_2 has also been identified, referred to as $iPLA_2$ (reviewed by Ackermann & Dennis, 1995; Balsinde & Dennis, 1997).

The key differences between the PLA_2 enzymes are that $sPLA_2$ is released extracellularly, has a low molecular weight (14–18 kDa), requires milli-molar calcium concentrations for catalysis and shows little fatty acid specificity. $sPLA_2$ is thought to be involved in the digestion of dietary lipids rather than in cell signalling (Murakami *et al*., 1997) since it fails to selectively hydrolyse arachidonyl containing phospholipids suggesting that its primary function is not to initiate the biosynthesis of PGs. In contrast, $cPLA_2$ functions intracellularly, has a high molecular weight (40–110 kDa), requires micro-molar levels of calcium for membrane translocation, exhibits a definite preference for the sn-2 AA position, and is phosphorylated by mitogen-activated protein kinases

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(MAPK). Thus, $cPLA_2$ is more likely to be involved in agonist induced AA release mediated by MAPK-dependent pathways.

Although $iPLA_2$ also occurs intracellularly and has high molecular weight (45–80 kDa) its activity appears to be independent of calcium-concentrations. Unlike the calcium-dependent PLA_2s, very little is known about its catalytic mechanism or intracellular role. $iPLA_2$ has no acyl specificity and has been suggested to function in steady state membrane remodelling and the control of intracellular AA levels rather than agonist induced AA release (Balsinde *et al*., 1995).

To investigate which PLA_2 enzymes are involved in AA release, PGE_2 generation and cell growth in A549 cells, the following PLA_2 inhibitors were used. For $sPLA_2$ inhibition, ONO-RS-82 or [2-(p-amylacinnamoyl)-amino-p-chlorobenzoic acid] and oleyloxyethylphosphocholine were used. ONO-RS-82 is a 14 kDa $sPLA_2$ inhibitor which inhibits 5-HT stimulated thromboxane production in human platelets (Konrad *et al*., 1992). Oleyloxyethylphosphocholine is a site specific inhibitor of porcine pancreatic PLA_2 (Augert *et al*, 1989). For $cPLA_2$ inhibition, a triflouromethyl ketone analogue of AA, arachidonyl triflouromethyl ketone (AA-$COCF_3$) was used. This is a potent, reversible and selective slow-binding inhibitor of human 85 kDa $cPLA_2$ (Street *et al*., 1993). It is active in intact platelets (Bartoli *et al*., 1994) and U937 cells (Riendeau *et al*., 1994) and is 1000-fold more selective for $cPLA_2$ than for $sPLA_2$ (Gelb *et al*., 1994). For $iPLA_2$ inhibition, both HELSS (haloenol lactone suicide substrate) or [E-6-(bromomethylene)tetrahydro-3-(1-napthalenyl)-2H-pyran-2-one haloenol lactone suicide substrate] and $PACOCF_3$ (palmitoyl trifluoromethyl ketone) were used. HELSS is a potent, irreversible $iPLA_2$ inhibitor (Ackermann *et al*., 1995) that binds covalently to the active site of the $iPLA_2$ enzyme and possesses a 1000-fold selectivity for $iPLA_2$ over $cPLA_2$ (Hazen *et al*., 1991). $PACOCF_3$ is also a selective inhibitor of $iPLA_2$ at concentrations up to 10 μM (Ackermann *et al*, 1995). Finally, for comparative purposes, MAFP (methyl arachidonyl fluorophosphate) a mixed inhibitor of both $cPLA_2$ and $iPLA_2$ (Balsinde & Dennis, 1997) was also tested.

It is known that $cPLA_2$ is catalytically activated by phosphorylation on serine-505 by a serine/threonine specific kinase, mitogen activated protein kinase (MAPK) (Lin *et al*., 1992). MAPKs are a group of protein kinases that are activated in response to a variety of extracellular stimuli including growth factors, cytokines and hormones. They mediate signal transduction from the cell surface to the nucleus. In mammalian cells, five distinct MAPK signalling cascades have been identified so far, each having several isoforms that respond synergistically to different upstream signals (Robinson & Cobb, 1997). These include the mitogen or extracellular signal regulated protein kinases (MAPK/ERKs) which include ERK1 (p^{44mapk}) and ERK2 (p^{42mapk}) which are activated by mitogenic stimuli from growth factor receptors and phorbol esters (Seger *et al*., 1991); stress activated protein kinases (SAPKs) or c-*jun* NH_2-terminal kinases (JNKs), which include JNK1 ($p^{45sapks\ \gamma}$) and JNK2 ($p^{54sapks\ \alpha/\beta}$) which are activated by stress inducing signals such as osmotic and heat shock, UV and γ-irradiation, protein synthesis inhibitors, metabolic poisons or pro-inflammatory cytokines IL-1β (Kyriakis & Avruch, 1996); the p38 MAPKs (α, β, βII, γ and δ) which are activated by UV and pro-inflammatory cytokines IL-1β (Brunet & Pouyssegur, 1996); as well as ERK3 and ERK5 that as yet, represent two poorly understood pathways. These kinases are in turn activated by distinct upstream MAPK/ERK kinases (MEKs, MKKs) which phosphorylate threonine and tyrosine residues. Several distinct MAPK kinases or MEKs have been identified in mammalian cells. MEK1/2 does not phosphorylate or activate p38 MAPK or JNKs whilst MEK1/2 is a strong activator of ERK1/2 (Derijard *et al*., 1995; Crews *et al*., 1992). The existence of all these various kinases presumably allows input into MAPK pathways from many different stimuli.

To identify the MAPK pathways involved in A549 cell signalling, the following MAPK and MEK inhibitors were used. SB 203580 ([4-(4-flourophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)imidazole]) is a member of a novel class of pyridinyl imidazoles. It is potent and specific inhibitor of p38 MAPK and p38β MAPK (Cuenda *et al*., 1997) but does not inhibit any other protein kinases including ERK1/2 MAPK or JNK (Goedert *et al*., 1997). The ERK1/2 MAPK inhibitor, apigenin (4′,5,7-trihydroxyflavone) is a plant flavonoid that reverses the transformed phenotypes of various rastransformed cells due to inhibition of MAPK associated signal transduction pathways (Kuo & Young, 1995). It also induces the dephosphorylation of ERK1/2 and thereby reduces ERK1/2 activity (Sato *et al*., 1994). The MEK1/2 inhibitor PD 98059 or ([2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one]) is a potent and reversible inhibitor that binds to the inactive form of MEK1/2 thereby inhibiting the phosphorylation and activation of ERK/MAPK (Dudley *et al*., 1995; Alessi *et al*., 1995). This highly selective MEK1/2 inhibitor was used to further evaluate the role of ERK1/2 in the control of A549 cell signalling.

EGF and IL-1β are known to stimulate AA release, PGE_2 production and increase cell growth in a variety of cell types including A549 cells (Carpenter & Cohen, 1979; Croxtall *et al*., 1993; Dinarello, 1996) and are therefore used as stimuli in this study. Here we attempt to evaluate the effects of PLA_2 and MAPK enzyme inhibitors on the AA release, PGE_2 generation and increase cell growth of A549 cells stimulated by EGF and IL-1β.

