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Cytokine-mediated induction of cyclo-oxygenase-2 by activation of tyrosine kinase in bovine endothelial cells stimulated by bacterial lipopolysaccharide

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1 The induction of cyclo-oxygenase-2 (COX-2) afforded by bacterial lipopolysaccharide (LPS, endotoxin) in bovine aortic endothelial cells (BAEC) is mediated by tyrosine kinase. LPS also causes the generation of several cytokines including interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). This study investigates whether endogenous IL-1β, TNF-α, EGF or PDGF contribute to the induction of COX-2 elicited by LPS in BAEC and if their action is due to activation of tyrosine kinase. Furthermore, we have studied the induction of COX-2 by exogenous cytokines.

2 Accumulation of 6-oxo-prostaglandin (PG) $F_{1\alpha}$ in cultures of BAEC was measured by radioimmunoassay at 24 h after addition of either LPS (1 μg ml^{-1}) alone or LPS together with a polyclonal antibody to one of the various cytokines. In experiments designed to measure 'COX activity', 6-oxo-$PGF_{1\alpha}$ generated by BAEC activated with recombinant human IL-1β, TNF-α, EGF or PDGF for 12 h was measured after incubation of washed cells with exogenous arachidonic acid (30 μM for 15 min). Western blot analysis determined the expression of COX-2 protein in BAEC.

3 The accumulation of 6-oxo-$PGF_{1\alpha}$ caused by LPS in BAEC was attenuated by co-incubation with one of the polyclonal antibodies, anti-IL-1β, anti-TNF-α, anti-EGF, anti-PDGF or with the IL-1 receptor antagonist, in a dose-dependent manner. Exogenous IL-1β, TNF-α or EGF also caused an increase in COX activity, while PDGF was ineffective. The increase in COX activity elicited by IL-1β (10 ng ml^{-1}), TNF-α (100 ng ml^{-1}) or EGF (1000 ng ml^{-1}) in BAEC was attenuated by erbstatin (0.005 to 5 μg ml^{-1}), as was the expression of COX-2 protein measured by Western blot analysis.

4 PDGF (10 ng ml^{-1}) significantly augmented the rise in COX activity and COX-2 protein caused by shorter incubation of BAEC with LPS (1 μg ml^{-1} for 3 h). Combination of PDGF (10 ng ml^{-1}) with a low concentration of IL-1β (1 ng ml^{-1}) for 12 h, also increased 'COX activity', but combination of PDGF and TNF-α (10 ng ml^{-1}) did not show any increased activity.

5 These results suggest that (*i*) the induction of COX activity and COX-2 protein elicited by LPS in BAEC is mediated by TNF-α with lesser contributions from PDGF, EGF or IL-1β; (*ii*) exogenous IL-1β, TNF-α or EGF alone induce COX-2 activity and protein in BAEC; (*iii*) PDGF synergizes with IL-1β, but not TNF-α, to cause expression of COX-2; and (*iv*) the induction of COX-2 protein and activity caused by these cytokines involves the activation of tyrosine kinase.

Keywords: Endotoxin; tumour necrosis factor; interleukin-1; platelet-derived growth factor; epidermal growth factor; erbstatin; cyclo-oxygenase

Introduction

Bacterial lipopolysaccharide (LPS) is a potent activator of the immune system which induces local or systemic inflammation and septic shock (Morrison & Ryan, 1979; Martich *et al.*, 1993). LPS stimulates the synthesis of arachidonic acid metabolites in a number of cell types including endothelial cells by causing the *de novo* synthesis of an 'inducible' isoform of cyclo-oxygenase (COX-2; Lee *et al.*, 1992; Mitchell *et al.*, 1993; Akarasereenont *et al.*, 1995b). The cellular mechanisms by which LPS causes the induction of COX-2 are largely unknown. Recently, we have shown that tyrosine phosphorylation is part of the signal transduction mechanism that mediates the induction of COX-2 elicited by LPS in endothelial cells (Akarasereenont *et al.*, 1994). LPS stimulates the release of a variety of inflammatory mediators including cytokines and growth factors *in vitro* and *in vivo* (Mantovani *et al.*, 1992; Hewett & Roth, 1993; see Henderson & Blake, 1992; Thiemermann, 1994). We chose four cytokines, interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), known to involve tyrosine kinase in their actions (Donato *et al.*, 1989; Ullrich & Schlessinger, 1990; Corbett *et al.*, 1993) and assessed their role in the response of BAEC to LPS. This study was designed to investigate whether or not (*i*) the release of IL-1β, TNF-α, EGF or PDGF by BAEC contributes to the induction of COX-2 caused by LPS in these cells (*ii*) exogenous IL-1β, TNF-α, EGF or PDGF cause the induction of COX-2 in BAEC and (*iii*) this effect is due to activation of tyrosine kinase. Some of these results have been communicated to the British Pharmacological Society (Akarasereenont *et al.*, 1995a).

Methods

Cell culture

Bovine aortic endothelial cells (BAEC) were obtained from fresh bovine aortae as previously described (de Nucci *et al.*, 1988) and cultured in 96-well plates (10^5 cells per well) with Dulbecco's Modified Eagle's Medium (DMEM; 200 μl/well) containing 10% foetal calf serum (Gibco) and 4 mM L-glutamine. The cells were characterized morphologically and by positive staining for von Willebrand Factor (98 ± 2%). These cells were used only between the 6th and 20th passage. To

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eliminate any possibility of contamination with other types of cells, a single BAEC was cloned by the spot technique (Doyle *et al.*, 1994) and used after the 6th passage; these cells were also positive for von Willebrand Factor (97 ± 3%). The agents, which were dissolved in distilled water or dimethylsulphoxide (DMSO final concentration less than 0.1%; v/v), were sterilized by filtration through a filter (pore size: 0.22 μm) before being added to the cells under sterile conditions. Cells were incubated at 37°C in a humidified incubator.

