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Activation of macrophage peroxisome proliferator-activated receptor-γ by diclofenac results in the induction of cyclooxygenase-2 protein and the synthesis of anti-inflammatory cytokines

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Abstract Cyclooxygenase-2 (COX-2) is an inducible isoform of the COX family of enzymes central to the synthesis of pro-inflammatory prostaglandins. Induction of COX-2 is mediated by many endogenous and exogenous molecules that include pro-inflammatory cytokines and bacterial lipopolysaccharide (LPS). It has been demonstrated that COX-2 can also be induced by diclofenac in cultured J774.2 macrophages. This induction was delayed compared to COX-2 induced by LPS and paracetamol selectively inhibited activity of this protein. The aim of the present study was to determine the transcription factor involved in the production of COX-2 after treatment of J774.2 cells with 500 μM diclofenac. Pre-treatment of cells with the peroxisome proliferator-activated receptor-γ (PPAR-γ) antagonists GW9662 (0.1–1 μM) or biphenol A Diglycidyl Ether (100–200 μM) resulted in reduction of the induction of COX-2 by diclofenac, but not by LPS. Induction of COX-2 by the PPAR-γ agonist 15deoxy$\Delta^{12,14}$prostaglandin J_2 was also reduced when the cells were pre-treated with the PPAR-γ antagonists BADGE or GW9662. On the other hand, pre-treatment of cells with the nuclear factor-kappa-B (NF-κB) Super-repressor IκBα (150–600 nM) reduced the induction of COX-2 by LPS, but not by diclofenac. We, therefore, have identified that PPAR-γ activation is a requirement for COX-2 induction after diclofenac stimulation of J774.2 cells. These results along with the finding that treatment of J774.2 macrophages with diclofenac resulted in the release of the anti-inflammatory cytokines, interleukin-10 and transforming growth factor-β suggest that the diclofenac-induced COX-2 protein may possess anti-inflammatory actions.

Keywords Cyclooxygenase-2 · Diclofenac · J774.2 · Lipopolysaccharide · Paracetamol · Peroxisome proliferator-activated receptor-γ

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Introduction

Cyclooxygenase (COX) enzymes catalyse the rate-limiting step in the synthesis of the prostanoids by metabolism of arachidonic acid. COX-1 was shown to be involved in the synthesis of prostanoids under physiological conditions and is expressed constitutively, whereas COX-2 discovered in 1991 was shown to be an inducible isoform [1] and to be involved in prostanoid biosynthesis in pathological conditions such as inflammation [2]. COX-2 was shown to be induced by various cytokines such as interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) [3] and various growth factors such as epidermal growth factor (EGF) [4]. Additionally, the products of infectious agents, such as the

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bacterial product lipopolysaccharide (LPS), induce COX-2 [5].

Induction of COX-2 at inflamed sites was demonstrated to be associated with an increase in the release of prostaglandin E_2 (PGE_2). Selective COX-2 inhibitors were shown to be effective anti-inflammatory drugs [6, 7], which potently reduced synthesis of pro-inflammatory prostaglandins locally [8, 9]. Recently, however, Gilroy et al. (1999) demonstrated that the induction of COX-2 protein during the later resolving phase of pleural inflammation in rats is associated with an increased release of prostaglandin D_2 (PGD_2) and its metabolite 15deoxy$\Delta^{12,14}$prostaglandin J_2 (15d-PGJ_2). Pharmacological inhibition of this resolving phase enzyme with COX-2 selective agents was shown to delay resolution of the inflammatory reaction, hence it was proposed that COX-2 contributes to the synthesis of anti-inflammatory prostaglandins [10].

Initially, induction of COX-2 protein was demonstrated to be mediated by the activation of the transcription factor nuclear factor-kappa-B (NFκB). However, recently it has been demonstrated that the transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) can also signal the induction of COX-2 protein in various cell lines including hepatocytes [11, 12], macrophages [12] and monocytes [13]. PPAR-γ is a member of the steroid nuclear receptor superfamily [14], of which three subtypes have been identified, namely PPARα, PPAR-β and PPAR-γ. When these receptors are activated, they form heterodimers with the retinoid-X receptor, which then bind to the PPAR response element (PPRE) on the promoter region of their target genes [15].

Simmons et al. [16] demonstrated that treatment of the J774.2 macrophage cell line with suprapharmacological concentrations of the non-steroidal anti-inflammatory drug (NSAID) diclofenac for 48 h resulted in the induction of a COX-2 immunoreactive protein, as opposed to 12 h induction with LPS. NSAIDs were more potent inhibitors of the LPS-induced COX-2 activity than of the diclofenac-induced COX-2 activity. On the other hand, paracetamol inhibited the diclofenac-induced COX-2 activity, but not the LPS-induced COX-2 activity [16].

The aim of the present study was to identify the transcription factor(s) involved in the production of COX-2 immunoreactive protein induced by diclofenac, which would help to predict the possible functions of this protein.