Methods

Cell culture

A549 cells (Flow) were maintained in continuous log phase growth in Dulbecco's modified Eagle medium/F-12 (DMEM/F-12) containing phenol red, 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (PS) in T-150 flasks (Greiner) at 37°C, 5% CO_2. The cells were not allowed to reach confluence at any time as this diminishes their response to growth factors, stimulators and inhibitors.

Measurement of arachidonic acid release

Sub-confluent A549 cells were seeded into 12-place multi-well plates (Falcon) at a density of 3×10^5 cells ml^{-1} $well^{-1}$ in DMEM/F-12, 10% FCS, 1% PS and incubated overnight. [^{3}H]-arachidonic acid [^{3}H]-AA in ethanol was evaporated to dryness under N_2 and resuspended in an appropriate volume of DMEM/F-12 (without phenol red) and after after vortex mixing left at 37°C for 1 h. After the cells had been washed with PBS, 9.25 KBq of [^{3}H]-AA in 0.5 ml DMEM/F-12 (without phenol red or FCS) was added to each well and incubated overnight. The media containing free [^{3}H]-AA was then removed and the cells washed three times with 1 ml DMEM/F-12 containing 1 mg ml^{-1} BSA. The cells thus labelled with [^{3}H]-AA were then treated for 3 h with various inhibitors and vehicle control (DMSO). Then either 10 nM EGF was added for 30 min or 1 ng ml^{-1} IL-1β was added for

3 h. After incubation, 0.4 ml of medium was removed from each well for scintillation counting.

Measurements of PGE_2 release

Sub-confluent A549 cells seeded into 12-place multi-well plates at a density of 5×10^4 cells ml^{-1} $well^{-1}$ in DMEM/F-12, 10% FCS and 1% PS. Following incubation overnight, the cells were washed in 2 mls of sterile PBS and 1 ml of fresh DMEM/F-12 (without the phenol red) containing 1% PS plus various inhibitors, EGF, IL-1β or vehicle control added. On day 2, cells were replenished with fresh experimental media containing test agents or vehicle control. On day 3, 0.5 mls of experimental media was removed and PGE_2 was measured in the samples using an enzymeimmunoassay (EIA) kit.

Cell proliferation experiments

Sub-confluent A549 cells were seeded into 12-place multi-well plates at a density of 5×10^4 cells ml^{-1} $well^{-1}$ in DMEM/F-12, 10% FCS and 1% PS. Following incubation overnight, the cells were washed in 2 mls of sterile PBS and 1 ml of fresh DMEM/F-12 (without the phenol red) containing 1% PS plus various inhibitors, EGF, IL-1β or vehicle control added. On day 2, cells were replenished with fresh experimental media containing test agents or vehicle control. On day 3, media was removed from each well and 1 ml of PBS containing 0.05% trypsin and 0.02% EDTA was added to each well. The dispersed cells then counted with a Coulter Multisizer II counter. The percentage inhibition of cell proliferation for PLA_2 inhibitor treated culture was calculated compared to control well. Trypan Blue was used to determine cell viability. The data reported are due to inhibition of cell proliferation and not cell death due to toxicity of the compounds tested.

Determination of the activation of MAPK's and $cPLA_2$

Following experimental treatment, the medium was aspirated and the A549 cell monolayer washed with PBS, 1 mM EDTA to remove adherent surface-bound proteins. The monolayer was dispersed with 0.05% trypsin in PBS, 10 mM EDTA and cell pellets were snap-frozen in 3 ml of PBS, 10 mM EDTA containing 1 mg ml^{-1} soyabean trypsin inhibitor, 0.01% leupeptin, 1 mM PMSF and 1 mM sodium orthovanadate. Once thawed, cell lysates were clarified by centrifugation at $13{,}000 \times g$ for 20 min. Protein concentrations were measured by Bradford assay and identical concentrations were used in each immunoprecipitation. 1 ml of cell lysate was incubated with 5 μg of precipitating monoclonal antibody for 16 h with continuous rocking. Then 20 mg Protein A-Sepharose was added for a further 2 h. The Protein A-Sepharose bound immunocomplexes were washed three times in PBS, 10 mM EDTA and then incubated with 250 μl sample buffer for 5 min at 90°C prior to SDS–PAGE analysis by Western blotting with the appropriate anti-phospho specfic monoclonal antibody (10 μg ml^{-1}) and detection by DAB. Western blots were scanned using a ScanMaker (Microtek, CA, U.S.A.) and the image composite transferred into Power Point (Micosoft, WA, U.S.A.) running on an Apple Macintosh. Densitometric analysis was performed with NIH Image 1.54 and relative band intensities reported as % changes within each blot. The values given are semiquantitative and are only meant to give some numerical guide to the *ratio* of band intensities. The blots presented are typical examples of at least three such experiments. Although overall band intensities varied between experiments, the *ratio* of band intensities remained the same.

Materials

EGF, ONO-RS-82, anti-phospho specific antibodies, DAB, DMEM/F-12, FCS and trypsin were purchased from Sigma Chemical Company (Poole, U.K.). [5,6,8,9,11,12,14,15-^{3}H-(N)]-arachidonic acid from NEN, Du Pont (Belgium). $AACOCF_3$, PD 98059 and HELSS compounds from Biomol Research Lab., Inc. (Plymouth, PA, U.S.A.). Apigenin, oleyloxyethylphosphocholine, MAFP, $PACOCF_3$ and SB 203580 compounds from Calbiochem-Novabiochem Ltd. (Nottingham, U.K.). Antibodies to COX2 (goat), ERK1/2 (rabbit), p38MAPK (mouse), JNK1 (rabbit) and $cPLA_2$ (mouse) were from Autogen Bioclear (Wilts, U.K.). PGE_2 (EIA) kit from Amersham (Buckinghamshire, U.K.). hrIL-1β was a generous gift from Dr Mauro Perretti (Dept of Biochemical Pharmacology, The William Harvey Research Institute, London, U.K.).

Statistical analysis

Each experiment was performed in triplicate ($n=3$) and each experiment is a typical example of at least three such experiments. Results were calculated as the mean $\pm$ s.e.mean and are presented as the per cent inhibition $\pm$ s.e.mean. Statistical differences were calculated on raw data using the ANOVA test with post analysis Bonferroni correction. A threshold value of $P<0.05$ was taken as significant.

Results

Effects of PLA_2 inhibitors, MEK, ERK and p38 MAPK inhibitors on EGF and IL-1β stimulated ^{3}H-AA release from A549 cells

Treatment of A549 cells with 10 nM EGF for 30 min typically stimulated release of ^{3}H-AA by 40% above control values. Such EGF stimulated AA release has been observed in several other different cell lines (Hack *et al*., 1991; Spaargaren *et al*., 1992). To investigate the isoforms of PLA_2 involved in the liberation of EGF stimulated AA release, A549 cells were pretreated with the specific inhibitors for each kind of PLA_2. Pre-treatment for 3 h with the $sPLA_2$ inhibitor ONO-RS-82 up to concentrations of 20 μM had no significant effect on EGF stimulated release (Figure 1). Similarly, oleyloxyethylphosphocholine up to concentrations of 20 μM was also without any significant effect (data not shown). However, pre-treatment with the $iPLA_2$ inhibitor, HELSS, at concentrations of 5 μM and above, significantly inhibited EGF stimulated ^{3}H-AA release. This inhibition was maximal (40%) at 10 μM with an IC_{50} of 5 μM (Figure 1). Further, $PACOCF_3$ also inhibited EGF stimulated release with an IC_{50} of 5 μM and a maximal effect at 10 μM (60%, data not shown). Similarly, the $cPLA_2$ inhibitor, $AACOCF_3$ at concentrations of 0.1 μM and above significantly inhibited EGF stimulated ^{3}H-AA release. This inhibition was maximal (80%) at 20 μM with an IC_{50} of 1 μM (Figure 1). Using MAFP, an inhibitor of both $iPLA_2$ and $cPLA_2$, resulted in the greatest inhibition of EGF stimulated ^{3}H-AA release with 45% inhibition at 0.1 μM and a maximal effect of 88% at 10 μM (data not shown). These results imply that the production of EGF stimulated AA release in A549 cells is apparently not mediated through the action of $sPLA_2$, but rather through the activation of both $iPLA_2$ and $cPLA_2$. This is in keeping with our previous findings that A549 cells appear to express undetectable levels of $sPLA_2$, but rather levels of the cytosolic isoform predominate instead (Tokumoto *et al*., 1993).