Measurement of the activity of cyclo-oxygenase

6-Oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) is the main COX metabolite released after activation of BAEC with LPS (1 μg ml^{-1}; Akarasereenont *et al.*, 1994b) and its accumulation was measured by radioimmunoassay (Salmon, 1978). In experiments involving cytokine antibodies, BAEC were treated with LPS together with a polyclonal antibody to either IL-1β (anti-IL-1β; 0.1, 1 or 10 μg ml^{-1}), TNF-α (anti-TNF-α; 0.1, 1 or 10 μg ml^{-1}), EGF (anti-EGF; 0.1, 1 or 10 μg ml^{-1}) or PDGF (anti-PDGF; 0.5, 5 or 10 μg ml^{-1}) for 24 h and the medium was subsequently assayed for 6-oxo-$PGF_{1\alpha}$. Identical conditions were used for assessing the effects of the recombinant human IL-1 receptor antagonist (IL-1ra; 1, 10 or 100 ng ml^{-1}).

Separate experiments were designed to measure the effects of exogenous cytokines on COX activity (in order to eliminate any effects of other enzymes involved in the arachidonic acid cascade induced or activated by cytokines). In these experiments, cells were treated with IL-1β (0.1, 1 or 10 ng ml^{-1}), TNF-α (1, 10 or 100 ng ml^{-1}), EGF (1, 10, 100 or 1000 ng ml^{-1}) or PDGF (1, 3 or 10 ng ml^{-1}) for 12 h after which time the cells were washed and fresh medium containing arachidonic acid (30 μM) was added for 15 min at 37°C. Subsequently, the supernatant was removed to measure 6-oxo-$PGF_{1\alpha}$.

To assess the involvement of protein tyrosine kinase, cells were treated with IL-1β (10 ng ml^{-1}), TNF-α (100 ng ml^{-1}) or EGF (1000 ng ml^{-1}) together with the tyrosine kinase inhibitor erbstatin (0.005, 0.05, 0.5 or 5 μg ml^{-1}; Imoto *et al.*, 1987; see also Akarasereenont *et al.*, 1994) for 12 h, after which time the cells were washed and fresh medium containing arachidonic acid (30 μM) was added for 15 min at 37°C and then assayed for 6-oxo-$PGF_{1\alpha}$. The same conditions, but with LPS stimulation only, were used to assess the effects of calphostin C (0.001, 0.01, 0.1 or 1 μg ml^{-1}; Kobayashi *et al.*, 1989) or staurosporine (0.0001, 0.001, 0.01 or 0.1 μg ml^{-1}; Tamaoki *et al.*, 1986) on COX activity.

To study the interactions of PDGF with other cytokines or LPS, BAEC were incubated with LPS (1 μg ml^{-1}) alone for 3 h. The medium was then replaced with fresh medium containing either no further addition or PDGF (10 ng ml^{-1}) alone or PDGF plus erbstatin (5 μg ml^{-1}) and incubation continued for a further 9 h, after which time COX activity was measured. BAEC were also incubated with IL-1β (1 ng ml^{-1}) alone or IL-1β with PDGF (10 ng ml^{-1}) and with TNF-α (10 ng ml^{-1}) alone or TNF-α with PDGF (10 ng ml^{-1}) for 12 h and COX activity measured.

Immunoblot (Western blot) analysis

BAEC were cultured in 6-well plates (37°C) and treated for 24 h with fresh medium alone (control) or containing LPS or LPS plus erbstatin (5 μg ml^{-1}). Similar experiments were done with IL-1β alone (10 ng ml^{-1}), TNF-α alone (100 ng ml^{-1}), EGF alone (1000 ng ml^{-1}) or with the cytokine plus erbstatin (5 μg ml^{-1}). Cells were also cultured with PDGF alone (10 ng ml^{-1}). In addition, cells were activated with LPS (1 μg ml^{-1}) for 3 h and subsequently the medium was removed and replaced with fresh medium containing PDGF (10 ng ml^{-1}) alone or PDGF (10 ng ml^{-1}) plus erbstatin (5 μg ml^{-1}) for a further 21 h. After 24 h, cells were washed with phosphate buffered saline (PBS; pH 7.4) and incubated (5 min) with 1 ml of extraction buffer (composition, mM: Tris 50, EDTA 10, Triton X-100 1% v/v, phenylmethylsulphonyl fluoride 1, pepstatin A 0.05 and leupeptin 0.2) with gentle shaking. The cell extract was then boiled (10 min) in a ratio of 1 : 1 with gel loading buffer (Tris 50 mM, SDS 10% w/v, glycerol 10% v/v, 2-mercaptoethanol 10% v/v and bromphenol blue 2 mg ml^{-1}). Samples were centrifuged at 10,000 *g* for 2 min before being loaded onto gradient gels (4–10% Tris-glycine) and subjected to electrophoresis (1 h at 125 V). The separated proteins were transferred to nitrocellulose (BIOR-AD; 1 h at 200 V). After transfer to nitrocellulose, the blot was incubated in blocking solution (dried minimal-fat milk 5% w/v and Tween-20 0.25% v/v in PBS solution) for 1 h and then primed (1 h) with a rabbit antibody raised to murine COX-2 (primary antibody, dilution 1 : 1000, Cayman Chemical Company, MI, U.S.A.; no detectable cross-reactivity with COX-1) which reacts with bovine COX-2 (Mitchell *et al.*, 1993). The blot was then incubated (1 h) with an anti-rabbit IgG developed in sheep (secondary antibody, dilution 1 : 1000, linked to alkaline phosphatase conjugate). Finally, the blot was developed (for approximately 5 min) with premixed solution containing (mM): 5-bromo-4-chloro-3-indolylphosphate (BCIP), 0.56 nitro blue tetrazolium (NBT) 0.48, Tris 10 and $MgCl_2$ 59.3 (pH 9.2). The detection limit of protein in cell extract was 1–10 ng of protein.

