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Prostaglandin E_2 requirement for transforming growth factor β_1 inhibition of elicited macrophage 14 kDa phospholipase A_2 release

Mark McCord, Brian Bolognese & [1]Lisa A. Marshall

SmithKline Beecham Pharmaceuticals, Department of Inflammation Pharmacology, 709 Swedeland Road, UW2532, King of Prussia, PA 19406-0939, U.S.A.

1 Cultured elicited-peritoneal macrophages release a soluble type II 14 kDa phospholipase A_2 (PLA_2) over time, reaching a plateau by 20-24 h of incubation and maintaining these levels over 72 h. Prostaglandin E_2 (PGE_2) is also produced but does not plateau until 48-72 h.

2 Transforming growth factor β_1 ($TGF\beta_1$) reduces cellular 14 kDa PLA_2 and its subsequent release by approximately half, but does not alter PGE_2 production. Co-incubation of $TGF\beta_1$ with indomethacin interfered, in a concentration-dependent manner, with the ability of $TGF\beta_1$ to reduce cellular 14 kDa PLA_2 and its subsequent release over 24 h. The regulation of $TGF\beta_1$ was not specific to indomethacin since other non-steroidal anti-inflammatory drugs had the same effect. This suggested that cyclo-oxygenase activity was essential for $TGF\beta_1$ to exert its effect and indeed, the addition of exogenous PGE_2 restored the $TGF\beta_1$ action.

3 PGE_2 alone exerted a concentration-dependent negative feedback action on elicited-macrophage 14 kDa PLA_2 release. The inhibitory concentration ($IC_{50} = \sim 180$ ng PGE_2 ml^{-1}) approximated the PGE_2 levels measured in the 24 h macrophage conditioned media (85-140 ng PGE_2 ml^{-1}) where PLA_2 release began to plateau. Further, incubation of cells with indomethacin over 48 h resulted in the enhancement of 14 kDa PLA_2 activity compared to that released from untreated cells. Forskolin failed to inhibit 14 kDa PLA_2 release, suggesting PGE_2 was not acting through an increase in adenylate cyclase.

4 Taken together, the data are consistent with the immunosuppressive aspects reported for both mediators during inflammation and demonstrates the requirement of PGE_2 for $TGF\beta_1$ action on the elicited macrophage.

Keywords: Phospholipase A_2; elicited-peritoneal macrophage; prostaglandin E_2; cyclo-oxygenase; transforming growth factor β_1; indomethacin

Introduction

Macrophages play an integral role at sites of chronic inflammation. They can be activated to release a variety of cytokines and growth factors as well as lipid mediators such as prostaglandins and leukotrienes (Hamilton & Adams, 1987; Riches *et al.*, 1988). During inflammation, prostaglandins facilitate the removal of foreign antigens or irritants by increasing capillary permeability, vasodilatation and cellular influx (Williams & Morley, 1973; Moncada *et al.*, 1973). As the condition becomes chronic, increased inflammatory cell populations (i.e. macrophages and T-lymphocytes) enhance local prostaglandin E_2 (PGE_2) release which then act by preventing lymphocyte activation (Gordon *et al.*, 1976; Weissmann, 1993), down-regulating macrophage functions (Gordon *et al.*, 1976; Bonta & Parnham, 1978; Weissmann, 1992) and affecting tissue events through inhibition of fibroblast growth (Johnson & Pastan, 1971) or function (Willoughby *et al.*, 1993). We have previously shown that cultured elicited macrophages from the peritoneal cavity of animals injected with a phlogistic agent, release PGE_2, but not leukotriene B_4 (LTB_4). This is accompanied by the synthesis and secretion of a type II 14 kDa phospholipase A_2 (PLA_2; EC 3.1.1.4, Marshall *et al.*, 1994). Phospholipase A_2 has a pivotal role in the generation of lipid mediators and when induced and released by cytokines or growth factors (Gilman & Chang, 1990; Oka & Arita, 1991; Spaargaren *et al.*, 1992), the soluble form is thought to mediate inflammation in a variety of diseases (Bomalaski *et al.*, 1991; Bomalaski & Clark, 1993). We have shown that the extracellular 14 kDa PLA_2 does not play a role in macrophage PGE_2 biosynthesis and proposed that a cell-associated 85 kDa PLA_2 may regulate this process (Marshall *et al.*, 1994). Alternatively, the possible regulatory role that PGE_2 may have in the production and release of PLA_2 has not been thoroughly explored in this system.

Macrophages respond to transforming growth factor β_1 ($TGF\beta_1$), a homodimeric polypeptide which is a mediator of wound healing, inflammation and angiogenesis (Massague *et al.*, 1992; Folkman & Brem, 1992). $TGF\beta_1$ has been shown to down-regulate mesangial cell cytokine-induced 14 kDa PLA_2 secretion which is accompanied by an increase in PGE_2 release (Schalkwijk *et al.*, 1992a) and 85 kDa PLA_2 (Schalkwijk *et al.*, 1992b) expression. We have reported that $TGF\beta_1$ also differentially regulates the two PLA_2 enzymes of peritoneal elicited-macrophages, reducing the production and release of the 14 kDa PLA_2 while enhancing the production of the 85 kDa PLA_2 (Bolognese *et al.*, 1995). However, PGE_2 was present but not altered in these studies.

The role of PGE_2 in $TGF\beta_1$-induced alteration of elicited-macrophage PLA_2 has not been fully studied. Here we show that PGE_2 itself inhibited soluble 14 kDa PLA_2 release in a concentration-dependent manner. In order for $TGF\beta_1$ to exert its effects on 14 kDa PLA_2 release or cell associated 85 kDa PLA_2, PGE_2 had to be present. This was evident when the co-administration of cyclo-oxygenase inhibitors with $TGF\beta_1$ failed to reduce 14 kDa PLA_2 but addition of exogenous PGE_2 restored the action of $TGF\beta_1$ despite the presence of cyclo-oxygenase inhibitors.