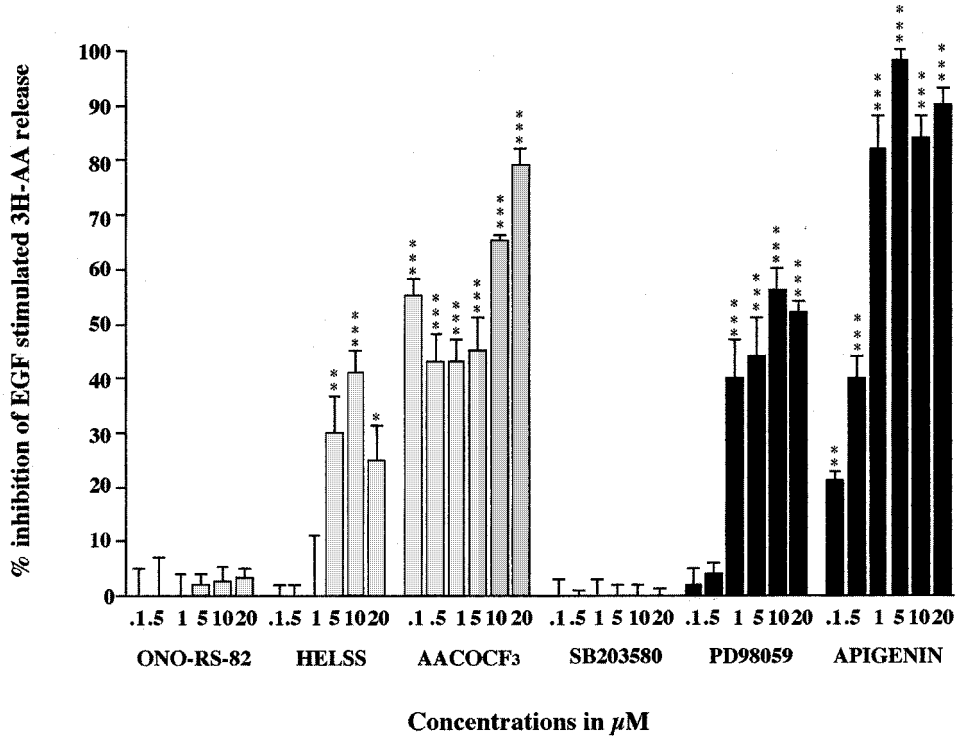

Figure 1 The effect of PLA_2, MEK and MAPK inhibitors on EGF stimulated ^{3}H-AA release from A549 cells. Cells were pre-incubated either with $sPLA_2$ inhibitor ONO-RS-82, $iPLA_2$ inhibitor HELSS, $cPLA_2$ inhibitor $AACOCF_3$, p38 MAPK inhibitor SB 203580, MEK inhibitor PD 98059 or with ERK inhibitor apigenin for 3 h, prior to stimulation by EGF (10 nM) for 30 min. The data are presented as mean $\pm$ s.e.mean, expressed as per cent inhibition of ^{3}H-AA release relative to EGF stimulation of 41% $\pm$ 4% above control values. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ inhibition compared to EGF stimulation.

Furthermore, the inhibition by the $iPLA_2$ inhibitor was surprising. The expression of this form of PLA_2 has not yet been characterized in A549 cells, however these results would appear to indicate its participation in AA release.

The MEK inhibitor PD 98059 and ERK inhibitor apigenin both inhibited EGF stimulated ^{3}H-AA release (Figure 1). Pre-treatment for 3 h with PD 98059 significantly inhibited EGF stimulated AA release, with maximal inhibition (56%) at 10 μM PD 98059 and an IC_{50} of 0.75 μM (Figure 1). This result confirms the involvement of MEK in the EGF signal transduction pathway leading to AA release and also indicates that the ERK pathway may also be involved since PD 98059 is also known to inhibit the MEK activation of ERK (Alessi *et al*., 1995).

Indeed, pre-treatment of A549 cells with the ERK inhibitor apigenin for 3 h also significantly suppressed EGF stimulated AA release, with maximal inhibition (98%) at 5 μM apigenin and an IC_{50} of 0.75 μM (Figure 1). Apigenin induces dephosphorylation of ERK1 and a reduction of MAPK activity in v-H-*ras*-transformed NIH-3T3 cells (Sato *et al*., 1994), and also inhibits MAPK-associated signal transduction pathways in various *ras*-transformed cells. Our results suggest that ERK is also involved in the EGF signal transduction pathway leading to the activation of PLA_2 and liberation of AA in A549 cells. In contrast, pretreatment for 3 h with the p38 MAPK inhibitor SB 203580 up to concentrations of 20 μM had no significant effect on EGF stimulated ^{3}H-AA release and therefore suggests that p38 MAPK does not play a part in the EGF-dependent activation of AA release in A549 cells.

IL-1β has also been shown to stimulate AA release from A549 cells (Newman *et al*., 1994). Typically, 1 ng ml^{-1} IL-1β stimulated AA release 110% above control values and pre-treatment for 3 h with the $sPLA_2$ inhibitor ONO-RS-82 up to concentrations of 20 μM had no significant effect on this stimulated ^{3}H-AA release (Figure 2). Likewise, oleyloxyethylphosphocholine also had no significance at concentrations up to 20 μM (data not shown). However, pre-treatment for 3 h with the $iPLA_2$ inhibitor, HELSS at concentrations 0.1 μM and above significantly inhibited IL-1β stimulated ^{3}H-AA release. This inhibition was maximal (40%) at 5 μM with an approximate IC_{50} of 0.75 μM (Figure 2). Further, $PACOCF_3$ also inhibited IL-1β stimulated release with an IC_{50} of 10 μM and a maximal effect of 57% inhibition at 20 μM (data not shown). Similarly, the $cPLA_2$ inhibitor, $AACOCF_3$ at concentrations of 0.1 μM and above significantly inhibited IL-1β stimulated ^{3}H-AA release. This inhibition was maximal (66%) at 20 μM (Figure 2). Using the mixed inhibitor MAFP produced the greatest inhibitory effect with an IC_{50} of 0.5 μM and a maximal effect of 98% at 10 μM (data not shown). This data shows the lack of involvement of $sPLA_2$ in IL-1β stimulated AA release and also again that $iPLA_2$ and $cPLA_2$ both appear to play a contributory role in A549 cells.

