British Journal of Pharmacology (2000) 131, 152–159
www.nature.com/bjp

Induction of COX-2 and PGE_2 biosynthesis by IL-1β is mediated by PKC and mitogen-activated protein kinases in murine astrocytes

[1]Eduardo Molina-Holgado, [1]Sergio Ortiz, [2]Francisco Molina-Holgado & *,[1]Carmen Guaza

[1]Neural Plasticity Department, Instituto Cajal, Consejo Superior de Investigaciones Cientificas, Avda. Dr. Arce 37, 28002 Madrid, Spain and [2]University of Manchester, School of Biological Sciences, Manchester

1 Interleukin-1 (IL-1) is an important mediator of immunoinflammatory responses in the brain. In the present study, we examined whether prostaglandin E_2 (PGE_2) production after IL-1β stimulation is dependent upon activation of protein kinases in astroglial cells.

2 Astrocyte cultures stimulated with IL-1β or the phorbol ester, PMA significantly increased PGE_2 secretion. The stimulatory action of IL-1β on PGE_2 production was totally abolished by NS-398, a specific inhibitor of cyclo-oxygenase-2 activity, as well as by the protein synthesis inhibitor cycloheximide, and the glucocorticoid dexamethasone. Furthermore, IL-1β induced the expression of COX-2 mRNA. This occurred early at 2 h, with a maximum at 4 h and declined at 12 h. IL-1 β treatment also induced the expression of COX-2 protein as determined by immunoblot analysis. In that case the expression of the protein remained high at least up to 12 h.

3 Treatment of cells with protein kinase C inhibitors (H-7, bisindolylmaleimide and calphostin C) inhibited IL-1β stimulation of PGE_2. In addition, PKC-depleted astrocyte cultures by overnight treatment with PMA no longer responded to PMA or IL-1. The ablation of the effects of PMA and IL-1β on PGE_2 production, likely results from down-regulation of phorbol ester sensitive-PKC isoenzymes. Immunoblot analysis demonstrated the translocation of the conventional isoform cPKC-α from cytosol to membrane following treatment with IL-1β.

4 In addition, IL-1β treatment led to activation of extracellular signal-regulated kinase (ERK1/2) and p38 subgroups of MAP kinases in astroglial cells. Interestingly, the inhibition of ERK kinase with PD 98059, as well as the inhibition of p38 MAPK with SB 203580, prevented IL-1β-induced PGE_2 release.

5 ERK1/2 activation by IL-1β was sensitive to inhibition by the PKC inhibitor bisindolylmaleimide suggesting that ERK phosphorylation is a downstream signal of PKC activation.

6 These results suggest key roles for PKC as well as for ERK1/2 and p38 MAP kinase cascades in the biosynthesis of PGE_2, likely by regulating the induction of cyclo-oxygenase-2, in IL-1β-stimulated astroglial cells.

British Journal of Pharmacology (2000) **131,** 152–159

Keywords: Interleukin-1β; prostaglandin E_2; protein kinase C; mitogen activated protein kinases; cyclo-oxygenase-2; murine astrocytes

Abbreviations: CNS, central nervous system; DMEM, Dulbecco's modified Eagle Medium; DMSO, dimethyl sulphoxide; DTT, dithiothreitol; ERK, extracellular signal regulated kinase; FCS, foetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFPA, glial fibrilar protein acidic; H-7, 1-(5-isoquinolinyl-sulphonyl)-2-methylpiperazine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; IL-1, interleukin-1; LPS, lipopolysacharide; MAPK, mitogen activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; PAGE, polyacrylamide gel electrophoresis; PGE_2, prostaglandin E_2; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; 4-α PMA, 4-α phorbol 12-myristate 13 acetate; PCR, polymerase chain reaction; RT, reverse transcription; SDS, sodium dodecyl sulphate; Thr; threonine; TPA, 12-O-tetradecanoyl-phorbol 13 acetate; Tyr, tyrosine