[1] Author for correspondence.

Methods

Elicited macrophage isolation and culture

Male guinea-pigs (Hazelton Research Animals, Denver, PA, U.S.A.) weighing 700–900 g were injected i.p. with 1 ml of horse serum (Gibco, Grand Island, NY, U.S.A.) 72 h prior to lavage. Animals were anaesthetized with Ketamine/Ropam (Hanna's Pharmaceutical Supply Co., Wilmington, DE, U.S.A.) and the peritoneal cavity was lavaged; 50 ml of sterile PBS (27°C) was injected i.p., the abdominal area was massaged and the lavage fluid was placed in 50 ml conical tubes as previously described (Marshall *et al.*, 1994). Cells were pelleted by centrifugation 400 *g*, 10 min, 27°C and the supernatant discarded. Peritoneal exudate cells from several animals were pooled, layered over a Percoll gradient and centrifuged at 400 *g* for 30 min (Colotta *et al.*, 1984). The mononuclear cells from the interface were recovered, washed once, placed in 5% FBS RPMI 1640 and then counted. Cells from 5–15 animals were routinely pooled to perform a single experiment.

Mononuclear cells were always incubated at a cell density of 5×10^6 per 35 mm diameter well using 6, 12 or 24 multiwell plates or 100×15 mm dishes (Nunc, Roskilde, Denmark). The cells in RPMI 1640 with 5% FBS were allowed to adhere for 1–2 h at 37°C in a 5% CO_2 atmosphere incubator. The non-adherent cells were removed followed by one wash with 1 ml RPMI 1640. The adherent macrophages (95–99% macrophages as determined by differential staining) were incubated in RPMI 1640 with 5% FBS over 20–24 h unless otherwise stated. Experiments were terminated by removing the macrophage conditioned media (MCM), which was then centrifuged at 1000 *g* for 10 min to remove cells, frozen in liquid nitrogen and stored at −20°C for analysis. Cyclo-oxygenase inhibitors were added in DMSO at a final concentration that was always less than 0.4% and used at concentrations in which PGE_2 synthesis and release was maximally inhibited. We have previously shown that TGFβ_1 can reduce the release of 14 kDa PLA_2 from elicited peritoneal macrophages cultured for 24 h in a concentration-dependent fashion (Bolognese *et al.*, 1995). TGFβ_1 was used at maximally effective concentrations (20–50 ng ml^{-1}) solubilized in RPMI 1640 with 5% FBS. Cell viability was monitored before and after experiments by trypan blue exclusion. No toxicity or changes in cell number were observed with any of the treatments.

Preparation of macrophage fractions

Macrophages from 5 animals were pooled to generate sufficient protein for activity and Western analysis and then treated as described above. After treatment the cells were scraped from the dishes and counted. The cells were resuspended in homogenization buffer, (at a concentration of 2×10^8 cells ml^{-1}) containing 0.34 M sucrose, 10 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM PMSF, 200 μM leupeptin, 20 $\mu g\ ml^{-1}$ soybean trypsin inhibitor, 20 $\mu g\ ml^{-1}$ aprotinin. EGTA was included to localize the 85 kDa PLA_2 predominantly to the cytosolic fraction (Channon & Leslie, 1990). The cell suspension was disrupted while on ice by sonication (5 s) with a Bransonic probe tip. The homogenate was centrifuged at 400 *g* for 10 min at 4°C to remove unbroken cells and debris and designated the homogenate. Fractions were prepared when the homogenate was centrifuged at 100,000 *g* for 60 min at 4°C to obtain a supernatant (cytosol) and particulate fraction. The particulate fraction was resuspended in homogenization buffer. All fractions and homogenates were frozen in liquid nitrogen and stored at −20°C for analysis. The particulate and cytosolic fractions were evaluated for *sn*-2 acylhydrolytic activity using [^{3}H]-AA *E. coli.* and vesicles of 1-palmitoyl-2-[^{14}C]-arachidonyl PC, respectively.

Measurement of eicosanoids

Prostaglandin E_2 was measured with enzyme immunoassay kits purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). Sample or standard dilutions were made with appropriate media and analyzed in triplicate. Results were obtained by interpolation from a standard curve prepared in the media calculated using Delta Soft v2.12 (Biomettalics Inc., Princeton, NJ, U.S.A.) and expressed as pg or ng ml^{-1} of sample volume or cell number.

Phospholipase A_2 enzymes

Recombinant human (rh) type II 14 kDa PLA_2 cloned from a human placenta cDNA library was expressed as an authentic processed enzyme in CHO cells and purified essentially as described (Stadel *et al.*, 1992). U937 85 kDa PLA_2 cDNA was subcloned into the baculovirus vector pAcCL29 and recombinant human U937 85 kDa PLA_2 (rh 85 kDa PLA_2) was expressed in Spotoptera frugiperde (SF21) cells as previously described (Amegadzie *et al.*, 1993).

Phospholipase A_2 enzyme assay

Phospholipase A_2 activity of isolated enzymes, MCM, or the cell particulate fraction (5–20 μg protein per assay) were measured by the acylhydrolysis of [^{3}H]-AA *E. coli.* The cell cytosolic fractions were assayed using vesicles of 1-palmitoyl-2-[^{14}C]-arachidonyl PC (Marshall & Roshak, 1993). The assay was initiated by addition of substrate and assays were incubated at 37°C for a time predetermined to be on the linear portion of a time versus hydrolysis plot (purified enzymes, used as positive controls, were assayed over 10 min whereas all other samples, MCM or cell fractions were assayed between 5–30 min). Results are calculated as percentage of free fatty acid hydrolyzed [(d.p.m. generated minus background d.p.m.) divided by (total d.p.m. added) × 100]. Background counts were never more than 1% of the total counts added. Data were expressed as percentage hydrolysis or as specific activity (pmol free fatty acid hydrolyzed $min^{-1}\ mg^{-1}$).