Materials and methods

Materials

LPS (serotype 0111:B4), paracetamol (4-acetamidophenol) and diclofenac (2-[2,6-dichlorophenyl)amino]benzeneacetic acid sodium salt) were purchased from Sigma, Poole, Dorset, UK. Polyclonal anti-COX-2 antibody, COX-2 electrophoresis standard protein and biphenol A Diglycidyl Ether (BADGE) were purchased from Cayman Chemicals, Ann Arbor, USA. Interleukin-10 (IL-10), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) ELISA kits were purchased from BD Biosciences, Cowley, Oxford, UK. Transforming growth factor-β (TGF-β) ELISA kit was purchased from Biosource, CA, USA. Percoll and PGE_2 enzyme immunoassay kits were purchased from GE Healthcare, Little Chalfont, Buckinghamshire, UK. Super-repressor IκBα (srIκBα) was a kind gift from Dr. Panos Kabouridis and Dr. Nathan Blackwell from the Bone and Joint Research Unit, William Harvey Research Institute, London. GW9662 (2-chloro-5-nitrobenzanilide) was a kind gift from Dr. David Bishop-Bailey from the Cardiac, Vascular and Inflammation Research Centre at the William Harvey Research Institute, London.

J774.2 macrophage cell culture

J774.2 murine macrophages (European Collection of Animal Cell Cultures, Salisbury, UK), were derived from a colonic tumour in BALB/c mice in 1968 [17]. They were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM), containing 10% foetal calf serum and 100 μg/ml gentamycin antibiotic, under standard culture conditions (37°C, 5% CO_2 and 95% air) and fed every 2 days.

Isolation of peritoneal macrophages

Naïve peritoneal macrophages were isolated from Wistar rats for stimulation in vitro. Peritoneal lavages were carried out with 50 ml of Dulbecco's phosphate buffered saline (DPBS). To 15 ml of 70% percoll (diluted in DMEM), 35 ml of peritoneal lavage was added slowly and then centrifuged at 400×*g* for 20 min at room temperature. The interface formed was carefully collected and then diluted in DPBS and centrifuged at 860×*g* for 5 min at room temperature and the supernatant discarded to remove excess percoll.

The macrophage cell pellet formed was re-suspended in complete DMEM and placed in standard cell culture conditions. The cells were given 30 min to become adherent and were then stimulated as described below. The formation of a red cell pellet after final centrifugation indicated the presence of erythrocytes. These cells were lysed by re-suspending the cell pellet in 9 ml of sterile water and 30 s later adding 1 ml of piperazine *N*,*N*′-bis(2-ethanesulphonic acid) (PIPES) followed by centrifugation at 860×*g* for 5 min at room temperature before re-suspension in DMEM as above.

Stimulation of cells to induce COX-2 protein

J774.2 macrophages were stimulated with 1 μg/ml LPS for 24 h (serotype 0111:B4) or 16 μM 15d-PGJ_2 or 500 μM diclofenac for 48 h to induce COX-2 protein. The cells were then scraped free and centrifuged at $860\times g$ for 10 min at 4°C and the cell pellet stored at −80°C.

In experiments where the effect of PPAR-γ antagonism or NF-κB inhibition on the induction of COX-2 protein by LPS or diclofenac was tested, J774.2 cells were pre-treated for 1 h with either BADGE (100–120 μM) or GW9662 (0.1–1 μM) to antagonise PPAR-γ or srIκBα (150–600 nM) to inhibit NF-κB. This was followed by stimulation with LPS for 24 h or diclofenac for 48 h. In addition, the effect of pre-treatment with BADGE (100–200 μM) or GW9662 (1–4 μM) on the induction of COX-2 protein by 16 μM 15d-PGJ_2 for 48 h was tested. Finally, the effect of pre-treatment of rat primary peritoneal macrophages with BADGE (100 and 200 μM) for 1 h on the induction of COX-2 protein by LPS (24 h) or diclofenac (48 h) was tested.

The activity of COX-2 protein induced by LPS or diclofenac was determined by measurement of the levels of PGE_2. The cells were stimulated with LPS or diclofenac as described above. At the end of the stimulation period the cells were washed with serum-free DMEM for 10 min and then 30 μM arachidonic acid in DMEM was added and incubated for 15 min at 37°C. The cells were then scraped and collected for protein concentration determination, whilst the culture medium was collected for measurement of prostaglandins by enzyme immunoassay (EIA). For inhibition studies, paracetamol (0.01–1,000 μM) was added for 30 min after aspirating the serum-free wash medium and prior to the addition of arachidonic acid for 15 min.

Western blotting

The protein concentration in cell pellets was determined using the method developed by Bradford [18]. Briefly, cells were sonicated for 20 s after re-constitution in 200 μl of protease inhibitory cocktail containing 4-(2-aminoethyl)benzenesulphonylflouride (AEBSF, 104 mM), aprotinin (0.08 mM), leupeptin (2.1 mM), bestatin (3.6 mM), pepstatin A (1.5 mM) and E-64 (1.4 mM), in 50 mM Tris buffer (pH 7.4). The protein concentration was measured using the Bradford reagent (Bio-Rad, Hemel Hempstead, Hertfordshire, UK).