Introduction

The pro-inflammatory cytokine, interleukin-1 (IL-1) is secreted by a variety of cells in response to infection, activated lymphocyte products, microbial toxins, and inflammatory stimuli (Dinarello, 1996). Besides the classical role of IL-1 as immune mediator, this cytokine has numerous effects in the central nervous system (CNS) (Berkenbosh *et al*., 1987; Hansen & Krueger, 1997; Rothwell, 1991; Hopkins & Rothwell, 1995). IL-1β has been shown to be produced in the CNS in response to a number of stimuli, such as peripheral lipopolysaccharide administration (Layé *et al*., 1994), traumatic brain injury (Fan *et al*., 1995) and brain viral infection (Marquette *et al*., 1996; Lledó *et al*., 1999). Under pathological situations involving CNS inflammation, IL-1 is mainly secreted by activated microglia/macrophages (Giulian *et al*., 1986; Benveniste, 1992), while astrocytes appear to be its main target as suggested by the presence of IL-1 receptors in their surface (Ban *et al*., 1993). In astroglial cells, IL-1 induces the expression of other cytokines, such as interleukin-6 and tumour necrosis factor-α, as well as other inflammatory mediators that may be implicated in the CNS response to injury (Merrill & Benveniste, 1996).

Arachidonic acid metabolites, such as prostaglandins are closely involved in the inflammatory responses following brain injury and bacterial or viral infections. High levels of prostaglandins have been measured in multiple sclerosis, AIDS-associated dementia, and other neurodegenerative disorders (Bolton *et al*., 1986; Fretland *et al*., 1992; Griffin *et*

*Author for correspondence; E-mail: cgjb@cajal.csic.es

al., 1994). Cyclo-oxygenase is the rate-limiting enzyme in prostanoids biosynthesis and exists as a widely constitutive isoform (COX-1), responsible for physiological levels of prostaglandins and as an inducible COX-2 encoded by an immediate early gene, rapidly induced after pro-inflammatory stimuli. COX-2 is expressed by inflammatory cells and is responsible for the high levels of prostaglandins present in acute and chronic inflammation, being inhibited by glucocorticoid hormones (O'Banion *et al*., 1992; Herschman 1996). Interestingly, systemic LPS and IL-1β injections have been shown to stimulate production of COX-2 within the mouse (Breder & Saper, 1996) and rat (Cao *et al*., 1996; Lacroix & Rivest, 1998) brain microvaculature. In addition, COX-2 expression is increased in frontal cortex of Alzheimer's disease brains (Pasinetti & Aisen, 1998). During the last years, the physiological role of COX-2 has been a topic of much interest (Vane *et al*., 1998).

In vitro studies have revealed the capacity of astrocytes to release prostaglandins and express mRNA COX-2 in response to IL-1β (Hartung *et al*., 1989; Katsuura *et al*., 1989; O Banion *et al*., 1996). However, the intracellular signalling mechanisms triggered by IL-1β and responsible for prostaglandin production are not completely defined. Protein kinase C (PKC) represents a family of closely related serine/threonine kinases that play a key role in different cellular signal transduction pathways (Nishizuka, 1986; 1992). A number of studies have demonstrated that phorbol esters, direct activators of PKC, stimulate the synthesis of prostaglandins (Crofford *et al*., 1994). However, in mesangial cells, prostaglandin biosynthesis induced by IL-1β seems to be independent on PKC activation (Conquer *et al*., 1992). Activation of mitogen-activated protein kinases (MAPK) cascade is one of the most rapid cellular responses to different stimuli, including IL-1 (Wilmer *et al*., 1997; O Neill & Greene 1998). Moreover, the cascade of MAPKs represent a point of convergence in intracellular signalling activated by protein Tyr kinases, by G-protein coupled receptors and by PKC. Several studies have linked prostaglandin biosynthetic pathway which mediate inflammatory responses, with activation of MAPK signalling cascade (Lin *et al*., 1993; Sanghera *et al*., 1996; Hambleton *et al*., 1996; Niiro *et al*., 1998). In mammalian cells, three subgroups of MAPKs have been detected and included the extracellular signal-regulated kinase, p42/44 (ERK1/2), the C-jun amino terminal kinases (JNKs) and p38 MAPKs (Cano & Mahadevan, 1995). These distinct sets of MAPKs can be activated by a variety of extracellular stimuli, and individual MAPK kinases may be implicated in the expression of a number of pro-inflammatory molecules depending on the cell type (Pouliot *et al*., 1997).