Measurement of the release of TNF-α from endothelial cells

BAEC or the cloned cells were cultured to confluence in 96-well plates as above and the medium replaced with fresh medium before use. Cells were incubated alone, treated with LPS (1 μg ml^{-1}) or treated with LPS plus erbstatin (5 μg ml^{-1}) for 24 h after which time the medium was removed and assayed for its content of TNF-α with ELISA Quantikine kits. The lower detection limit for TNF-α is 4.4 pg ml^{-1}.

Measurement of cell viability

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Mosmann, 1983). At the end of each experiment, cells in 96-well plates were incubated (37°C; 1 h) with MTT (0.2 mg ml^{-1}) dissolved in culture medium, after which time, the medium was removed by aspiration and cells were solubilized in DMSO (200 μl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of optical density at 650 nm (OD 650) using a Molecular Devices microplate reader (Anthos, Salzburg, Austria).

Positive controls for loss of cell viability were provided by experiments designed to determine limiting concentrations of DMSO. Cell viability was 80 ± 1% with 0.1%, 67 ± 4% with 1% and 40 ± 5% with 10% DMSO (v/v), relative to the control untreated cells over a 24 h incubation period.

Statistical analysis

Results are shown as mean ± s.e.mean from triplicate determinations (wells) from 3 separate experimental days ($n = 9$). ANOVA analysis was used to determine the significance of differences between means and P values of less than 0.05 were taken as statistically significant.

Materials

Escherichia coli lipopolysaccharide (serotype: 0111:B4), DMSO, phosphate buffered saline (PBS; pH 7.4), Trizma base, EDTA, Triton X-100, phenylmethylsulphonyl fluoride (PMSF), pepstatin A, leupeptin, glycerol, 2-mercaptoethanol, bromphenol blue, sulphanilamide, naphthylethylenediamide, phosphoric acid, sodium nitrite, sodium dodecyl sulphate (SDS), calphostin C, staurosporine, anti-rabbit IgG antibody,

goat IgG, premixed BCIP/NBT solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 6-oxo-$PGF_{1\alpha}$ and its respective antibody were supplied by Sigma Chemical Company (Poole, Dorset). [^{3}H]-6-oxo-$PGF_{1\alpha}$ was purchased from Amersham International (Little Chalfont, Bucks). Dulbecco's modified Eagle's medium (DMEM) was obtained from Flow Laboratories. L-Glutamine was obtained from B.D.H. (Dagenham, Essex) and foetal calf serum was obtained from Gibco BRL (Paisley, Renfrewshire). Recombinant human TNF-α and recombinant human EGF were purchased from Genzyme (West Malling, Kent) and recombinant human IL-1β, recombinant human PDGF and recombinant human IL-1 receptor antagonist were purchased from R&D systems (Abingdon, Berks). The cytokine antibodies, all goat IgG, anti-hTNF-α (no cross-reactivity with TNF-β and other cytokines), anti-IL-1β (no cross-reactivity with IL-1α and other cytokines), anti-hEGF (no cross-reactivity with TGF-α and other cytokines), anti-hPDGF (>10% cross-reactivity with PDGF-AA subtype, nearly 100% cross-reactivity with PDGF-AB as well as PDGF-BB subtype and no cross-reactivity with other cytokines) and the TNF-α ELISA Quantikine kits were purchased from R&D systems. Pure nitrocellulose membrane (0.45 μm) and filter paper were purchased from BIO-RAD (Hemel Hempstead, Hertfordshire). Erbstatin (erbstatin analogue) was obtained from Calbiochem Novabiochem (Nottingham, UK).

Results

Effects of antibodies to cytokines on the induction of COX activity in BAEC activated by LPS

Untreated BAEC released only small amounts of 6-oxo-$PGF_{1\alpha}$ (1–2 ng ml^{-1}) over 24 h. After exposure to LPS (1 μg ml^{-1}) for 24 h, significantly higher amounts of 6-oxo-$PGF_{1\alpha}$ were accumulated (50–60 ng ml^{-1}; Akarasereenont *et al.*, 1995b). When BAEC were treated with LPS given together with anti-IL-1β (0.1, 1 or 10 μg ml^{-1}), anti-TNF-α (0.1, 1 or10 μg ml^{-1}), anti-EGF (0.1, 1 or 10 μg ml^{-1}) or anti-PDGF (0.5, 5 or 10 μg ml^{-1}), the accumulation of 6-oxo-$PGF_{1\alpha}$ was inhibited in a dose-dependent manner with a maximum inhibition of 70±3% for anti-TNF-α, 50±5% for anti-PDGF, 35±3% for EGF and 25±5% for anti-IL-1β (all at 10 μg ml^{-1}; Figure 1).

Cloned BAEC released over 24 h only small amounts of 6-oxo-$PGF_{1\alpha}$ (0.05±0.02 ng ml^{-1}; n=9). After exposure of these cloned BAEC to LPS (1 μg ml^{-1} for 24 h), significantly higher amounts of 6-oxo-$PGF_{1\alpha}$ accumulated (52±5 ng ml^{-1}; n=9; P<0.05). The anti-TNF-α antibody (0.1, 1 or 10 μg ml^{-1}), inhibited the LPS-induced accumulation of 6-oxo-$PGF_{1\alpha}$ in a dose-dependent manner with a maximum inhibition of 75±3% for anti-TNF-α (at 10 μg ml^{-1}).