Immunoblot analysis

Cell fractions (50 μg protein) and/or recombinant enzymes used as standards were analyzed by SDS–PAGE (10–20% gradient gels; Integrated Separation Systems, Natick, MA, U.S.A.). Proteins were transferred to nitrocellulose paper, incubated with the appropriate rabbit antiserum (diluted 1:1000–2000) and then incubated with donkey anti-rabbit IgG conjugated to horseradish peroxidase (diluted 1:5000; Boehringer Mannheim, Indianapolis, IN, U.S.A.). Detection of immunoreactive bands was carried out by the enhanced chemiluminescence technique Western blotting system (Amersham International, Arlington Heights, IL, USA). Rabbit polyclonal antiserum against the 85 kDa PLA_2 was prepared as previously described (Amegadzie *et al.*, 1993). Scanning densitometry was performed using an UVP Imagestore 5000 (San Gabriel, CA, USA) as previously described (Roshak *et al.*, 1994).

Solubilization of cellular 14 kDa PLA_2 by acid extraction

14 kDa PLA_2 is known to resist acid treatment and this has been used as an initial purification step to liberate the 14 kDa PLA_2 and reduce overall total protein (Kramer *et al.*, 1989; Marshall *et al.*, 1991; Marshall & Roshak, 1993). Samples were subjected to acid extraction by exposure to equal volumes of 0.36 N H_2SO_4 for 1 h at 4°C. After treatment the samples were readjusted to pH 7.4 by the addition of 2 M Tris (pH 10) and to 150 mM NaCl and the precipitate was removed by centrifugation (10,000 *g*, 5 min). The supernatant was then directly analyzed by an ELISA assay to measure the concentration of 14 kDa PLA_2.

Quantification of type II 14 kDa PLA$_2$ by ELISA

We previously demonstrated than an anti-rh type II 14 kDa PLA$_2$ monoclonal antibody could recognize the guinea-pig macrophage soluble 14 kDa PLA$_2$ that was released into the media (Marshall *et al.*, 1994; Bolognese *et al.*, 1994). Mouse anti-rh Type II 14 kDa PLA$_2$ monoclonal antibodies were prepared as previously described (Marshall *et al.*, 1994; Roshak *et al.*, 1994) and demonstrated no cross-reactivity with either type I pancreatic 14 kDa PLA$_2$, 85 kDa PLA$_2$ animal or human albumin or various inflammatory mediators such as tumour necrosis factor, interleukin-1, platelet activating factor. Microtitre plates (Nunc Immuno Plate Maxisorp F96, Roskilde, Denmark) were coated with the monoclonal antibody SK088-3C6.16.2 (100 μl, 2 μg ml^{-1}) in 50 mM sodium phosphate, 150 mM NaCl, 0.02% NaN_3, pH 7.4 for 18 h at 4°C. The plates were washed 4 times (wash buffer composition: 10 mM Tris, 150 mM NaCl, 0.02% NaN_3, 0.05% Tween 20, pH 7.4) and then blocked with 200 μl 1% BSA in 50 mM Tris, 150 mM NaCl, 0.02% NaN_3, pH 7.4 for 5 min at 37°C. MCM samples, and the corresponding standards (50 μl), were diluted in assay medium (RPMI 1640, 5% FBS) or acid extracted samples and corresponding standards were prepared in acid extracted homogenization buffer. Samples or standards were co-incubated with 50 μl conjugate (2 μg ml^{-1} biotinylated monoclonal antibody SK097-1E8.5.2) for 1 h at 37°C. The plates were washed 4 times with wash buffer, followed by the addition of 100 μl per well of the streptavidin-alkaline phosphatase conjugate (diluted 1:2000 in streptavidin buffer, 0.5% bovine gamma globulin, 50 mM Tris, 150 mM NaCl, 0.02% NaN_3 1 mM $MgCl_2$, pH 7.4) and incubated for 30 min at 37°C. The plates were washed again followed by the addition of 100 μl substrate per well (*p*-nitrophenyl phosphate, 1 mg ml^{-1}) and incubated for 30 minutes at 37°C after which the plate was read at 405 nm using a MR7000 plate reader (Dynatech Laboratories Inc., Chantilly, VA, USA). Purified rh type II 14 kDa PLA$_2$ was used to generate a standard curve ranging from 5 pg to 200 pg per 50 μl. Standard curves and results were calculated using Delta Soft v2.12 (Biometallics Inc., Princeton, NJ, U.S.A.).

Protein determination

All protein concentrations were determined by Bradford protein analysis kits (BioRad, Richmond, CA, U.S.A.).