In the current study, we described that IL-1β induced the expression of mRNA COX-2 and the protein in astrocytes. In addition, we analysed pharmacologically the possible participation of PKC activation and MAPKs cascade in IL-1β-induced PGE_2 production by mouse astrocyte cultures.

Methods

The following reagents were obtained from the indicated suppliers: Dulbecco's modified Eagle medium, foetal calf serum (FCS), penicillin/streptomycin mix from Gibco-BRL (Barcelona, Spain); recombinant mouse interleukin-1β (IL-1β) from Genzyme (Cambridge, MA, U.S.A.); monoclonal anti-GFAP, lipopolysaccharide (LPS) (from *E. Coli* 026:B6), H-7 1-(5-isoquinolinylsulphonyl)-2-)-2-methylpiperazine), 12-O-tetradecanoylphorbol 13 acetate (TPA); 4-α phorbol 12-myristate 13 acetate (4-αPMA), actinomycine D and cycloheximide from Sigma (St. Louis, MO, U.S.A.); bisindolylmaleimide I, NS-398, calphostin-C, PD 98059, SB 203580, from Calbiochem (La Jolla, CA, U.S.A.); PGE_2 enzymeimmunoassay system BIOTRAK, Hybond ECL-nitrocellulose membrane and ECL Western blotting detection reagents from Amersham Pharmacia Biotech (London, U.K.); culture flasks and dishes were from Falcon (Franklin Lakes, NJ, U.S.A.); Affinity-purified rabbit anti-phospho p42/44 and anti-phospho p38 were from New England Biolabs (Beverly, MA, U.S.A.); rabbit polyclonal anti-PKC-α was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); COX-2 antibody from Cayman Chemicals (MI), Mac-1 antibody from Serotec (Oxford, U.K.) and the secondary antibody peroxidase-conjugated goat anti-rabbit IgG was from Jackson Immuno Research Laboratories (West Grove, PA, U.S.A.). Secondary antibodies for immunofluorescence were from Southern Biotechnology (Birmingham, AL, U.S.A.). All other reagents were obtained from standard suppliers.

Astrocyte cultures

Primary astrocyte cultures were generated from the cerebral cortex of 1-day-old neonatal mice (Balb/c Cajal Institute, Madrid, Spain) as described by McCarthy & de Vellis (1980) with our modifications (Molina-Holgado *et al*., 1995). Cells were plated on 75 cm^2 flasks in Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated foetal calf serum plus 50 units ml^{-1} penicillin and 50 $\mu g\ ml^{-1}$ streptomycin (growth medium). After 10 days, the cultures were shaken for 18 h at 240 r.p.m. at 37°C to remove microglia and oligodendrocyte progenitors. The astrocyte monolayers were then trypsinized and plated at an approximate density of 30,000 cells cm^{-2} (6 or 24 cm well dishes and 90 cm^2 culture plates) and subcultured for 3–4 days before experiments. The cultures were immunocytochemically characterized by specific markers for different cell types. More than 95% of the cells reacted positively with the monoclonal anti Glial Fibrilar Acidic Protein (GFAP), a marker of astrocytes and less than 5% were A2B5 oligodendrocyte progenitors or Mac-1 positive microglia. Cultures with more than 5% of cells positive for A2B5 or Mac-1 were discarded.

Astrocyte stimulation

Cells were stimulated in 1 ml of growth media containing mouse recombinant IL-1β, LPS or TPA for different periods of time up to 24 h, and supernatants collected for PGE_2 determination. The protein synthesis inhibitor, cycloheximide, the COX-2 blocker NS-398 and the protein kinase inhibitors used were prepared as 1000 times stock solutions in DMSO and added 60 min before stimulation to the wells. Control cells were only exposed to equivalent amounts of DMSO.