Incubation with goat IgG (10 μg ml^{-1}), the relevant control for the cytokine-antibodies used, did not affect the accumulation of 6-oxo-$PGF_{1\alpha}$ following LPS (93±3% of LPS, n=9).

The IL-1ra was also assessed as an inhibitor of LPS-induced accumulation of 6-oxo-$PGF_{1\alpha}$. Over the range of concentrations used, (1, 10 or 100 ng ml^{-1}), IL-1ra caused weak, but dose-dependent, inhibitions of 8±3%, 14±3% and 20±4%, respectively (n=9 for each dose).

Incubation of BAEC or cloned BAEC with LPS for 24 h did not affect cell viability as measured by the MTT assay (viability: 86±2% of control). In addition, anti-IL-1β, anti-TNF-α, anti-EGF or anti-PDGF (up to 10 μg ml^{-1}) did not reduce cell viability, when given either alone or in combination with LPS (viability: 83±5% of control).

Effects of exogenous IL-1β, TNF-α, EGF or PDGF on COX activity

Incubation of BAEC with IL-1β (0.1, 1 or 10 ng ml^{-1}), TNF-α (1, 10 or 100 ng ml^{-1}) or EGF (1, 10, 100 or 1000 ng ml^{-1}) for 12 h resulted in a dose-related increase in COX activity (Figure 2). By contrast, incubation of BAEC with PDGF (1, 3 or 10 ng ml^{-1}) did not result in a significant increase in COX activity (Figure 2).

Effects of the tyrosine kinase inhibitor, erbstatin, on the induction of COX activity caused by exogenous IL-1β, TNF-α or EGF

In BAEC activated with TNF-α, the increase in COX activity elicited by TNF-α was inhibited by erbstatin in a dose-depen-

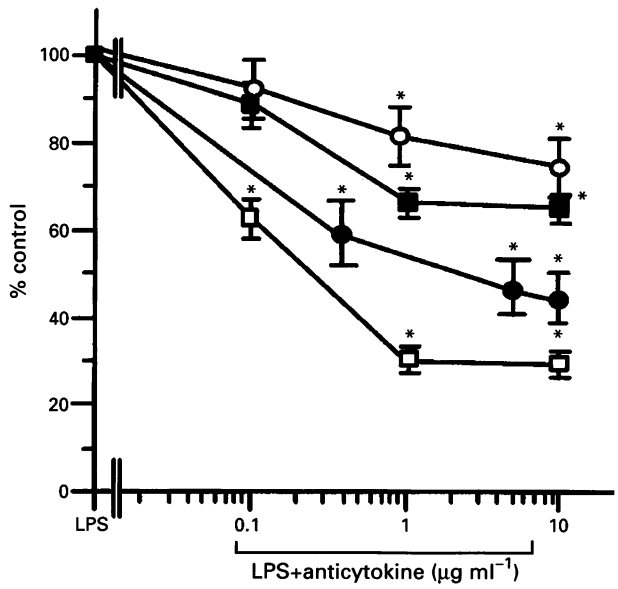

Figure 1 The effects of anti-IL-1β (○), anti-TNF-α (□), anti-EGF (■) or anti-PDGF (●) on the induction of COX in BAEC activated with LPS (1 μg ml^{-1}) measured at 24 h by the accumulation of 6-oxo-$PGF_{1\alpha}$ in the culture medium. For ease of comparison, the values for each antibody have been calculated as a percentage of the corresponding value for LPS alone. Typical values for the accumulation in control cultures were 1±0.03 ng ml^{-1} and after LPS treatment 56±7 ng ml^{-1}, n=9. Data are expressed as mean±s.e.mean from triplicate determinations (wells) from 3 separate experimental days (n=9). *P<0.05 when compared to LPS-treated cells at 24 h (LPS). For abbreviations in this and subsequent figures, see text.

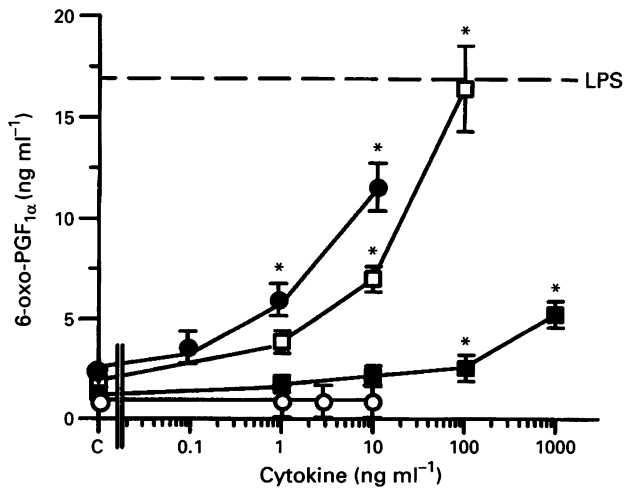

Figure 2 Dose-dependent increase of COX activity in BAEC activated with IL-1β (●), TNF-α (□), EGF (■) or PDGF (○) for 12 h measured by the formation of 6-oxo-$PGF_{1\alpha}$ in the presence of exogenous arachidonic acid (30 μM; 15 min). Data are expressed as mean±s.e. mean from triplicate determinations (wells) from 3 separate experimental days (n=9). *P<0.05 when compared to untreated cells at 12 h (C). Dashed line shows the level of COX activity in BAEC activated with LPS (1 μg ml^{-1}) for 12 h.

dent manner (Figure 3). The inhibition was first significant at a concentration of erbstatin of 0.5 μg ml^{-1}. Similarly, erbstatin caused a dose-dependent inhibition of the increase in COX activity elicited by IL-1β or EGF in BAEC (Figure 3). This inhibition was also first significant with erbstatin at 0.5 μg ml^{-1}. Calphostin C (0.001, 0.01, 0.1 or 1 μg ml^{-1}) or staurosporin (0.0001, 0.001, 0.01 or 0.1 μg ml^{-1}) did not affect increased COX activity induced by LPS ($n=9$).