Using the Bio-Rad mini protean II gel electrophoresis system, 15 μg of sample or 10 μg of COX-2 standard protein were electophoresed in 10% sodium dodecyl sulphate-polyacrylamide gels. The proteins were separated electrophoretically at 180 V and then transferred onto nitrocellulose membranes electrophoretically at 100 V for 1 h. The blots were blocked with 5% (w/v) non-fat milk and 0.2% (w/v) bovine serum albumin (BSA; essentially IgG free) in Tris buffered saline containing 0.1% (v/v) Triton X100 (TTBS) overnight at 4°C. The membranes were then washed for 20 min in TTBS and incubated with polyclonal anti-COX-2 (2 μg/ml) antibody at room temperature for 1 h, washed again and incubated with 0.2 μg/ml of goat anti-rabbit horseradish peroxidase conjugated secondary antibody. The blots were finally washed and developed on X-ray films using ECL plus reagent (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Prostaglandin E_2 enzyme immunoassay kit

The levels of PGE_2 in the culture medium collected from J774.2 macrophages stimulated with LPS or diclofenac were determined using a commercial enzyme immunoassay kit from GE Healthcare. The kit was used according to the manufacture's protocol. Culture medium was incubated on a goat anti-mouse IgG-coated plate along with anti-PGE_2 antibody and horseradish peroxidase-labelled PGE_2. The blue colour developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was read colorimetrically at 630 nm. The concentration of PGE_2 in the samples was determined by comparing the optical density of PGE_2 in the samples with a PGE_2 standard curve (0.05–6.4 ng/ml).

Cytokine enzyme-linked immunosorbant assays (ELISAs)

TNF-α, IL-10 and IL-6 determinations were carried out using ELISA kits performed according to the manufacturer's instructions. A 96-well plate was coated with 100 μl of capture antibody overnight at 4°C. The plate was aspirated and washed three times with wash buffer. The plate was then blocked with 200 μl of assay diluent (10% foetal calf serum in phosphate buffered saline) and incubated for 1 h at room temperature. After washing, one hundred microliters of cell culture supernatant, diluted as necessary, in assay diluent and standard (1,000–31.3 pg/ml) were added to the plate and incubated for 2 h at room temperature. After washing the plate five times, 100 μl of detection antibody and avidin-horseradish peroxidase conjugate (diluted 1:125) was added to each well and incubated for 1 h at room temperature. After further washes, the last incubation containing 100 μl of substrate solution was left for 30 min at room temperature and in the dark to develop the plate. At the end of this incubation period, 50 μl of stop solution (1 M sulphuric acid) was added and the plate read in a plate reader at 450 nm.

TGF-β cytoset antibody pair ELISA was also performed according to the manufacturer's instructions. One 96-well plate was coated with 100 μl of 1 μg/ml coating antibody in coating buffer and incubated for 18 h at 4°C. The plate was then washed with wash buffer and blocked for 2 h at

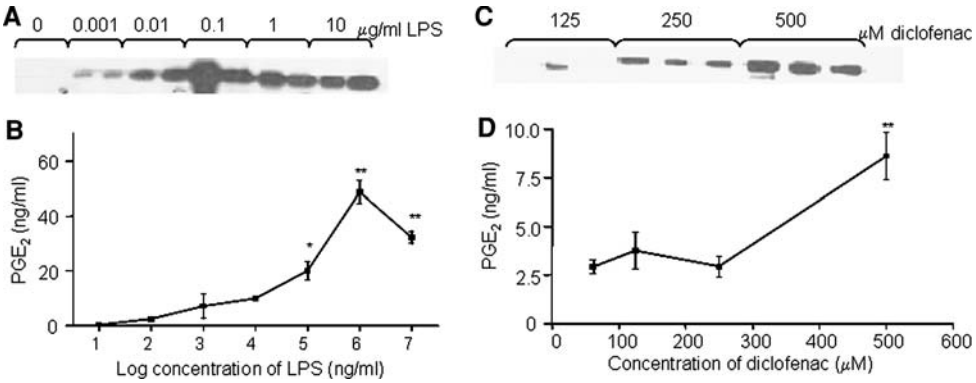

Fig. 1 Concentration response to LPS (**a**) and diclofenac (**c**) on the induction of COX-2 protein after 24 and 48 h, respectively, and on PGE_2 release by LPS (**b**) and diclofenac (**d**) in J774.2 macrophages. $*P < 0.05$, $**P < 0.01$. Data represent mean ± SEM. $n = 3$

room temperature with 300 µl blocking solution (the same buffer used for coating but containing 0.5% w/v bovine serum albumin, pH 7.4). After three washes, seven standards (4,000–75 pg/ml) and diluted samples (diluted in assay buffer consisting of coating buffer with 0.5% w/v bovine serum albumin and 0.1% v/v Tween 20, pH 7.4) were added at 100 µl/well. This was followed immediately by the addition of 50 µl of biotinylated antibody (1 µg/ml in assay buffer) and incubation for 2 h at room temperature with continual shaking. Prior to addition to the plate, 500 µl of sample was treated with 20 µl of 1 N HCl to adjust to pH 3, incubated for 15 min at room temperature and then neutralised with 15 µl of 1 N NaOH. The plate was then washed three times and incubated with 100 µl of streptavidin-HRP (diluted 1:2,500 in assay buffer) for 30 min at room temperature with continual shaking. The plate was then developed with 100 µl TMB solution for 30 min. Finally, the plate was read spectrophotometrically at an absorbance of 450 nm.