Materials and chemicals

Transforming growth factor β_1 (TGFβ_1, ultrapure from human platelets) was purchased from Genzyme Co. (Cambridge, MA, U.S.A.). 1-Palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine (PC, 52 mCi mmol^{-1}) and [^{3}H]-arachidonic acid-(AA)-labelled *E. coli* (0.5 μCi 5 nmol^{-1} P_i) were purchased from New England Nuclear (NEN, Boston, MA, U.S.A.). Hanks balanced salts solution (HBSS) with and without Ca^{2+}/Mg^{2+}, RPMI 1640, foetal bovine serum (FBS, mycoplasma-free), and phosphate-buffered saline (PBS) were obtained from GIBCO (Grand Island, NY, U.S.A.). Percoll solution, bovine serum albumin (BSA), dithiothreitol (DTT), bovine gamma globulin, *p*-nitrophenyl phosphate (PNP), ethyleneglycol-*bis*-(*β*-aminoethyl ether)-*N,N,N′,N′* tetracetic acid (EGTA) Tris buffer (Tris), phenylmethylsulphonyl fluoride (PMSF), leupeptin, soybean trypsin inhibitor (SBTI), aprotinin and ibuprofen were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Aminopropyl solid phase silica columns (No. 9070, 100 mg ml^{-1}) were obtained from Burdick and Jackson (Muskeogon, MI, U.S.A.). Aspirin, meclofenamic acid and indomethacin were synthesized by the Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals.

Calculations and statistics

Data are expressed as mean ± standard deviation (s.d.) of 3 determinations ($n=3$) unless otherwise stated and subjected to one way analysis of variance and Duncan's multiple range test ($P<0.05$) for statistical evaluation where indicated. All experiments were performed 2–4 times on 2–4 different cell preparations.

Results

Effect of indomethacin on TGFβ_1-induced inhibition of 14 kDa PLA$_2$ release into the 24 h MCM

Co-incubation of a maximal concentration of TGFβ_1 (20 ng ml^{-1}) with indomethacin (1 μM) abolishes TGFβ_1-induced inhibition of 14 kDa PLA$_2$ release (Figure 1). Indomethacin itself had no effect on hydrolysis levels measured in the 24 h MCM. In a subsequent study, indomethacin reversed the TGFβ_1 inhibitory effect on 14 kDa PLA$_2$ in a concentration-dependent manner (no treatment, 8.2 ± 1.6%; TGFβ_1 (50 ng ml^{-1}), 4.3 ± 0.9%; TGFβ_1 and indomethacin at the following concentrations; 1 μM, 7.9 ± 1.3%; 0.1 μM, 8.5 ± 1.1%; 0.03 μM, 6.0 ± 1.4% and 0.01 μM, 5.0 ± 1.4% hydrolysis of [^{3}H]-AA *E. coli* per 30 μl of MCM in 30 min ($n=3$)).

Effect of indomethacin on TGFβ_1-induced changes in cell-associated 14 kDa or 85 kDa PLA$_2$ production

Similar effects of indomethacin on TGFβ_1 were demonstrated on cell-associated 14 kDa PLA$_2$ activity. Acylhydrolytic activity of the particulate fraction of cells treated with TGFβ_1 alone was reduced by approximately 60% when compared to the non-treated controls (Figure 2). Neither the combination of TGFβ_1 and indomethacin nor indomethacin alone altered activity when compared to non-treated control cell levels.

Two studies, each using a pool of 5 animals per treatment, demonstrated that the cytosolic 85 kDa PLA$_2$ activity of cells treated with TGFβ_1 (242.5 ± 11.3 pmol in 30 min per 20 μg protein) was significantly higher ($P<0.05$) than the control (188.3 ± 6.7 pmol in 30 min per 20 μg protein, mean ± s.d. of data from 2 experiments). Activity in the cytosol of cells co-treated with TGFβ_1 and indomethacin (183.8 ± 15.9 pmol in

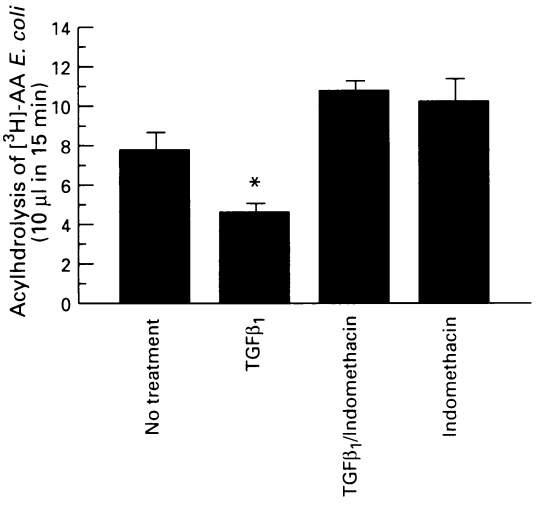

Figure 1 Effects of TGFβ_1 and indomethacin or indomethacin alone on the release of 14 kDa *sn*-2 acylhydrolytic activity in cell-free MCM of elicited macrophages cultured over 20–24 h. The concentrations of TGFβ_1 and indomethacin were 20 ng ml^{-1} and 1 μM, respectively. Data are expressed mean percentage hydrolysis ± s.d. and represents one of three experiments. *Significantly different from the control ($P<0.01$).

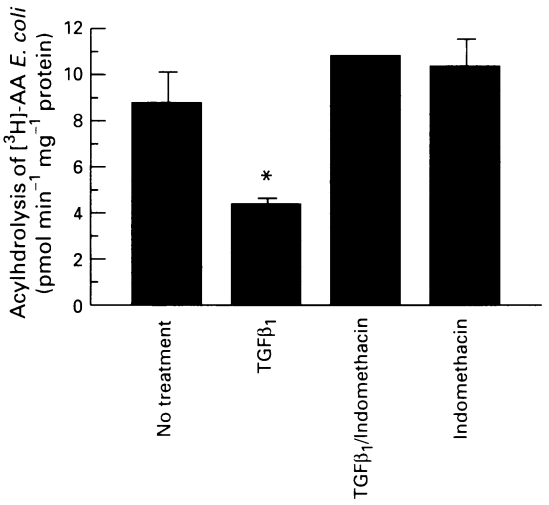

Figure 2 Effects of TGFβ_1, TGFβ_1 and indomethacin or indomethacin alone on 14 kDa *sn*-2 acylhydrolytic activity measured in the particulate fraction of elicited macrophages. The concentrations of TGFβ_1 and indomethacin were 50 ng ml^{-1} and 1 μM, respectively. Data are expressed as mean specific activity $\pm$ s.d. ($n=2$ determinations of a single pooled sample from 5 animals) derived from percentage hydrolysis of 5 μg protein in 5 min and represents one of three experiments. *Significantly different from the control ($P<0.05$).