Likewise, the MEK inhibitor PD 98059 and ERK inhibitor apigenin both significantly inhibited IL-1β stimulated AA release, whereas, p38 MAPK inhibitor SB 203580 upto concentrations of 20 μM had no effect (Figure 2). Pre-treatment for 3 h with PD 98059 at concentrations of 1 μM and above significantly inhibited IL-1β stimulated AA release, with maximal inhibition (47%) at a 20 μM concentration (Figure 2). This suggests that MEK plays a role in the signal transduction pathway. Furthermore, pre-treatment for 3 h with ERK inhibitor apigenin at concentrations of 0.1 μM and above significantly inhibited IL-1β stimulated AA release, with maximal inhibition (68%) at a concentration of 20 μM (Figure 2), suggesting that ERK pathways are also involved in IL-1β stimulated AA release. All this appears to indicates that MEK

and ERK pathways are involved in IL-1β stimulated AA release in A549 cells, whereas the p38 MAPK pathway does not appear to play a significant part.

The use of PLA_2 inhibitors, MEK, ERK and p38 MAPK inhibitors on EGF and IL-1β stimulated PGE_2 release from A549 cells

Previously we have demonstrated that PGE_2 release plays an important role in the control of cell growth in A549 cells (Croxtall & Flower, 1992). To further investigate which isoforms of PLA_2 are involved in the generation of PGE_2, we have used the panel of PLA_2 inhibitors in a PGE_2 release assay. Figure 3 shows that untreated control A549 cells after 72 h in culture, typically release between 50 and 150 pg ml^{-1} of PGE_2 to the media. 10 nM EGF typically stimulated this PGE_2 release by between 2–3-fold pre-treatment for 3 h with $sPLA_2$ inhibitor ONO-RS-82 up to concentrations of 1 μM had no significant effect on EGF-stimulated PGE_2 release. Similarly, oleyloxyethylphosphocholine had no effect on significant effect on PGE_2 release (data not shown). However, pre-treatment for 3 h with the $iPLA_2$ inhibitor HELSS at concentrations ranging from 0.1 μM and 1 μM inhibited EGF-stimulated PGE_2 release (Figure 3) as did $PACOCF_2$ (IC_{50} 1 μM, data not shown). Concentrations above 1 μM for both ONO-RS-82 and HELSS appears to have a toxic effect on the cells probably due to longer contact times. Similarly, the $cPLA_2$ inhibitor AACOCF$_3$ at concentrations of 5 μM and above significantly inhibited EGF-stimulated PGE_2 release (Figure 3). Moreover, the mixed inhibitor MAFP significantly inhibited PGE_2 release at all concentrations tested (0.1 μM and above, $P<0.001$ all points, data not shown). This reinforces the concept that the level of PGE_2 release from A549 cells stimulated by EGF is mediated largely through the concerted actions of $iPLA_2$ and $cPLA_2$ whereas $sPLA_2$ plays no role.

The EGF stimulated PGE_2 release was also significantly inhibited by treatment with SB 203580 at concentrations of 5 μM and above, PD 98059 at concentrations of 1 μM and above and by apigenin at concentrations of 10 μM and above (Figure 3). This indicates not only the involvement of MEK and ERK signalling pathways in EGF stimulated PGE_2 generation in A549 cells but also that the p38 MAPK pathway may play a role.

We have also shown that IL-1β induced A549 cell growth is also mediated, at least in part, by elevated PGE_2 levels (Newman *et al*., 1994). Figure 4 illustrates that 1 ng ml^{-1} IL-1β typically stimulated PGE_2 release by 1500–5000% above control values and this was not affected by ONO-RS-82 (and oleyloxyethylphosphocholine not shown), whereas co-treatment with HELSS (and $PACOCF_3$ and MAFP not shown) at concentrations 0.1 μM and above and AACOCF$_3$ at concentrations of 5 μM and above significantly inhibited PGE_2 release. Concentrations of ONO-RS-82 and HELSS above 1 μM appeared to be toxic. Therefore, again the active forms of PLA_2 which are involved in IL-1β stimulated PGE_2 generation appear to be $iPLA_2$ and $cPLA_2$ and not $sPLA_2$.

IL-1β stimulated PGE_2 release from A549 cells was also significantly inhibited by co-incubation with SB 203580 at concentrations 1 μM and above, PD 98059 at concentrations 1 μM and above and apigenin at concentrations 0.5 μM and above (Figure 4). This implies that not only MEK and ERK signalling pathways play a role but also that p38 MAPK is also involved in IL-1β stimulated PGE_2 generation in A549 cells.

Cell growth and the effect of PLA_2 inhibitors, MEK, ERK and p38 MAPK inhibitors on EGF and IL-1β stimulated A549 cells

Following 72 h in culture, basal cell numbers increased from 50,000 (day 0) to 81,000, which is an increase of 62% ± 2%.

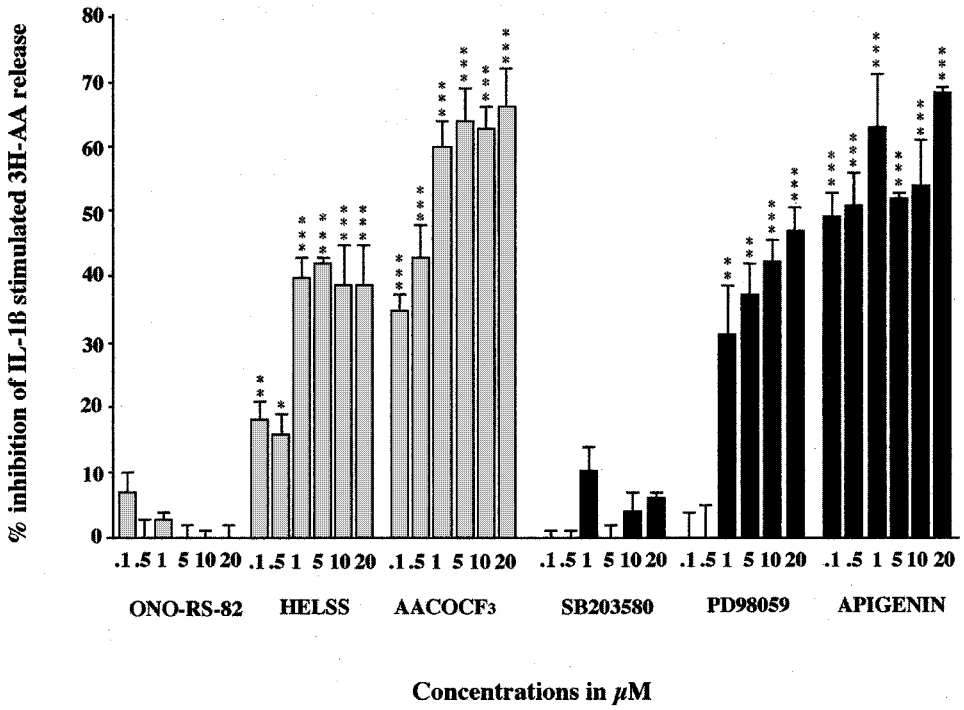

Figure 2 The effect of PLA_2, MEK and MAPK inhibitors on IL-1β stimulated ^{3}H-AA release from A549 cells. Cells were pre-incubated either with $sPLA_2$ inhibitor ONO-RS-82, $iPLA_2$ inhibitor HELSS, $cPLA_2$ inhibitor AACOCF$_3$, p38 MAPK inhibitor SB 203580, MEK inhibitor PD 98059 or with ERK inhibitor apigenin for 3 h, prior to stimulation by IL-1β (1 ng ml^{-1}) for 3 h. The data are presented as mean ± s.e.mean, expressed as per cent inhibition of ^{3}H-AA release relative to IL-1β stimulation of 110% ± 6% above control values. *$P<0.05$, **$P<0.01$, ***$P<0.001$ inhibition compared to IL-1β stimulation.