Statistical analysis

The results were expressed and presented graphically as means ± standard error of the mean (SEM). The results were statistically analysed using ANOVA followed by post-hoc Dunnett's test to compare the vehicle with drug treatment groups. Any P value ≤ 0.05 was considered to be statistically significant.

Results

Comparison of the concentration and time-profiles of the expression and activity of the LPS and diclofenac-induced COX-2 proteins in J774.2 macrophages

The effect of different concentrations of LPS on the induction of COX-2 protein in J774.2 macrophages after 24 h incubation was estimated (Fig. 1a). This increase in COX-2 protein was coupled to increased biosynthesis of PGE_2 and its release into the culture medium (Fig. 1b). It was found that maximal protein induction as well as PGE_2 release occurred with 1 µg/ml of LPS, therefore, this concentration of LPS was used in subsequent experiments for comparison with the diclofenac induction of COX-2.

Diclofenac after a 48 h incubation, produced a concentration dependent increase in COX-2 protein induction (Fig. 1c), which was also coupled to increased PGE_2 release (Fig. 1d). Therefore, as 500 µM diclofenac was found to cause maximal induction of COX-2 coupled with a high level of PGE_2 production, this concentration was used in subsequent experiments.

The time-profiles for COX-2 protein induction by 1 µg/ml LPS and 500 µM diclofenac were compared. COX-2 protein was induced by LPS from 6 h, became more pronounced at 9 h and continued to increase thereafter until 48 h (Fig. 2, top panel). On the other hand, the diclofenac-induced COX-2 protein appeared between 24 and 48 h (Fig. 2, bottom panel).

Comparison of the inhibitory effect of paracetamol on the LPS and diclofenac-induced COX-2 proteins in J774.2 macrophages

The inhibitory effect of paracetamol on the enzymatic activity of the LPS and diclofenac-induced COX-2 activities was examined by measurement of PGE_2 levels in the culture medium after addition of exogenous arachidonic acid. Both LPS and diclofenac increased PGE_2 release significantly from stimulated cells in comparison to

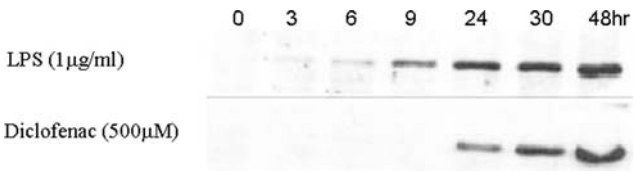

Fig. 2 Time-course of induction of COX-2 by 1 µg/ml LPS (*top panel*) and 500 µM diclofenac (*bottom panel*) in J774.2 macrophages

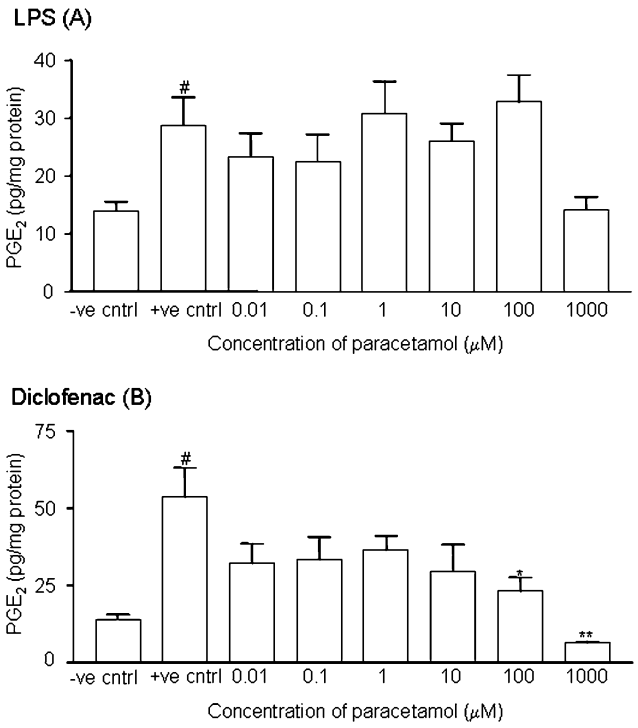

Fig. 3 Concentration response to the inhibitory effect of paracetamol (0.01–1,000 μM) on the COX-2 activity induced by 0.1 μg/ml LPS for 24 h (**a**) or 500 μM diclofenac for 48 h (**b**) in J774.2 macrophages. Data represent mean ± SEM. Panel **a**, $^{\#}P < 0.05$ for LPS stimulation (+ve control) compared to the unstimulated cells (−ve control). Panel **b**, $^{\#}P < 0.01$ for diclofenac stimulation (+ve control) compared to the unstimulated cells (−ve control); $^{*}P < 0.05$; $^{**}P < 0.01$ for 100 and 1,000 μM paracetamol treatment, respectively, compared to stimulated cells (+ve control) $n = 6$

unstimulated cells. Paracetamol failed to reduce the levels of PGE_2 except for a non-significant reduction at a concentration of 1,000 μM in the LPS-induced COX-2 activity (Fig. 3a). On the other hand, paracetamol produced a significant reduction in the levels of PGE_2 ($P < 0.05$, ANOVA) with a more pronounced reduction with 100 and 1,000 μM paracetamol ($P < 0.05$ and $P < 0.01$, respectively; Dunnett's post-hoc test) in the diclofenac stimulated J774.2 cells (Fig. 3b).