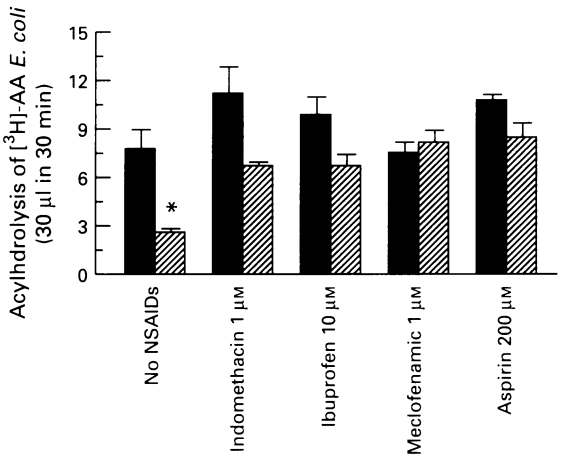

Figure 4 Effects of structurally distinct cyclo-oxygenase inhibitors on 14 kDa *sn*-2 acylhydrolytic activity released by elicited macrophages cultured over 20–24 h. Cells were incubated in the absence (solid columns) or presence (hatched columns) of TGFβ_1 (50 ng ml^{-1}) and exposed to vehicle or indomethacin (1 μM), ibuprofen (10 μM), meclofenamic acid (1 μM) and aspirin (200 μM). Data are mean percentage hydrolysis $\pm$ s.d. ($n=3$) and represent one of three experiments. *Significantly different from the control ($P<0.05$).

30 min per 20 μg) was not significantly different from the control. The fractions were also subjected to sodium dodecylsuphate-polyacrylamide gel electrophoresis with equal amounts of protein (40 μg) loaded per lane and probed with an anti-85 kDa PLA_2 rabbit serum for immunoblot analysis. Figure 3 illustrates the 85 kDa PLA_2 protein migrating at approximately 105 kDa and that cells exposed to TGFβ_1 possess enhanced levels of 85 kDa PLA_2 versus the control untreated group, confirming our previous report (Bolognese *et al.*, 1995). The protein band for the TGFβ_1 and indomethacin treated cells remained near the control, indicating that the differences in activity measurements were due to changes in enzyme protein levels. Two immunoblots from two separate studies conducted identically were evaluated by densitometry (study I: no treatment, 62; TGFβ_1, 146 and TGFβ_1/indomethacin, 46 pixels and study II: no treatment, 327; TGFβ_1, 723 and TGFβ_1/indomethacin, 363 pixels).

Effect of other cyclo-oxygenase inhibitors on TGFβ_1-induced inhibition of 14 kDa PLA_2 release

To determine if this effect was unique to indomethacin, elicited macrophages were exposed to a variety of structurally distinct cyclo-oxygenase inhibitors. Neither cell number not viability was altered by the treatment. Figure 4 shows that the inhibitors alone (solid columns) did not significantly affect the release of 14 kDa PLA_2 into the media, although there was a trend for minor enhancement. Treatment of cells with TGFβ_1 significantly reduced 14 kDa PLA_2 release, but when cells were exposed to TGFβ_1 and an inhibitor (hatched columns), in all cases 14 kDa PLA_2 release was similar to the untreated control (Figure 4). The PGE_2 levels were measured in the same 24 h MCM. Untreated cells and cells exposed to TGFβ_1 alone released (139.1 $\pm$ 6.8 ng PGE_2 ml^{-1} and 139.3 $\pm$ 15.6 ng PGE_2 ml^{-1} ($n=3$)), respectively, while cells treated with indomethacin, (12.4 $\pm$ 2.6 ng PGE_2 ml^{-1}), ibuprofen (12.9 $\pm$ 1.7 ng PGE_2 ml^{-1}), meclofenamic acid, (12.4 $\pm$ 0.9 ng PGE_2 ml^{-1}) or aspirin (10.1 $\pm$ 1.2 ng PGE_2 ml^{-1}) alone reduced PGE_2 production by 91 $\pm$ 1.3%, 91 $\pm$ 1.3%, 91 $\pm$ 0.7% and 93 $\pm$ 1%, respectively. Similarly in cells exposed to TGFβ_1, cyclo-oxygenase inhibitors produced greater than 90% inhibition of PGE_2 levels (indomethacin, 9.9 $\pm$ 1.4 ng PGE_2 ml^{-1}; ibuprofen, 9.9 $\pm$ 2.0 ng PGE_2 ml^{-1}; meclofenamic acid, 9.2 $\pm$ 0.6 ng PGE_2 ml^{-1} and aspirin, 2.0 $\pm$ 0.2 ng PGE_2 ml^{-1}; ($n=3$)).

To confirm the requirement of PGE_2 in the TGFβ_1 reduction of 14 kDa PLA_2 release, elicited macrophages were incubated with TGFβ and indomethacin (1 μM) with or without PGE_2 for 24 h. The concentration of PGE_2 (200 ng ml^{-1}) chosen approximates the amount that accumulates in the MCM when cells were cultured over 20 h. The addition of PGE_2 during the treatment with both TGFβ_1 and indomethacin resulted in a 76 $\pm$ 1.3% reduction of 14 kDa PLA_2 activity in the MCM indicating that PGE_2 was needed for the TGFβ_1-induced effect (Figure 5). Co-incubation of PGE_2 and TGFβ_1 reduced activity in the MCM to the same extent as TGFβ_1 alone. Prostaglandin E_2 alone reduced PLA_2 activity by 73 $\pm$ 0.9%.