Incubation of BAEC with erbstatin did not affect cell viability, when given either alone or in combination with LPS (viability: 81 ± 5% of control).

Characterization of COX isoforms present in BAEC treated with LPS, IL-1β, TNF-α, EGF or PDGF

Figure 4 shows that extracts of untreated BAEC contained no detectable COX-2 protein, as determined by Western blot analysis. In contrast, after incubation for 24 h with LPS (1 μg ml^{-1}), TNF-α (100 ng ml^{-1}), EGF (1000 ng ml^{-1}), or IL-1β (10 ng ml^{-1}), the respective cell extracts contained a protein of approximately 70 kDa, which was recognised by a specific antibody to COX-2. Incubation of BAEC with PDGF (10 ng ml^{-1}) did not increase the expression of COX-2 protein (data not shown). Moreover, the induction of COX-2 protein afforded by LPS, TNF-α, EGF or IL-1β in BAEC was prevented by erbstatin (5 μg ml^{-1}).

Effects of the combination of cytokines on COX activity and COX-2 protein expression

Experiments were designed to elucidate whether PDGF, which by itself was inactive (see above), could act synergistically with LPS, IL-1β or TNF-α to increase COX activity and protein. Treatment of BAEC with LPS (1 μg ml^{-1}) for 3 h followed by incubation with medium alone for the next 9 h did not result in a significant increase in COX activity at 12 h (Figure 5). When BAEC were activated with LPS for 3 h and the medium was replaced with fresh medium containing PDGF (10 ng ml^{-1}) for a further 9 h, a significant increase in COX activity (almost three-fold) was observed at 12 h (Figure 5). This increase in COX activity caused by PDGF after LPS was abolished by pretreatment of BAEC with erbstatin (5 μg ml^{-1}). In addition, treatment of BAEC with PDGF after LPS caused a substantial expression of COX-2 protein (Figure 4) which was greater than the one caused by LPS alone (Figure 4b; lane 5). The increase in COX-2 protein caused by PDGF after LPS was also inhibited by erbstatin (5 μg ml^{-1}; Figure 4).

In separate experiments we looked for synergism between exogenous cytokines, using PDGF with sub-maximal concentrations of other cytokines (Figure 5). Here, PDGF (10 ng ml^{-1}) given together with IL-1β for 12 h significantly enhanced the level of COX activity induced by IL-1β (1 ng ml^{-1} for 12 h) alone. In contrast, PDGF (10 ng ml^{-1}; given together with TNF-α for 12 h) did not augment the rise in COX activity afforded by TNF-α (10 ng ml^{-1}) alone.

The release of TNF-α from BAEC in culture

Untreated BAEC released only small amounts of TNF-α, as measured by ELISA (Table 1). Treatment of BAEC with LPS (1 μg ml^{-1} for 24 h) resulted in a 4 fold rise in TNF-α levels in the supernatant. This release of TNF-α caused by LPS was not inhibited by erbstatin (5 μg ml^{-1}; Table 1). Table 1 additionally shows that the cloned BAEC also released TNF-α following exposure to LPS.

Discussion

We have demonstrated the expression of COX-2 activity in three ways: (*i*) by accumulation over 24 h of 6-oxo-PGF$_{1\alpha}$, (*ii*) by the ability of the cells to convert exogenous arachidonic

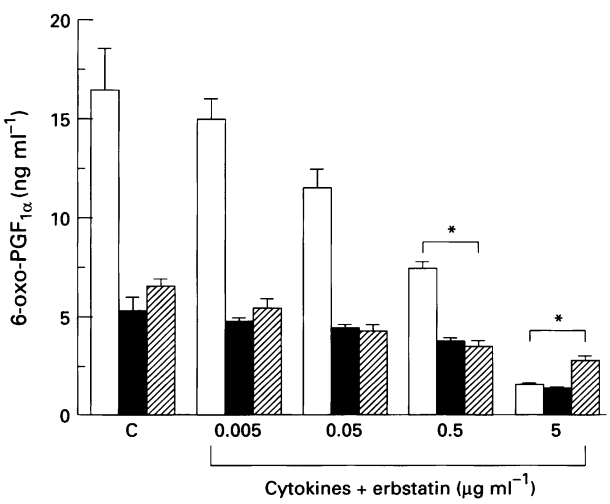

Figure 3 Dose-dependent inhibition of COX activity by erbstatin in BAEC treated with TNF-α (100 ng ml^{-1}; open columns), EGF (1000 ng ml^{-1}; solid columns) or IL-1β (10 ng ml^{-1}; hatched columns), measured at 12 h by the formation of 6-oxo-PGF$_{1\alpha}$. Values are shown for the effects of cytokines alone (C) and for cytokines with increasing concentrations of erbstatin; note that for all three cytokines, erbstatin inhibition was significant at concentrations of 0.5 μg ml^{-1} or higher. Data are expressed as mean ± s.e. mean from triplicate determinations (wells) from 3 separate experimental days ($n=9$). *$P<0.05$ when compared to cytokine alone (C).