The role of PPAR-γ in the induction of COX-2 protein by LPS, diclofenac and 15d-PGJ_2 in J774.2 macrophages

The effect of two PPAR-γ antagonists, GW9662 and BADGE, on the induction of COX-2 protein by diclofenac or LPS in J774.2 macrophages was tested. The cells were pre-treated with either of the two antagonists for 1 h and then stimulated with either LPS (1 μg/ml) for 24 h or diclofenac (500 μM) for 48 h. The PPAR-γ antagonists were used at concentrations above and below their reported IC_{50} values for antagonism of PPAR-γ [19, 20].

GW9662 at concentrations between 0.1 and 1 μM failed to reduce the induction of COX-2 protein induced by LPS (Fig. 4, top panel). However, the induction of COX-2 protein by diclofenac was clearly reduced by pre-treatment of the cells with GW9662 (Fig. 4, bottom panel).

BADGE had a similar effect to GW9662 on COX-2 induction in J774.2 macrophages as no effect was seen in LPS stimulated cells (Fig. 5, top panel), whereas the induction of COX-2 protein by diclofenac was concentration dependent reduced when the cells were pre-treated with BADGE at concentrations between 100 and 120 μM (Fig. 5, bottom panel).

15d-PGJ_2 has been identified as an endogenous agonist of the PPAR-γ receptor [21]. Stimulation of J774.2 macrophages with 16 μM 15d-PGJ_2 for 48 h resulted in the induction of COX-2 protein. This induction was reduced by pre-treatment of the cells for 1 h with 1–4 μM GW9662 (Fig. 6, top panel) as well as with BADGE at concentrations of 100 and 200 μM (Fig. 6, bottom panel).

The role of NF-κB in the induction of COX-2 protein by LPS and diclofenac in J774.2 macrophages

The pre-treatment of J774.2 macrophages for 1 h with the NF-κB inhibitor srIκBα (150–600 nM), resulted in

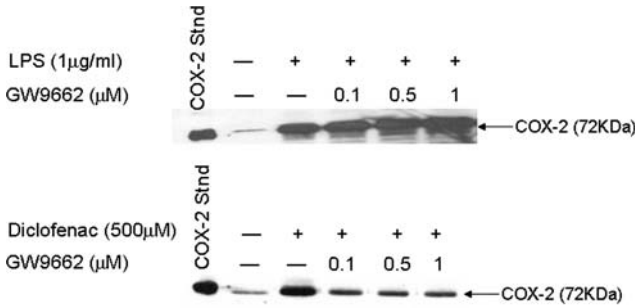

Fig. 4 The effect of pre-treatment of cultured J774.2 macrophages with the PPAR-γ antagonist GW9662 for 1 h on the induction of COX-2 protein by either 1 μg/ml LPS after 24 h (*top panel*) or 500 μM diclofenac after 48 h (*bottom panel*)

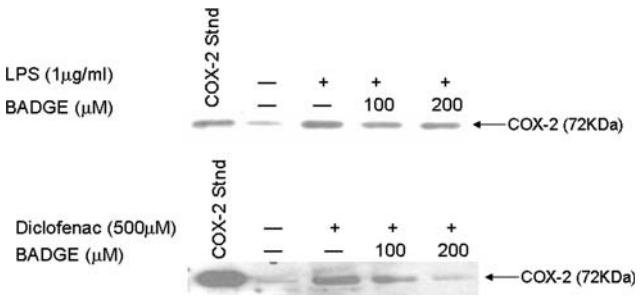

Fig. 5 The effect of pre-treatment of cultured J774.2 macrophages with the PPAR-γ antagonist BADGE for 1 h on the induction of COX-2 protein by either 1 μg/ml LPS after 24 h (*top panel*) or 500 μM diclofenac after 48 h (*bottom panel*)

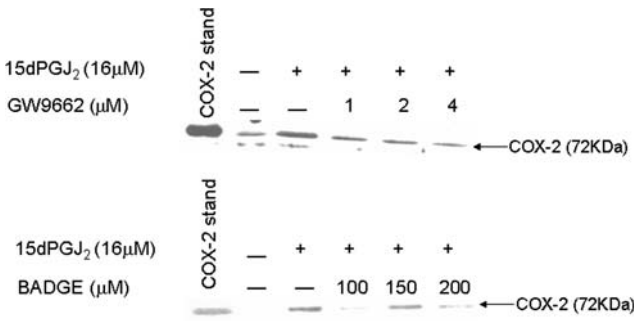

Fig. 6 The effect of pre-treatment of cultured J774.2 macrophages with the PPAR-γ antagonists GW9662 (*top panel*) or BADGE (*bottom panel*) for 1 h on the induction of COX-2 protein by 16 μM 15-deoxy-PGJ_2 after 48 h