To verify that the changes observed were not due to altered enzyme activity or reduced secretion, cell-associated homogenate 14 kDa PLA_2 was quantified by ELISA. Cells from 10–15 animals were pooled and cultured as previously described for each study. Table 1 shows that acid extracted homogenates from untreated cells yielded 17 or 16 pg PLA_2 in 85 μg total homogenate protein for study I and study II, respectively. The TGFβ_1 treatment significantly ($P<0.05$) reduced the 14 kDa PLA_2 mass compared to that measured in the control homogenates. Indomethacin had no effect on enzyme mass while PGE_2 alone significantly ($P<0.05$) reduced the 14 kDa PLA_2 enzyme. When PGE_2 was added back to cells treated with TGFβ_1 and indomethacin, 14 kDa PLA_2 protein was reduced to the same extent as with TGFβ_1 alone.

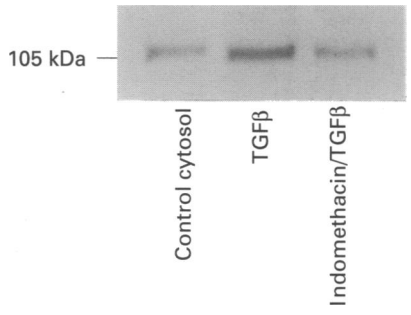

Figure 3 Western analysis of cytosolic fraction of elicited macrophages after treatment with TGFβ_1 (50 ng ml^{-1}), TGFβ_1 and indomethacin (1 μM) or indomethacin alone. Samples were loaded with equal amounts of protein (40 μg). Immunoblots show bands migrating identically to the rh 85 kDa PLA_2 standard (mol. wt. $\sim$105 kDa).

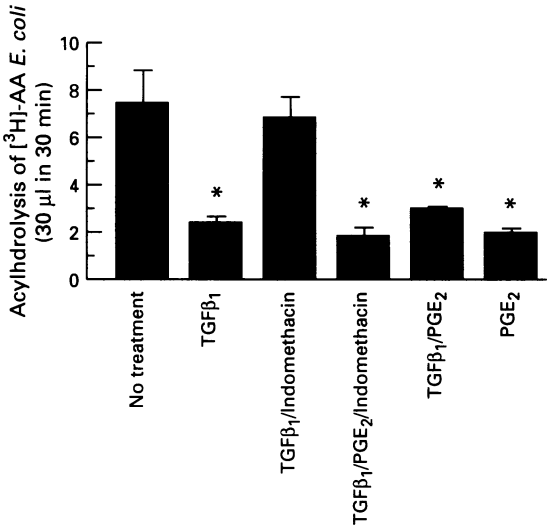

Figure 5 Effect of PGE_2 on modulation of TGFβ_1 action by indomethacin. The concentrations of TGFβ_1 or PGE_2 or indomethacin were 50 ng ml^{-1}, 200 ng ml^{-1} or 1 μM, respectively. Data are expressed as mean percentage hydrolysis ± s.d. Graph represents one of three experiments. *Significantly different from the control ($P<0.05$).

Table 1 Quantification of cell-associated 14 kDa PLA_2 from cultured elicited macrophage homogenates following incubation with TGFβ_1, PGE_2 and indomethacin

	14 kDa PLA_2 (pg per 85 μg homogenates)	
	Study I	*Study II*
No treatment	17 ± 4.2	16 ± 1.7
TGFβ_1	8 ± 0.0*	10 ± 1.0*
Indomethacin	12 ± 0.6	16 ± 2.3
PGE_2	4 ± 0.7*	6 ± 1.9*
TGFβ_1/PGE_2/indomethacin	7 ± 1.3*	5 ± 0.1*

In 2 different studies elicited macrophages were collected from 10–15 animals and pooled as described in Methods. Cells were cultured alone, with TGFβ_1 (50 ng ml^{-1}), indomethacin (1 μM), PGE_2 (200 ng ml^{-1}), or TGFβ_1, indomethacin and PGE_2 after which the cells were homogenized by acid extraction as described in Methods and analyzed by ELISA. All data are mean ± s.d. *Indicates significantly different from the control ($P<0.05$).

Evaluation of PGE_2 action on 14 kDa PLA_2 release by cultured elicited macrophages

Elicited macrophages were cultured with increasing concentrations of PGE_2 (0, 2, 20, or 200 ng ml^{-1}) with or without indomethacin over 24 h. Figure 6 shows one representation of 3 studies. PGE_2 reduced PLA_2 activity detected in 24 h MCM in a concentration-dependent manner generating IC_{50} s ranging from 180–200 ng PGE_2 ml^{-1} in the absence (a) and in the presence of indomethacin IC_{50} = 3–20 ng PGE_2 ml^{-1} (b). The differences in the extent of inhibition between the two protocols (a vs. b) is most likely due to the indomethacin-mediated total depletion of all prostanoids (e.g., prostacyclin and thromboxane as well as PGE_2) released by these cells and indicates that other prostanoids may influence PGE_2 function to an extent. The direct effect of PGE_2 on 14 kDa PLA_2 24 h MCM acylhydrolytic activity was directly assessed and concentrations as high as 200 ng ml^{-1} (570 μM) had no effect (data not shown).