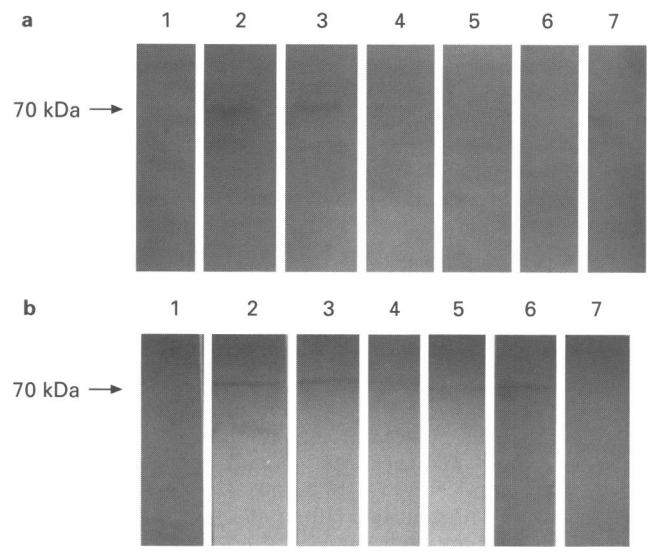

Figure 4 The figure shows Western blots using polyclonal antibodies to COX-2 of cell extracts from mitogen-treated and untreated BAEC. (a) Equal amounts of protein were loaded in all lanes (30 μg/lane). Control untreated BAEC (lane 1) contained no detectable COX-2 protein. In contrast, BAEC activated with LPS (1 μg ml^{-1} for 24 h; lane 2), TNF-α (100 ng ml^{-1} for 24 h; lane 3) or EGF (1000 ng ml^{-1} for 24 h; lane 4) contained COX-2 protein. The induction of COX-2 protein by LPS, TNF-α or EGF in BAEC was prevented by erbstatin (5 μg ml^{-1}; lane 5, 6 and 7, respectively). (b) Equal amounts of protein were loaded in all lanes (10 μg/lane). Control, untreated BAEC (lane 1) contained no detectable COX-2 protein. In contrast, BAEC activated with LPS (lane 2), IL-1β (10 ng ml^{-1} for 24 h; lane 3) contained COX-2 protein. The induction of COX-2 protein by IL-1β in BAEC was prevented by erbstatin (5 μg ml^{-1}; lane 4). Lower levels of COX-2 protein were also detected in BAEC treated with LPS for 3 h (lane 5) and these were increased after subsequent treatment with PDGF (10 ng ml^{-1} for 21 h; lane 6). This increase in COX activity by PDGF was also inhibited by erbstatin (5 μg ml^{-1}; lane 7). Similar results were obtained using cell extracts from 3 separate batches of cells.

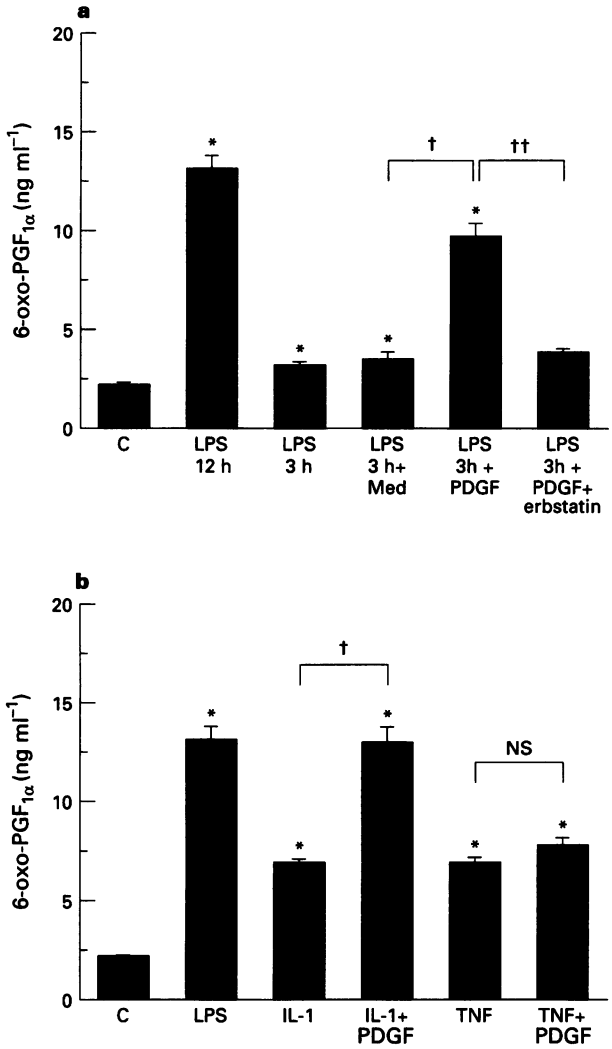

Figure 5 (a) Increase in COX activity (measured by the formation of 6-oxo-$PGF_{1\alpha}$) in BAEC treated with PDGF ($10\,ng\,ml^{-1}$ for 9 h) after prior LPS incubation ($1\,\mu g\,ml^{-1}$ for 3 h); this increase was inhibited by erbstatin ($5\,\mu g\,ml^{-1}$; together with PDGF). *$P<0.05$ when compared to untreated cells at 12 h (C); †$P<0.05$ when compared to LPS-treated cells at 3 h and containing medium alone for a further 9 h incubation; ††$P<0.05$ when compared to LPS treated cells at 3 h and containing PDGF alone for further 9 h. (b) Synergistic increase in COX activity (measured by the formation of 6-oxo-$PGF_{1\alpha}$ in BAEC treated with IL-1β ($1\,ng\,ml^{-1}$; 12 h) plus PDGF ($10\,ng\,ml^{-1}$; together with IL-1β) when compared to BAEC treated with IL-1β alone. In contrast, BAEC treated with TNF-α ($10\,ng\,ml^{-1}$; 12 h) plus PDGF ($10\,ng\,ml^{-1}$; together with TNF-α) did not increase COX activity when compared to BAEC treated with TNF-α alone. *$P<0.05$ when compared to untreated cells at 12 h (C); †$P<0.05$ when compared to IL-1β treated cells at 12 h. Data are expressed as mean ± s.e. mean from triplicate determinations (wells) from 3 separate experimental days ($n=9$).