complete loss of the expression of COX-2 protein induced by 1 μg/ml LPS at concentrations of srIκBα between 300 and 600 nM (Fig. 7, top panel). On the other hand, the expression of COX-2 protein induced by diclofenac was not affected by inhibition of NF-κB with srIκBα (Fig. 7, bottom panel). srIκBα binds irreversibly to NF-κB preventing its phosphorylation and subsequent translocation into the nucleus [22]. It is transported into cells through its prior combination with the protein transducing domain (PTD) of the human immunodeficiency virus, which is rich in positively charged amino acids that interact with negatively charged phospholipids in cell membranes thus facilitating its entry into cells [22].

Comparison of the release of pro-inflammatory and anti-inflammatory cytokines by LPS and diclofenac in J774.2 macrophages

Determination of the concentration of pro-inflammatory and anti-inflammatory cytokines in J774.2 macrophages stimulated with LPS or diclofenac was carried out to elucidate a possible role for the diclofenac-induced COX-2 protein during the inflammatory process.

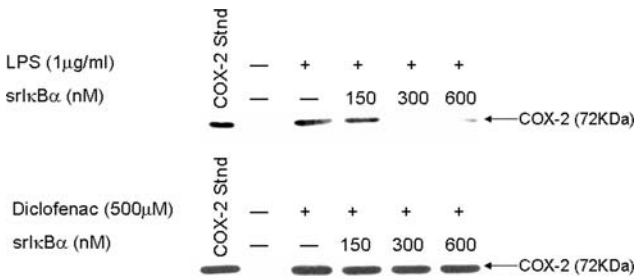

Fig. 7 The effect of pre-treatment of cultured J774.2 macrophages with the NFκB inhibitor, srIκBα for 1 h on the induction of COX-2 protein by either 1 μg/ml LPS for 24 h (*top panel*) or 500 μM diclofenac for 48 h (*bottom panel*)

LPS caused a concentration dependent and significant increase ($P < 0.001$) in the levels of the pro-inflammatory cytokines TNF-α (Fig. 8a) and IL-6 (Fig. 8c) 9 h following stimulation. Although there was a general increase in the levels of the anti-inflammatory cytokines IL-10 (Fig. 8b) and TGF-β (Fig. 8d) following LPS treatments (9 h), this was not as pronounced as the increase in TNF-α and IL-6 and failed to reach significance.

Whilst diclofenac treatment of J774.2 macrophages had no effect on the accumulation of IL-6 after 48 h incubation (Fig. 9c), a significant reduction in the basal release of TNF-α was shown with the levels of TNF-α falling below the detection limits of the assay (Fig. 9a). However, diclofenac produced a significant increase in the levels of IL-10 (Fig. 9b; $P < 0.05$) and TGF-β (Fig. 9d; $P < 0.01$). The increase in the concentration of IL-10 and TGF-β was most pronounced with 500 μM diclofenac, which coincided with the highest induction of COX-2 protein and greatest PGE_2 release (Fig. 1c, d).

The role of PPAR-γ in the induction of COX-2 protein by diclofenac in rat primary peritoneal macrophages

Primary rat peritoneal macrophages were isolated and stimulated with either 1 μg/ml LPS or 500 μM diclofenac. The aim of this experiment was to determine whether the induction of COX-2 protein by diclofenac through the activation of PPAR-γ occurs only in the J774.2 macrophage cell line. As shown in lanes 2 and 3 in Fig. 10, clear induction of COX-2 protein by LPS and diclofenac, respectively, was seen. The expression of the COX-2 protein induced by diclofenac (at 48 h) was reduced in a concentration-dependent manner by the pre-treatment of the cells with BADGE (lanes 4 and 5).

Discussion

Further research on the COX-2 protein induced by supra-pharmacological concentrations of diclofenac in the J774.2 murine macrophage cell line, identified by Simmons et al. [16], is certainly required as it may help to identify previously unknown actions by COX-2. Thus, in the present study, we have demonstrated that induction of COX-2 by diclofenac in J774.2 cells requires the activation of the transcription factor PPAR-γ, since the pre-treatment of the cells with the PPAR-γ receptor antagonists, BADGE or GW9662 [19, 20] reduced the induction of the COX-2 protein. Therefore, treatment of J774.2 cells with diclofenac resulted transcriptional activation of PPAR-γ, but had no effect on the expression of PPAR-γ (data not shown). In contrast, the induction of COX-2 by LPS was unaffected. However, the NFκB inhibitor, srIκBα [22], had no effect