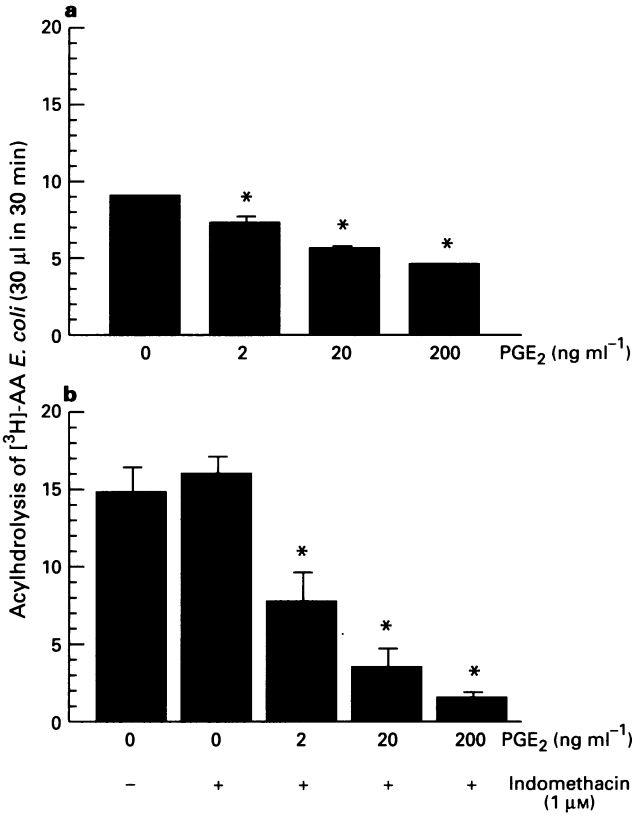

Figure 6 Effect of various concentrations of PGE_2 on *sn*-2 accylhydrolytic activity released into the MCM. Cells were cultured at 5×10^6 ml^{-1} in the presence of vehicle alone or increasing concentrations of PGE_2 (a) or increasing concentrations of PGE_2 with indomethacin (1 μM, b) for 20 h after which the cell-free MCM was collected and assessed for *sn*-2 acylhydrolytic activity as described in Methods. All data are mean percent hydrolysis ± s.d., ($n=3$). *Significantly different from the control ($P<0.05$). Both (a) and (b) represent data from one of three experiments. IC_{50} for PGE_2-induced inhibition of acylhydrolysis measured in the MCM was 180 ng ml^{-1} (a) and 3 ng ml^{-1} (b) for non-treated and indomethacin treated, respectively.

PGE_2 and its inhibition of elicited macrophage 14 kDa PLA_2 release over an extended culture period

To put the role of PGE_2 into physiological perspective, additional observations were carried out over 24, 48 or 72 h culture periods. The amount of PLA_2 activity measured in the MCM of untreated cells incubated over 48 and 72 h (2.0 ± 0.2, and 1.8 ± 0.0% hydrolysis per 20 μl MCM per 15 min, respectively) was not altered from the activity measured in 24 h MCM (1.7 ± 0.1%) confirming our previous findings (Bolognese *et al.*, 1995). In this study the measurement of PGE_2 accumulation was expanded showing that PGE_2 levels continued to increase from 85.8 ± 4.3 ng PGE_2 ml^{-1} ($n=3$) at 24 h to 1196.6 ± 2.7 ($n=3$) and 1420.6 ± 5.2 ng ($n=3$) at 48 and 72 h, respectively.

In 3 separate studies, cells cultured for 48 h were exposed to indomethacin (1 μM) for 48 h and compared to untreated controls. PGE_2 release from cells treated with indomethacin was totally abolished (data not shown) while the PLA_2 activity in the media increased significantly ($P<0.05$) (Study I, 5.7 ± 2.2%; Study II, 4.7 ± 0.8% or Study III, 7.3 ± 0.9% hydrolysis per 30, 30 or 70 μl MCM, respectively, in 40 min, ($n=3$)) compared to untreated controls (Study I, 2.5 ± 0.3; Study II, 3.0 ± 0.1% or Study III, 3.6 ± 0.5% hydrolysis per 30, 30 or 70 μl MCM, respectively, in 40 min, ($n=3$)).

Effect of forskolin on 14 kDa PLA_2 release

PGE_2 is known to function through second messenger pathways, such as adenylate cyclase (AC), to regulate cellular activity. To investigate whether the inhibitory effect of PGE_2 is mediated through the increase of AC, forskolin, an activator of adenylate cyclase was evaluated for its effect on 14 kDa PLA_2 release by elicited macrophages (Seamon & Daly, 1986). Forskolin exposure through 24 h of culture at 1 and 10 μM ($10.1 \pm 1\%$ and $7.2 \pm 2\%$ hydrolysis per 30 μl in 30 min ($n=3$), respectively) did not significantly alter 14 kDa PLA_2 release. PGE_2 alone significantly reduced ($3.7 \pm 0.3\%$ hydrolysis per 30 μl in 30 min ($n=3$)) 14 kDa PLA_2 activity compared to the control ($7.2 \pm 1\%$ hydrolysis per 30 μl in 30 min ($n=3$)).

Discussion

The elicited macrophage represents the major population at 3–5 days in the inflamed peritoneum of animals previously exposed to a variety of phlogistic agents (Marshall *et al.*, 1992). The study of these cells in culture differs from the study of other cell systems where a cytokine or growth factor is added to initiate the responses studied (Oka & Arita, 1991; Crowl *et al.*, 1991; Schalkwijk *et al.*, 1992b). These cells actively produce and release 14 kDa PLA_2 and PGE_2 when cultured, having already been exposed to the battery of mediators and cytokines produced *in vivo* (Gans *et al.*, 1989; Marshall *et al.*, 1994). Here, we expanded our previous observations (Bolognese *et al.*, 1995) and noted that while 14 kDa PLA_2 secretion plateaued at 24 h of incubation and remained steady up to 72 h, the PGE_2 release continued to accumulate reaching levels that were approximately 10 fold greater than that measured at 24 h. The possible functional role of PGE_2 in this system and its effects on TGFβ_1 regulation of PLA_2 was further investigated.