acid and (*iii*) by Western blot analysis of the COX-2 protein. We shall refer to all these concordant results as an increase in COX-2 activity. Pretreatment of BAEC with polyclonal antibodies to TNF-α, IL-1β, EGF or PDGF attenuated the increase of COX-2 activity caused by LPS in BAEC. In addition, exogenous TNF-α, IL-1β or EGF, but not PDGF, enhanced COX-2 activity. These effects were largely attenuated by the tyrosine kinase inhibitor, erbstatin. Although incubation with PDGF alone did not increase COX-2 activity, PDGF acted synergistically with IL-1β or LPS, but not with TNF-α, in enhancing COX-2 activity. Thus, the induction of COX-2 afforded by LPS in BAEC is mediated by endogenous TNF-α and to a lesser extent by PDGF, EGF and IL-1β and is due to activation of tyrosine kinase by these cytokines.

We undertook these experiments as a result of our earlier work (Akarasereenont *et al.*, 1994) demonstrating that tyrosine kinase was involved in the increased output of prostaglandins from BAEC following exposure to LPS. We inferred from those results that cytokines acting through a tyrosine kinase were the mediators of the expression of COX-2 caused by LPS in endothelial cells. A variety of cells secrete a number of cytokines in response to any given stimulus (Mantovani *et al.*, 1992; Henderson & Blake, 1992) and the complexity of the cytokine system is compounded by the possibilities of interactions between cytokines (Pober & Cotran, 1990; Floege *et al.*, 1990). We chose to study four cytokines which either are secreted by endothelial cells or are known to activate tyrosine kinase, namely TNF-α, EGF, IL-1β and PDGF (Libby *et al.*, 1986; Ullrich & Schlessinger, 1990; Pober & Cotran, 1990; Kohno *et al.*, 1990).

Our experiments with selective antibodies to these cytokines showed clearly that TNF-α played the most important role in mediating the induction of COX-2 afforded by LPS, anti-TNF-α producing about 70% inhibition. Although polyclonal antibodies to PDGF, EGF or IL-1β also attenuated the rise in COX-2 activity caused by LPS, their effects were less pronounced. Exogenous cytokines incubated with the cells in the absence of LPS were also active in inducing COX-2 with TNF-α almost 100 fold more potent than EGF. These results were not unexpected, for TNF-α mediates most of the consequences of LPS in animals and man, including induction of COX-2 (Wheeler *et al.*, 1992; Fletcher, 1993; Hewett & Roth, 1993; Tracey & Cerami, 1993). Furthermore, EGF increases COX activity in human endothelial cells (Kawakami *et al.*, 1986; Ristimäki *et al.*, 1988; Zavoico *et al.*, 1989).

We found that exogenous IL-1β was very effective in inducing COX-2 activity in BAEC, as it is in human cells (Endo *et al.*, 1988; Maier *et al.*, 1990a). However, the comparatively weak inhibition by IL-1β antibody of the increase of COX activity caused by LPS in BAEC suggests that this cytokine was not secreted in adequate amounts following LPS. There are at least two other explanations for the discrepancy. Firstly, it is possible that the antibody we used, to human IL-1β, did not cross-react fully with bovine IL-1. However, this is less likely, for the receptors on the BAEC appeared to recognize the exogenous human IL-1β adequately. Secondly, it is conceivable that LPS stimulates the formation of IL-1β, but this

Table 1 The release of immunoreactive tumour necrosis factor-α (TNF-α) in the medium of cultured endothelial cells treated with lipopolysaccharide (LPS, 1 $\mu g\ ml^{-1}$) alone or LPS plus erbstatin (5 $\mu g\ ml^{-1}$) at 24 h incubation

Bovine aortic endothelial cells (BAEC)	*TNF-α (pg ml^{-1})*
Untreated	10.4 ± 0.4
With LPS (1 $\mu g\ ml^{-1}$)	37.4 ± 0.8
With LPS (1 $\mu g\ ml^{-1}$) plus erbstatin (5 $\mu g\ ml^{-1}$)	39.1 ± 0.7
Cloned	< 4.4
Cloned with LPS (1 $\mu g\ ml^{-1}$)	122.0 ± 1.2
Cloned with LPS (1 $\mu g\ ml^{-1}$) plus erbstatin (5 $\mu g\ ml^{-1}$)	124.4 ± 1.4

Data are expressed as mean ± s.e.mean from triplicate determinations (wells) from 3 separate experimental days ($n=9$).

cytokine could be functioning either intracellularly (Maier *et al.*, 1990b) or bound tightly to its membrane receptors (Kurt-Jones *et al.*, 1987). In either of these locations the IL-1β may be less accessible to the antibody.