Typically, 10 nM EGF stimulated this cell growth by 70% ± 2% over control values at 72 h. Our previous work has shown that this EGF-stimulated proliferation of A549 cells is dependent (at least in part) upon elevated PGE_2 levels (Croxtall & Flower, 1992). The data presented here show that increased cell proliferation of A549 cells by EGF was inhibited by HELSS, $PACOCF_3$ (not shown) and $AACOCF_3$ at concentrations of 0.5 μM and above and by MAFP (0.1 μM and above, not shown), whereas ONO-RS-82 (and oleyloxyethylphosphocholine, not shown) was without effect (Figure 5). This again shows

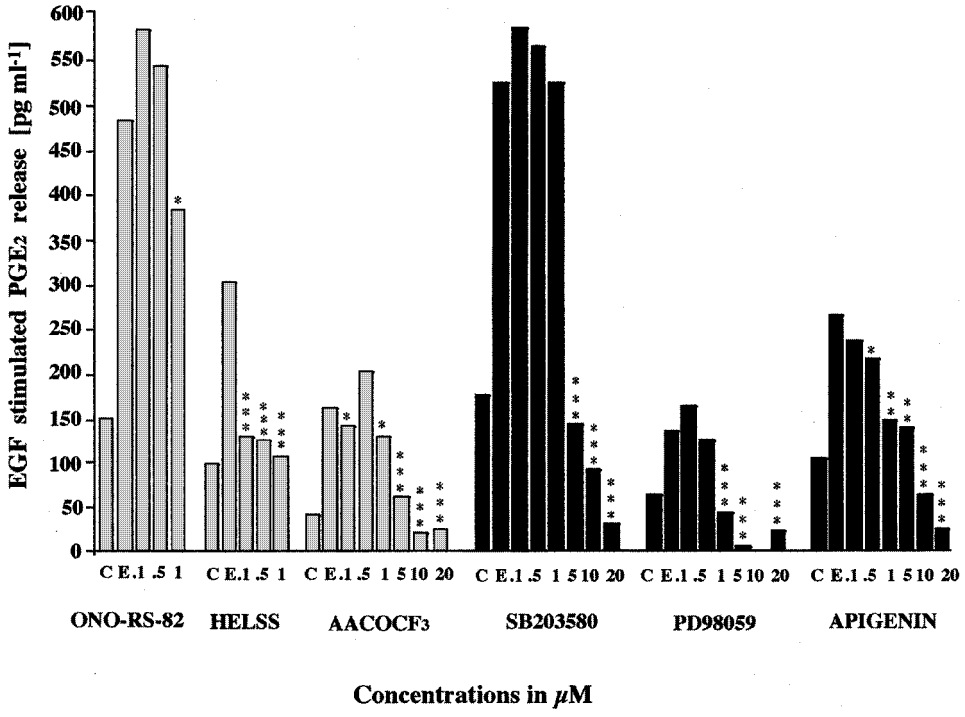

Figure 3 The effect of PLA_2 inhibitors, MEK and MAPK inhibitors on EGF stimulated PGE_2 release from A549 cells. Cells were treated with EGF (E, 10 nM) plus either with $sPLA_2$ inhibitor ONO-RS-82, $iPLA_2$ inhibitor HELSS, $cPLA_2$ inhibitor $AACOCF_3$, p38 MAPK inhibitor SB 203580, MEK inhibitor PD 98059, ERK inhibitor apigenin or untreated as control (C) for 72 h. The data are presented as mean ± s.e.mean, the concentrations of PGE_2 released in the media in pg ml^{-1}.

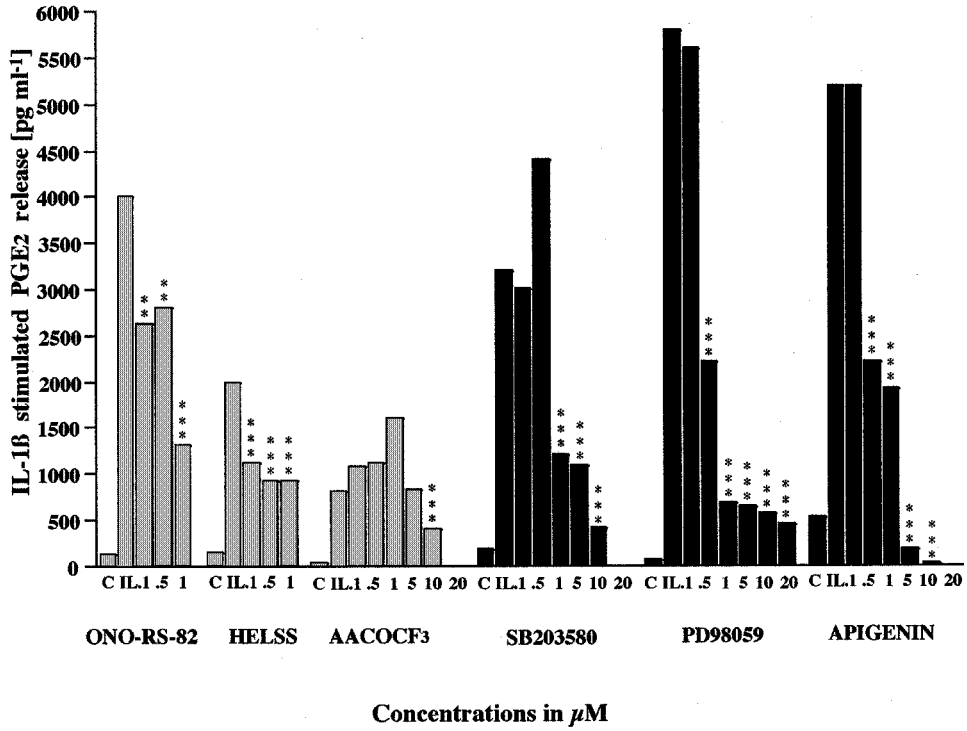

Figure 4 The effect of PLA_2 inhibitors, MEK and MAPK inhibitors on IL-1β stimulated PGE_2 release from A549 cells. Cells were treated with IL-1β (IL, 1 ng ml^{-1}) plus with either $sPLA_2$ inhibitor ONO-RS-82, $iPLA_2$ inhibitor HELSS, $cPLA_2$ inhibitor $AACOCF_3$, p38 MAPK inhibitor SB 203580, MEK inhibitor PD 98059, ERK inhibitor apigenin or untreated as control (C) for 72 h. The data are presented as mean ± s.e.mean, the concentrations of PGE_2 released in the media in pg ml^{-1}.

the contributory effect of both $iPLA_2$ and $cPLA_2$ to EGF-induced cell growth, whereas $sPLA_2$ plays no role.