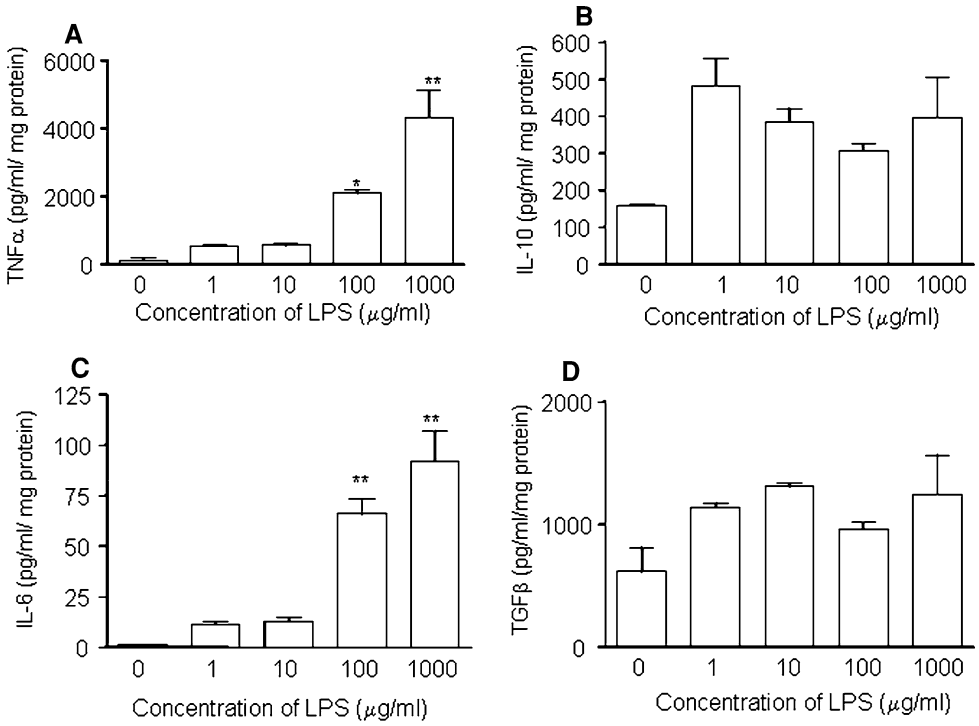

Fig. 8 Concentration response to the effect of LPS on the level of TNF-α (**a**), IL-10 (**b**), IL-6 (**c**) and TGF-β (**d**) after 9 h in J774.2 macrophages. $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 3$

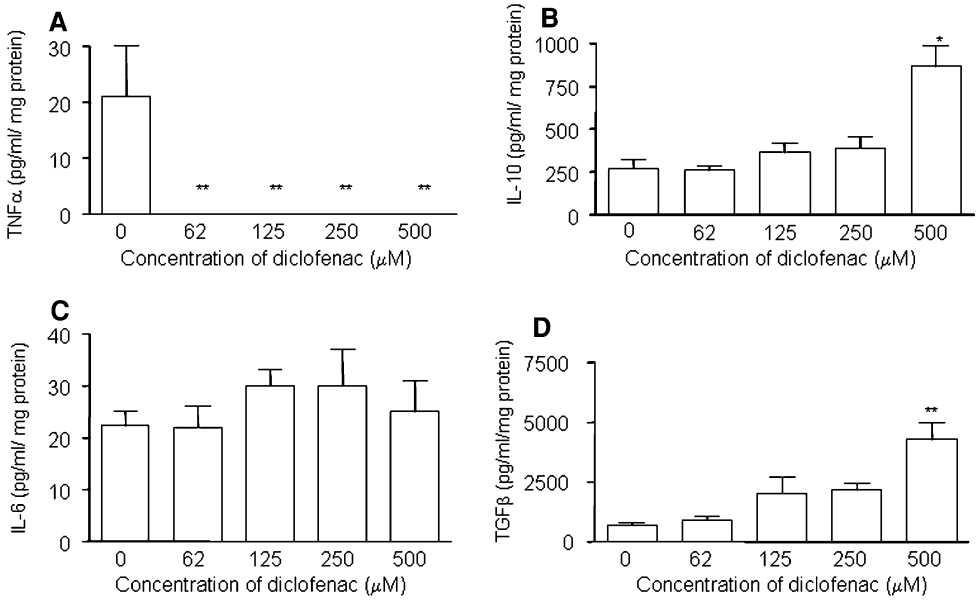

Fig. 9 Concentration response to the effect of diclofenac on the level of TNF-α (**a**), IL-10 (**b**), IL-6 (**c**) and TGF-β (**d**) after 48 h in J774.2 macrophages. $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 3$

Fig. 10 Stimulation of COX-2 protein by 1 μg/ml LPS (24) and 500 μM diclofenac (48 h) in primary rat peritoneal macrophages and the effect of the PPAR-γ antagonist BADGE on the induction of COX-2 protein by diclofenac after 48 h

on the COX-2 protein induction by diclofenac, whilst COX-2 protein induced by LPS was greatly reduced. Further confirmation that COX-2 can be induced by PPAR-γ activation is provided by the fact that both BADGE and GW9662 prevented COX-2 induction by the PPAR-γ agonist 15d-PGJ_2 [21]. To confirm that the induction of COX-2 protein by diclofenac with the involvement of PPAR-γ is not a transformed cell line specific effect, primary rat peritoneal macrophages were isolated and shown to induce COX-2 following treatment with diclofenac. This

COX-2 induction was also reduced by concomitant treatment with BADGE.