The addition of exogenous PGE_2 over the 24 h culture period resulted in a concentration-dependent suppression of the release of 14 kDa PLA_2 from elicited macrophages. This was accompanied by a reduction in cellular 14 kDa PLA_2 activity indicating an action on protein production. The concentrations of PGE_2 ($IC_{50} = 180$ ng ml^{-1}) required to inhibit PLA_2 approximated those measured in the MCM (85–140 ng PGE_2 ml^{-1}) at 24 h where the synthesis and release of 14 kDa PLA_2 plateaued. The further accumulation of PGE_2 in the MCM beyond 24 h of culture is in line with maintenance of the cessation of PLA_2 release by 24 h suggesting that PGE_2 could be exerting a negative feedback inhibition. Finally, the enhanced 14 kDa PLA_2 release by cells exposed to indomethacin over 48 h of culture further supports direct PGE_2 modulation of 14 kDa PLA_2 release.

PGE_2 has been reported to act though a G-protein coupled receptor affecting the levels of cyclic AMP in a variety of cell types (Lim *et al.*, 1983; Takenawa *et al.*, 1986). Gene transcription can be initiated through interaction of cyclic AMP with the cyclic AMP responsive element binding (CREB) protein (Spaulding, 1993). The possibility that PGE_2 acts though this mechanism suppressing gene transactivation and the subsequent formation of 14 kDa PLA_2 message cannot be ruled out. Forskolin did not mimic the PGE_2 inhibition suggesting that PGE_2 is not acting through the direct upregulation of adenylate cyclase. This is not unusual, since similar examples exist such as a recent report which showed that PGE_2 down regulation of nitric oxide synthase in mesangial cells was not mediated through the activation of adenylate cyclase (Tetsuka *et al.*, 1994). The exact mode of action of PGE_2 on 14 kDa PLA_2 formation and release remains to be elucidated.

TGFβ_1 is involved in the initial recruitment and activation of macrophage chemoattractants and induces macrophage production of a variety of inflammatory cytokines (Wahl *et al.*, 1987). It is pleiotropic in that it has both immunosuppressive actions and diverse inhibitory effects and can play a role in resolution of inflammatory episodes (Wahl *et al.*, 1989). We had previously demonstrated that TGFβ_1 could induce a 50% reduction in type II 14 kDa PLA_2 formation and secretion from elicited macrophage with no effect on PGE_2 accumulation (Bolognese *et al.*, 1994). When TGFβ_1-treated elicited macrophages were co-incubated with indomethacin, the inhibitory effect of TGFβ_1 on 14 kDa PLA_2 production and secretion was abrogated. This effect was not unique to indomethacin, since other cyclo-oxygenase inhibitors acted identically and suggested that PGE_2 was needed for TGFβ_1 to exert its inhibitory action. The addition of PGE_2 reversed the effect of indomethacin on TGFβ_1-treated cells, supporting the requirement for PGE_2. We have previously shown that TGFβ_1 reduces cell-associated 14 kDa PLA_2 levels and that the 85 kDa PLA_2 was increased by TGFβ_1 (Bolognese *et al.*, 1994). We found that both of these cell-associated responses to TGFβ_1 were negated in the presence of indomethacin. Further, the addition of exogenous PGE_2 to TGFβ_1-treated cells, exposed to indomethacin, reduced cell-associated 14 kDa PLA_2 protein in a manner similar to the reduction produced by TGFβ_1 alone. This indicates an action of PGE_2 on the TGFβ_1 modulation of the 14 kDa PLA_2 enzyme and rules out an ancillary effect such as blockade of 14 kDa PLA_2 secretion.

Our studies do not support an indirect role of TGFβ_1 on 14 kDa PLA_2 such as acting through the induction of PGE_2. We were not able to demonstrate an effect of TGFβ_1 on PGE_2 levels at the 24 h time point where the TGFβ_1-induced inhibition of 14 kDa PLA_2 was exhibited, nor were we able to show an alteration in the accumulation of PGE_2 before 24 h with TGFβ_1 treatment (data not shown). The signal transduction pathway of TGFβ_1 is not fully understood but thought to be mediated, in part, through activation of a tyrosine kinase (Sporn *et al.*, 1987). There is a possibility that TGFβ_1 could influence the PGE_2-mediated signal pathway though modifications such as induction of serine/threonine kinase-mediated phosphorylation. Indeed, a report exists demonstrating that TGFβ_1 induces the phosphorylation of the CREB protein (Kramer *et al.*, 1991). Potential PGE_2-induced modification of TGFβ_1-mediated processes is also possible. Further work is needed to understand fully the interaction of PGE_2 with TGFβ_1 and its modulation of TGFβ_1 action.

Overall, the data demonstrate that PGE_2 exerts a negative feedback regulatory role on the release of 14 kDa PLA_2 by activated macrophages. Further, it appears to be essential for the effect that TGFβ_1 has on elicited macrophage PLA_2(s), both 14 and 85 kDa. The concept that prostanoids play both pro and anti-inflammatory roles is gaining more appreciation (Goodwin, 1991; Willoughby *et al.*, 1993; Weissman, 1993). These data add additional support to the idea that the use of cyclo-oxygenase inhibitors could interfere with the normal modulatory processes mediated by PGE_2 either directly or indirectly through its interaction with TGFβ_1 during ongoing inflammation.

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