Co-incubation with SB 203580 up to concentrations of 20 μM had no significant effect on EGF-stimulated A549 cell growth. However, both PD 98059 and apigenin at concentrations 5 μM and above significantly inhibited EGF stimulated A549 cell growth. Therefore, it seems that both MEK and ERK pathways appear to be involved in the control of EGF induced A549 cell growth. However, inhibition of the p38 MAPK pathway does not appear to inhibit cell growth despite inhibiting EGF-stimulated PGE_2 release.

IL-1β (1 ng ml^{-1}) also typically stimulated cell growth by 35% $\pm$4% over control values at 72 h (Figure 6). This IL-1β induced cell proliferation was significantly inhibited by co-incubation with HELSS at concentrations 0.5 μM and above and by $AACOCF_3$ at concentrations of 10 μM and above. Similarly, $PACOCF_3$ at 0.1 μM and above and MAFP at 0.1 μM also inhibited IL-1β stimulation (data not shown). Whereas ONO-RS-82 at concentrations up to 1 μM had no effect (and oleyloxyethylphosphocholine had no effect up to 0.5 μM, data not shown). Concentrations above 1 μM of ONO-RS-82 and HELSS appear to be toxic for these cells. This again shows that IL-1β stimulated A549 cell proliferation is mediated through the actions of both $iPLA_2$ and $cPLA_2$, whereas $sPLA_2$ plays no role.

Co-incubation with SB 203580 up to concentrations of 20 μM had no significant effect on IL-1β stimulated A549 cell proliferation. However, both PD 98059 and apigenin at concentrations 5 μM and above significantly inhibited IL-1β stimulated A549 cell growth (Figure 6). This data again suggests that inhibition of the p38 MAPK signalling pathway does not appear to inhibit cell proliferation stimulated by IL-1β. Whereas it seems that both MEK and ERK pathways are involved in this process.

Effect of EGF and IL-1β on MAPK activation

Lysates of A549 cells treated either with 10 nM EGF or 1 ng ml^{-1} IL-1β for the indicated times were immunoprecipitated with specific monoclonal antibodies to several members of the MAPK family. Western blotting of the selected immunoprecipitates with anti-phospho specific antibodies enabled the activation status of each MAPK to be determined. Figure 7 shows increased activation of ERK1, p38 and JNK after 5 min of treatment with EGF (ERK2 remained unchanged, not shown). Generally, longer contact times were required for IL-1β to activate these kinases with maximal activation occuring between 1–3 h. These results are in accord with our previous observations on the activation of $cPLA_2$ (Croxtall *et al*., 1996a,b) and arachidonic acid release (Croxtall *et al*., 1995) by these factors.

Effect of MAPK inhibitors on $cPLA_2$ activation and COX2 expression

Results shown in Figure 8 demonstrate that A549 cells pre-incubated for 3 h with either PD 98059 or apigenin fail to activate ERK1 and $cPLA_2$ in response to EGF or IL-1β. Whereas, the induction of COX2 remains unaffected. However, in contrast, pre-incubation with SB 203580 does not effect activation of ERK1 or $cPLA_2$ but significantly attenuates the induction of COX2. These results reinforce the premise of this study that EGF and IL-1β stimulate proliferation of A549 cells by activation of the ERK1 pathway and subsequently $cPLA_2$ resulting in enhanced arachidonic acid release. Inhibition of this pathway results in a significant attenuation of cell growth. However, it appears that COX2 is differentially activated by an alternative but parallel pathway involving p38MAPK.

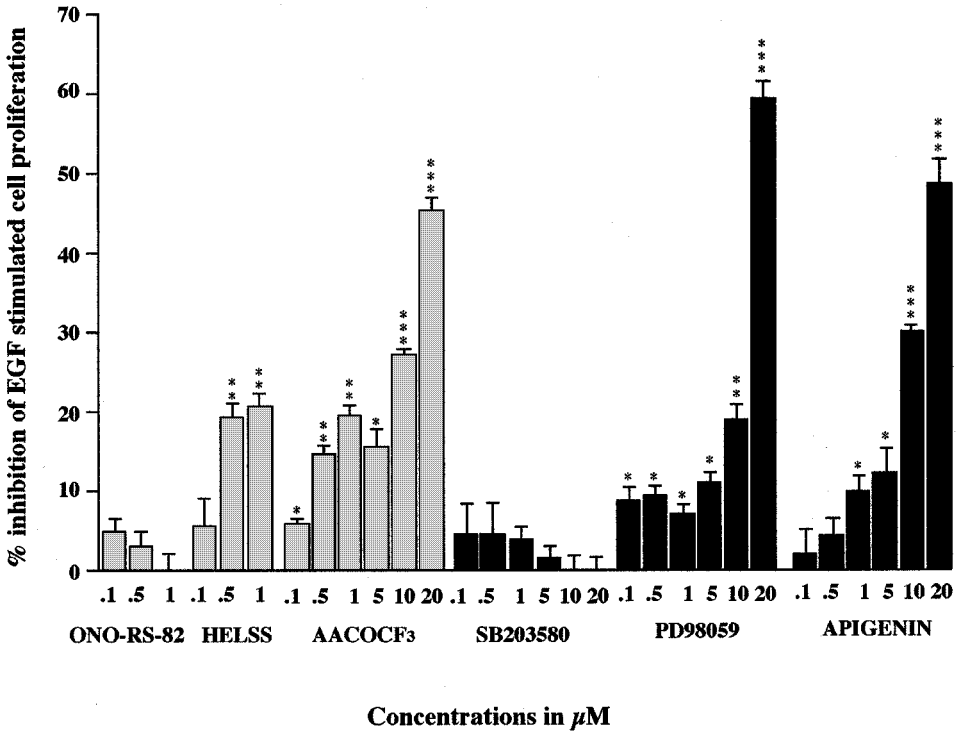

Figure 5 The effect of PLA_2 inhibitors, MEK and MAPK inhibitors on EGF stimulated A549 cell growth. Cells were treated with EGF (10 nM) plus with either $sPLA_2$ inhibitor ONO-RS-82, $iPLA_2$ inhibitor HELSS, $cPLA_2$ inhibitor $AACOCF_3$, p38 MAPK inhibitor SB 203580, MEK inhibitor PD 98059, ERK inhibitor apigenin or untreated as control (C) for 72 h. The data are presented as mean $\pm$ s.e.mean, expressed as per cent inhibition of cell growth with respect to EGF stimulation of 72% $\pm$ 3% above control values. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ inhibition compared to EGF stimulation.