Surprisingly, exogenous PDGF was completely inactive in inducing COX-2 in contrast to the clear inhibitory effects of anti-PDGF on the expression of COX-2 by LPS. Others have also found that PDGF increased COX activity and COX-2 protein in several cell types (Lin *et al.*, 1989; Floege *et al.*, 1990; Pomerantz *et al.*, 1993). One explanation of the lack of activity of exogenous PDGF in our model is that this cytokine needs another factor to be present in order to induce COX-2. This additional factor is neither normally present in our BAEC cultures, nor is it induced in BAEC by PDGF, but it is released by LPS. Synergy or co-operation between cytokines has been frequently reported (Pober & Cotran, 1990). For instance, IL-1β potentiates many fold the stimulation of PGI_2 induced by PDGF in human mesangial cells (Floege *et al.*, 1990). In our BAEC cultures such synergism was clearly possible between PDGF and IL-1β. It is less likely with LPS as a stimulus, for the IL-1β antibody should have reduced markedly the overall effect if PDGF were a major mediator only through synergism with IL-1β. The lack of synergism between TNF-α and PDGF demonstrates some divergence between IL-1β and TNF-α, cytokines which frequently exert common effects (Pober & Cotran, 1990), and that TNF-α and PDGF act independently in changing COX levels in BAEC although both pathways involve tyrosine kinase.

For both EGF and PDGF, tyrosine kinase activity is linked to the receptors (Ullrich & Schlessinger, 1990) and although TNF-α can in tumour cells activate the tyrosine kinase of the EGF receptor (Donato *et al.*, 1989), it is clear from the relative activities of EGF and PDGF that activation of the receptor-linked tyrosine kinase *per se* is not crucial for the induction of COX-2. In monocytes, LPS directly activates a tyrosine kinase linked to its membrane-bound CD14 receptor (Wright *et al.*, 1990). However, a direct activation of this tyrosine kinase by LPS cannot explain our results in endothelial cells, for these cells lack the membrane-bound CD14 receptor (Pugin *et al.*, 1993; Goldblum *et al.*, 1994). From the assay of immuno-reactive TNF-α in cell supernatants after treatment with LPS it is clear that although the tyrosine kinase inhibitor, erbstatin, totally prevented the increase in COX-2 activity (Akarasereenont *et al.*, 1994), it did not prevent TNF-α secretion. This finding together with the efficacy of erbstatin against all exogenous cytokines tested (TNF-α, EGF, IL-1β and PDGF after LPS), would imply that the protein tyrosine phosphorylation was common to all effective stimuli, subsequent to secretion of the cytokines. This scheme might be more likely to involve a common group of tyrosine kinases rather than those more specifically associated with each cytokine or its receptor. One possible candidate is the src family of tyrosine kinases as the viral enzyme, pp60-v-src, is known to induce COX-2 protein (Xie *et al.*, 1991). The crucial substrate for tyrosine kinase is also unknown, although in human endothelial cells, the LPS-induced activation of the nuclear transcription factor, NF-κB, is decreased by inhibitors of tyrosine kinase (Read *et al.*, 1993), as is the IL-1 induced activation of NF-κB in lymphoid cells (Iwasaki *et al.*, 1992). For both these stimuli, activation of NF-κB actually represents phosphorylation of the corresponding inhibitor protein, I-κB (Cordle *et al.*, 1993). The promoter region of the gene for COX-2 contains elements responsive to NF-κB (Kosaka *et al.*, 1994).

Which of the four cytokines we have used are the most likely mediators of the effects of LPS in our model? Three of them, IL-1β, PDGF and EGF, are either known to be present in, or secreted from, endothelial cells following LPS or IL-1β (Libby *et al.*, 1986; Hajjar *et al.*, 1987; Suzuki *et al.*, 1989; Albelda *et al.*, 1989; Pusztai *et al.*, 1993; Hewett & Roth, 1993; Tracey & Cerami, 1993; Wilson *et al.*, 1994). However, Pober & Cotran (1990) in their review state that there is no convincing demonstration of TNF release from endothelial cells and neither Libby *et al.* (1986) nor Palkama *et al.* (1993) were able to show TNF release from endothelial cells. Clearly, this cytokine was produced by our monoculture of endothelial cells on stimulation by LPS. Although the BAEC were derived from a primary culture which could be contaminated by macrophages or monocytes (the normal source of TNF-α), the endothelial cells used in these experiments were passaged at least six times before use. This we would argue, as did Libby *et al.* (1986), decreases considerably the possibility of such contamination. Furthermore, LPS under identical conditions strongly induces NO synthase activity in macrophages (Salvemini *et al.*, 1993; Akarasereenont *et al.*, 1994; 1995b), while the release of nitrite from our BAEC cultures activated with LPS was less than 5% of that observed in LPS-activated macrophages (Akarasereenont *et al.*, 1995b). Finally in a clone of endothelial cells, the antibody to TNF-α was as effective as it was in the standard BAEC cultures, in inhibiting the increase in COX-2 activity elicited by LPS. This clone also released immunoreactive TNF-α following LPS.

Thus, the increase in COX-2 activity caused by LPS in BAEC is mediated by cytokines released by LPS from the same cells. The major mediator is TNF-α, with PDGF, EGF and IL-1β making successively lesser contributions. As already noted for LPS, the effects of these individual cytokines involve a tyrosine kinase, sensitive to inhibition with erbstatin. The induction of NO synthase caused by LPS in aortic smooth muscle cells or in J774.2 macrophages is also susceptible to inhibitors of tyrosine kinase (Marczin *et al.*, 1993; Akarasereenont *et al.*, 1994). The essential part played by tyrosine kinase in the induction of both these enzymes, critical components of the response to LPS *in vivo* (see Thiemermann, 1994), suggest that the new generation of tyrosine kinase inhibitors (Levitzki *et al.*, 1992) may have real therapeutic potential in septic shock (Novogrodsky *et al.*, 1994) and other pathological conditions which are associated with the induction of COX-2 and/or NO synthase.

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