For PKC or MAPK immunoblotting cells were serum starved for 24 h and then stimulated for the indicated times with mouse recombinant IL-1β or pharmacological agents as described in the Figure legends.

Western blot analysis

After culture in serum-free medium for 24 h, quiescent astrocytes were stimulated with test agents for the indicated times. Cells were washed with ice-cold PBS and lysed in 150 μl of TBS, pH 7.6, containing 10% glycerol, 1% NP-40, 0.5 mM EDTA, 1 mM PMSF Western blot, 50 $\mu g\ ml^{-1}$ leupeptin, 10 $\mu g\ ml^{-1}$ aprotinin, 5 mM benzamidine, 100 mM sodium

ortovanate, 2 mM NaF and 5 mM DTT. Lysates were added 5× Laemmli sample buffer before boiling for 4 min. Then, equal amounts of protein (20 μg) were resolved on 10% SDS-polyacrylamide gel electrophoresis and electroblotted at 4°C for 1 h to ECL nitrocellulose. The membranes were blocked for 1 h at room temperature in 5% (w v^{-1}) dry skim milk (Sveltese, Nestlé) in TBS (pH 7.6) plus 0.1% Tween-20 (TTBS) and then blots rinsed in TTBS. The membranes were incubated overnight with activation state-specific antibodies for the MAPKs (rabbit anti-phospho p42/44 and p38, New England BioLabs) at 1:1000 dilution or specific COX-2 antibody (Cayman Chemicals, MI, U.S.A.) at 1:2000 dilution. After extensive washing in 5% milk TTBS solution, blots were incubated with peroxidase-conjugated goat anti-rabbit IgG1 at 1:10,000 dilution. Blots were washed again before developing with an enhanced chemiluminiscence (ECL) kit.

To determine changes in the subcellular distribution of PKC isoenzymes, the cells were fractionated into cytosol and membrane fractions according to the following protocol. Astrocytes grown into 100 cm^2 were stimulated with IL-1β for different times and harvested in 1 ml of ice-cold homogenization buffer (20 mM K-HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 50 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, 5 mM benzamidine, 100 mM sodium ortovanate, 2 mM NaF and 5 mM DTT) and disrupted with a teflon homogenizer. The cytosolic fraction was obtained as supernatant after centrifugation at 100,000 × *g* for 60 min. To prepare a membrane fraction, the pellets were resuspended in 400 μl of the same buffer plus 1% Triton X-100 and collected after centrifugation at 100,000 × *g* for 30 min. Lysates (20 μg) were resolved on 10% SDS–PAGE and immunoblotted with rabbit polyclonal anti-PKC-α (1:6000) overnight at 4°C as described above.

RT–PCR analysis of COX-2

Astrocytes were plated in 35 mm culture dishes and stimulated with or without IL-1β (10 ng ml^{-1}) for different time periods. The cells were washed with PBS, and total RNA was isolated by the guanidinium isothiocyanate/phenol/chloroform method (Chomczynski & Sacchi, 1987). RNA concentration was quantified spectrophotometrically and the isolated RNA was treated with DNase to digest any contaminant genomic DNA. RT–PCR was performed in one step using Titan™ one tube RT–PCR system according to the manufacturer's instructions (Roche Molecular Biochemicals). RT–PCR amplification was carried out with 2 μg of RNA using the primer pair 5′-CCATGTCAAAACCGTGGTGAATG-3′ and 5′-ATGG-GAGTTGGGCAGTCATAG-3′ (Nogawa *et al*., 1997). The steps of amplification were 94°C for 30 s, 55°C for 45 s, 68°C for 45 s for 30 cycles. PCR products were resolved on 2% agarose gel with 0.5 μg ml^{-1} of ethidium bromide. Only one PCR product of the expected size was obtained and COX-2 transcript was identified as a 374 bp band. The mouse glyceraldehyde 3 phosphate dehydrogenase (GAPDH) gene (a houskeeping gene) was used as an internal standard gene.