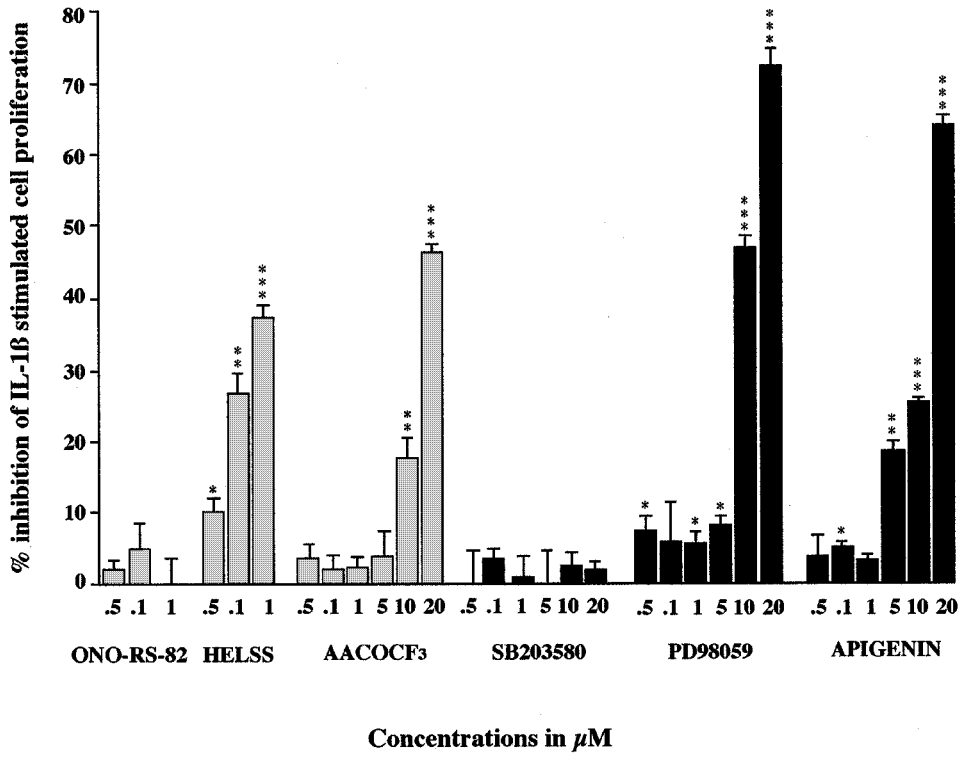

Figure 6 The effect of PLA_2 inhibitors, MEK and MAPK inhibitors on IL-1β stimulated A549 cell growth. Cells were treated with IL-1β (1 ng ml^{-1}) plus with either $sPLA_2$ inhibitor ONO-RS-82, $iPLA_2$ inhibitor HELSS, $cPLA_2$ inhibitor $AACOCF_3$, p38 MAPK inhibitor SB 203580, MEK inhibitor PD 98059, ERK inhibitor apigenin or untreated as control (C) for 72 h. The data are presented as mean$\pm$s.e.mean, expressed as per cent inhibition of cell growth with respect to IL-1β stimulation of 35%$\pm$2% above control values. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ inhibition compared to IL-1β stimulation.

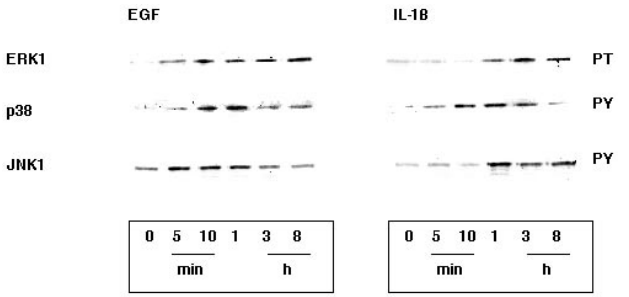

Figure 7 Time course of the activation of ERK1, p38MAPK and JNK1. A549 cells were treated for the times indicated by either 10 nM EGF or 1 ng ml^{-1} IL-1β. Immunoprecipitates of the selected MAPK's were examined for activation status by Western blotting with antibodies to either phosphothreonine (PT) or phosphotyrosine (PY). EGF induced a 20 fold activation of ERK1 from 5 min, a 10-fold activation of p38MAPK from 10 min and a 2 fold activation of JNK1 from 5 min. Whereas, IL-1β induced a 5 fold activation of ERK1 at 1 h, a 10 fold activation of p38MAPK at 1 h and a 30 fold activation of JNK1 at 1 h.

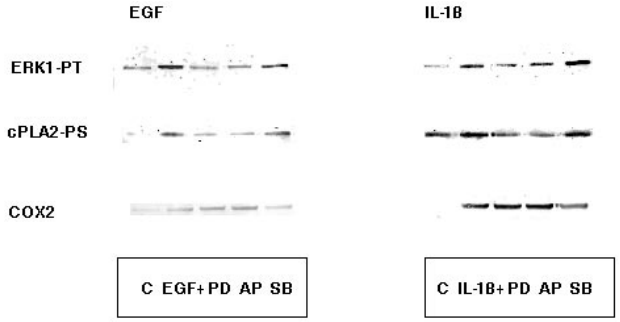

Figure 8 The effect of MAPK inhibitors on $cPLA_2$ activation and COX2 induction. Cells were pre-treated with either 10 μM PD 98059 (PD), 10 μM apigenin (AP) or 10 μM SB 203580 (SB) for 3 h prior to treatment with 10 nM EGF for 30 min or 1 ng ml^{-1} IL-1β for 3 h. Immunoprecipitates of ERK1 and $cPLA_2$ were examined for activation status by Western blotting with antibodies to phosphothreonine (PT) and phosphoserine (PS) respectively. COX2 expression was assessed Western blotting the total cell lysate with COX2 antibody. The activation of ERK1 and $cPLA_2$ by either EGF or IL-1β was prevented by both PD and AP but unaffected by SB. Whereas the induction of COX2 was unaffected by PD and AP but reveresed by SB.

Inhibition of this pathway attenuates PGE_2 release but fails to arrest cell proliferation.

Discussion

From the results obtained in this study it is apparent that both EGF and IL-1β increase AA release, up-regulate PGE_2 production and increase cell proliferation in A549 cells. EGF and IL-1β stimulated AA release was followed by an increase in PGE_2 production and a subsequent increase in cell proliferation. This demonstrates the close association of enhanced PGE_2 release with elevated A549 cell growth. Data obtained showed that EGF stimulates AA release by 40% over control values, up-regulates PGE_2 production by 450% over control values and increases cell proliferation by 70% over control values, whereas IL-1β stimulates AA release by 110% over control values, up-regulates PGE_2 production by 4000% over control values and increases cell proliferation by 35% over control values in A549 cells. However, the extent of EGF

stimulated cell growth was much greater than IL-1β, despite a much lower increase in PGE_2. This perhaps indicates that PGE_2 may not be the only mediator involved and that other PG's may have a role to play in A549 cell proliferation. This is supported by our previous work (Croxtall & Flower, 1992) where dexamethasone suppression of cell growth was only partially reversed by the addition of exogenous PGE_2, implying that other mediators are necessary.

The PLA_2 inhibitor studies revealed that $cPLA_2$ inhibitor $AACOCF_3$ has a profound inhibitory effect on both EGF and IL-1β stimulated AA release on, EGF or IL-1β stimulated PGE_2 release, and also on cell proliferation of A549 cells. The crucial role of $cPLA_2$ in the rapid release of AA after stimulus-coupled cell activation is now well established. $cPLA_2$ preferentially liberates AA following translocation from the cytosol to the membrane fraction in response to stimulus-initiated transient increase in cytoplasmic Ca^{2+} concentrations and phosphorylation by MAPK (Wissing *et al*., 1997). This increase in AA release results in up-regulation of PGE_2 release with concomitant enhanced cell growth. $AACOCF_3$ has been used by several workers to inhibit $cPLA_2$ activity in platelets, U937 monocytes and messengial cells (Riendeau *et al*., 1994; Bartoli, *et al*., 1994). It also potently depressed the $cPLA_2$ activity and eicosanoid production in rat corpora lutea (Kurusu *et al*., 1997). Consistently, results obtained from our study also showed that EGF and IL-1β stimulation of AA release, up-regulation of PGE_2 release and enhanced cell growth are all mediated, in part, by the activation of $cPLA_2$, whereas $sPLA_2$ plays no significant role.