Our data, therefore, suggest that the transcription factor activated to induce COX-2 protein by diclofenac is PPAR-γ. This finding confirms similar previously published observations. First, a number of NSAIDs have been reported to induce COX-2 protein via activation of PPAR-γ [23–26], including diclofenac itself at relatively high concentrations similar to those used in this study [27]. Second, PPAR-γ activation has been shown to result in the induction of apoptosis in various cell lines [28–30]. Thus, we suggest that activation of PPAR-γ by diclofenac in J774.2 macrophages mediates the induction of apoptotic cell death in these cells.

Diclofenac is a classical NSAID commonly used for the treatment of pain and inflammation [31]. Its mechanism of action is dependent on the inhibition of COX activity resulting in reduction of prostaglandin synthesis [32, 33]. In the present study, diclofenac has been used as an experimental tool to induce COX-2 protein in order to investigate novel pathways leading to the induction of COX-2. In order to prevent the inhibition of the diclofenac-induced COX-2 protein by diclofenac itself, the cells were washed with serum-free medium for 10 min in order to wash out diclofenac, which is a reversible inhibitor of COX activity [34]. This allows for the investigation of potential inhibitors of this enzyme.

We propose that the diclofenac-induced COX-2 protein, if demonstrated to exist in vivo, could be involved in anti-inflammatory processes. This hypothesis is supported by the fact that a concentration dependent increase in the release of the anti-inflammatory cytokines IL-10 and TGF-β was shown after 48 h treatment with diclofenac along with reduction in the release of the pro-inflammatory cytokine TNF-α, which correlates with the peak of COX-2 protein induction.

The late-induced COX-2 protein produced by macrophages during the resolving phase of the carrageenan-induced pleural inflammation, which has been demonstrated to play an anti-inflammatory role was suggested to be induced by activation of PPAR-γ [10]. In addition, induction of neutrophil and macrophage apoptosis during resolution [35] is driven by the PPAR-γ agonist 15d-PGJ_2 [21]. This provides further support that activation of PPAR-γ by diclofenac in J774.2 cells and the subsequent induction of COX-2 protein along with the release of IL-10 and TGF-β as well as induction of apoptosis [16] could represent macrophages in an "anti-inflammatory state".

Macrophages are known to be important inflammatory cells through the production of pro-inflammatory cytokines. However, emerging evidence supports an important anti-inflammatory role for macrophages during the resolution of acute inflammation through the release of anti-inflammatory cytokines [36] and induction of apoptosis [35].

Induction of inflammatory resolution through 15d-PGJ_2 mediated activation of PPAR-γ has been demonstrated in various animal models of inflammation [37–39]. PPAR-γ activation by 15d-PGJ_2 and ciglitazone in macrophages was shown to suppress the LPS mediated release of the pro-inflammatory cytokines TNF-α, IL-6 and IL-12 [40]. This is consistent with our results showing suppression of TNF-α release after diclofenac treatment, further supporting involvement of PPAR-γ. In addition, to the suppression of pro-inflammatory cytokines release by PPAR-γ activation, Alleva et al. [40] show an increase in the release of IL-10, which is also consistent with our results.

TGF-β has been reported to be involved in the induction of apoptosis [41], which in our experimental setting is likely to be linked to PPAR-γ activation. In addition, TGF-β was shown to have opposite effects to TNF-α on inflammatory cells [42], which may contribute to the suppression of TNF-α levels after diclofenac treatment. The induction of COX-2 protein after treatment of J774.2 cells with diclofenac may also be mediated by TGF-β as TGF-β has previously been shown to result in the induction of COX-2 [43].

Paracetamol which is a potent analgesic and antipyretic drug possesses weak anti-inflammatory activity and is also a weak inhibitor of COX-1 and COX-2 activity [44]. A precise explanation of how paracetamol produces its pharmacological effects is still lacking. However, it is thought to act through the selective inhibition of central nervous system (CNS) COX activity [45]. The recently identified COX-1 variant, named COX-3 [46], is a possible target for paracetamol [47, 48], if its existence in human cells is confirmed [49]. To our knowledge, there is no evidence that paracetamol has an effect on the resolution of acute inflammation. However, we have demonstrated, despite not having effects on the onset of pleural inflammation induced by carrageenan in rats, that administration of 200 mg/kg paracetamol orally at the resolving phase of inflammation prevented resolution (our unpublished observations). We, therefore, speculate that selective inhibition of the late-induced anti-inflammatory COX-2 protein during pleurisy [10] could explain the effect produced by paracetamol during the resolution of inflammation, which also suggests that the late-induced COX-2 protein could be biochemically different to COX-2 induced at the onset of inflammation and by LPS. It is possible that the "anti-inflammatory COX-2" (represented by the diclofenac-induced COX-2 in vitro and the late-induced COX-2 in vivo) is differentially glycosylated to the "pro-inflammatory COX-2" (represented by the LPS-induced COX-2 in vitro and the early induced COX-2 in vivo). Differential glycosylation of COX-2 has already been described [50].

Finally, the evidence provided in this study shows that the diclofenac-induced COX-2 protein is induced by activation of PPAR-γ. This COX-2 protein is likely to be functionally related to that which plays an important role in the resolution of inflammation.

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