Data obtained from this study also demonstrate the ability of the $iPLA_2$ inhibitors HELSS and $PACOCF_3$ to significantly inhibit either EGF or IL-1β stimulated cell proliferation, PGE_2 generation and AA release from A549 cells, albeit to a lesser extent than $AACOCF_3$. This raises the question of the significance of $iPLA_2$ mediated cell proliferation in this cell line. Recent reports suggest its presence in most cell lines and is crucial for membrane remodelling and controlling the amount of AA released (Balsinde & Dennis, 1997). Normally, small amounts of free AA are constantly generated in the cell and this step is thought to be mediated by $iPLA_2$ (Balsinde *et al*., 1995). Surette and colleagues (1999) have shown using HL-60 cells that $iPLA_2$ plays a significant role in the control of cellular AA levels, cell proliferation and survival. Therefore, $iPLA_2$ may also act as a housekeeping enzyme in regulating phospholipid turnover and cell growth in A549 cells. The lack of inhibition of EGF or IL-1β stimulated cell proliferation, basal PGE_2 generation and EGF or IL-1β stimulated AA release from A549 cells by the $sPLA_2$ inhibitor ONO-RS-82 supports data from many groups that this isoform appears to play little role in these processes.

MAPKs play a pivotal role in the phosphorylation and activation of $cPLA_2$ (Lin *et al*., 1992). Components of MAPK pathways have also been implicated as mediators of phosphorylation of intracellular substrates such as protein kinases and transcription factors as well as regulators of cell growth and differentiation (Johnson *et al*., 1996). So far, all known mitogens including EGF exert intracellular responses through the induction of MAPK cascades (Seger & Krebs, 1995). To investigate the involvement of MAPK cascades by EGF and IL-1β in stimulating AA release, PGE_2 generation and cell growth in A549 cells, the ERK inhibitor apigenin, the MEK inhibitor PD 98059 and the p38 MAPK inhibitor SB 203580 were used in these assays.

Results obtained from this study showed that ERK inhibitor apigenin significantly inhibited in a concentration dependent manner EGF or IL-1β stimulated cell proliferation, PGE_2 generation and AA release from A549 cells and also blocked the activation of $cPLA_2$. This is highly suggestive of a role for the ERK pathway in controlling the acivity of $cPLA_2$ and thus the release of AA and PGE_2 as a means of regulating cell growth. Many groups have reported the concomitant activation of MAPK and $cPLA_2$ in a variety of stimulated cells (Lin *et al*., 1993; Qiu & Leslie, 1994; Durstin *et al*., 1994; Xing *et al*., 1994; Sa *et al*., 1995; Ambs *et al*., 1995) and we can now add the concomitant activation of PGE_2 release and cell growth.

To further evaluate the role of ERK in the control of A549 cell signalling, the MAPK kinase (MEK) inhibitor PD 98059 was used. PD 98059 also significantly inhibited in a concentration dependent manner EGF or IL-1β stimulated cell proliferation, PGE_2 generation and AA release from A549 cells and again also blocked the activation of $cPLA_2$. This suggests that EGF and IL-1β actions are mediated through MAPK-activating enzyme MEK and, which thereby regulates acivity of the MAPK cascades and thus the phosphorylation and activation of $cPLA_2$ resulting in AA release, PGE2 generation and enhanced cell growth.

Zhang and colleagues (1997) have reported that aggregation of the high affinity IgE receptor in the RBL-2H3 mast cell line resulted in the release of AA. Treatment of these cells with PD 98059 inhibits activation of ERK and the release of AA. Cybulsky and McTavish (1997) also reported that EGF stimulated the activity and tyrosine phosphorylation of ERK leading to cell proliferation in a rat glomerular epithelial cell line. Likewise, Dudley and coworkers (1995) demonstrated that PD 98059 prevented activation of MAPK and inhibited stimulation of cell growth when stimulated by EGF or PDGF in BALB-3T3 mouse fibroblasts and rat kidney cells. Furthermore, dominant negative or constitutively active mutants of MEK inhibit or accelerate cell proliferation of NIH-3T3 cells respectively (Seger *et al*., 1994). Also mutants of ERK and its antisense cDNA cause an inhibition of cell proliferation (Pages *et al*., 1993). All these data implicate the importance of MEK/ERK cascades in the control of cell proliferation. Our data also supports the importance of this pathway and suggests that one key feature of their action may be the activation of AA release by these kinases.

The p38 MAPK inhibitor SB 203580 had no significant effect on EGF and IL-1β stimulated AA release, and on EGF or IL-1β stimulated cell proliferation in A549 cells. However, SB 203580 significantly inhibited EGF and IL-1β stimulated PGE_2 production and blocked the induction of COX2 (Figure 8). This perhaps suggest that PGE_2 production in this A549 cell line is under the dual control of ERK1 and p38 MAPK through the concerted regulation of AA release and COX-2 expression respectively and that cell growth arrest is accomplished by blocking both pathways. Both EGF and IL-1β induce COX-2 and a role for p38 MAPK in COX-2 induction explains the effect of SB 203580 compound in EGF and IL-1β stimulated PGE_2 release.

Recently, it has been shown that EGF also activates the SAPK/JNK pathway which is mediated by Rac 1 and CDC42 following Ras stimulation (Coso *et al*., 1995; Minden *et al*., 1995). The Raf/ERK pathway is well known in EGF induced cell proliferation and intracellular signalling and the role of SAPK/JNK pathway as a mediator of EGF stimulated cell growth in A549 cells has been recently suggested by Bost and colleagues (1997). Furthermore, EGF induced stimulation of JNK activity has also been observed in two other lung cancer cell lines, M103 cells and H1011 cells (Bost *et al*., 1997). All this indicates that SAPK/JNK may be another important growth promoting pathway in human non-small lung cancer

cells. However, to date, there is no evidence to suggest that p38 MAPK pathways are involved in EGF stimulated cell proliferation in A549 cells as yet or in any other cell lines. Nevertheless, since this pathway shares many similar stimuli with the SAPK/JNK pathway it cannot be completely ruled out as a possible role in EGF or IL-1β stimulated cell growth under some circumstances.

In summary, we show that both EGF and IL-1β stimulate proliferation of A549 cells by a mechanism including the activation of MAPK pathways which in turn leads to the activation of $cPLA_2$ and not $sPLA_2$. However, the involvement of $iPLA_2$ also appears to play an important role. It is evident that MEK and ERK pathways are involved in the modulation of EGF and IL-1β induced AA release A549 cell growth in A549 cells. The MEK and ERK pathways appear to be more active in EGF than IL-1β stimulated A549 cells in controlling arachidonic acid release rather than the p38 MAPK pathway. Whereas, in EGF and IL-1β stimulated PGE_2 generation and COX2 expression, it appears that p38 MAPK, ERK and MEK all participate in the regulation of PGE_2 release. A better understanding of the signalling pathways that lead to activation of AA release may help in the application of selective kinase inhibitors to control abberant cell proliferation and inflammatory diseases.

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