Measurement of PGE_2

The cell-free supernatants were collected and stored at −70°C until measurement of PGE_2. The PGE_2 concentration in the culture medium was determined by a PGE_2 enzyme-immunoassay system (BIOTRAK, Amersham Pharmacia Biotech, U.K.). PGE_2 standard curve range from 2.5 to 320 pg $well^{-1}$. The sensitivity of the assay was 2 pg $well^{-1}$. The cross-reactivity with PGE_1 is 7.0%, with $PGF_2\alpha$ 0.04% and 6-Keto-PGF_1 α <0.1 and with Arachidonic acid <0.001%. The within assay precision was <7.6% and between assay was <14%.

The evaluation of the protein content in the cultures was determined by a BCA protein assay reagent (Pierce, IL, U.S.A.) with bovine serum albumin as the standard.

Statistical analysis

The data are presented as mean±s.e.mean values of *n* independent determinations, and were triplicated within each experiment. Comparisons were analysed by using one-way analysis of variance (ANOVA) followed by the *posteriori* Student-Newman-Keuls' *t*-test. A value of $P<0.05$ was considered significant.

Results

IL-1β stimulates prostaglandin biosynthesis and induces the expression of COX-2 mRNA and protein in mouse cultured astrocytes

Treatment of astrocyte cultures with IL-1β (10 ng ml^{-1}) caused a time-dependent increase in PGE_2 release, that was nearly 2 fold and more than 3 fold increased in comparison to control cultures at 8 and 12 h respectively (Figure 1). Exposure of the cells to IL-1β also induced the expression of mRNA COX-2 as well as COX-2 protein (Figure 2A–C). The kinetic profile of the expression of COX-2 mRNA by RT–PCR analysis revealed a time-dependent induction of COX-2 mRNA by IL-1β. COX-2 mRNA was detected as early as 2 h, peaked at 4 h and then, started to decline by 12 h (Figure 2A). The earliest induction of COX-2 protein occurred at 2 h and remained elevated up to 12 h (Figure 2B).

Dexamethasone (1 μM) prevented IL-1β-induced PGE_2 release (Figure 1), while both PMA (100 nM) and LPS (1 μg ml^{-1}) increased PGE_2 secretion after 24 h treatment (Figure 3). As expected, the inactive phorbol 4-αPMA (100 nM) failed to stimulate PGE_2 release. Figure 3 also shows that 2 μM of NS-398, a specific inhibitor of COX-2, (Futaki *et al*., 1993), totally abolished the stimulatory action of IL-1β on PGE_2 secretion, clearly indicating that prostaglandin produc-

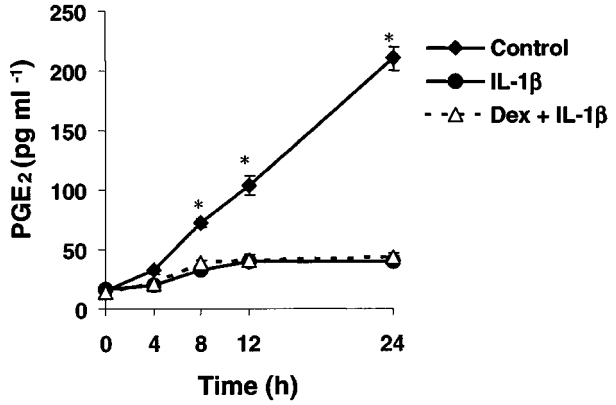

Figure 1 Time course of IL-1β (10 ng ml^{-1}) induced release of PGE_2 from cultured mouse astrocytes in the presence or not of dexamethasone (1 μM). Supernatants were collected at the indicated times. Levels of PGE_2 in the medium were determined by enzyme-immunoassay system. Data are mean±s.e.mean (bars) values for three to four independent experiments in triplicate. Statistical significance: *$P<0.001$ compared with non-treated and dexamethasone treated cells after the corresponding incubation period.

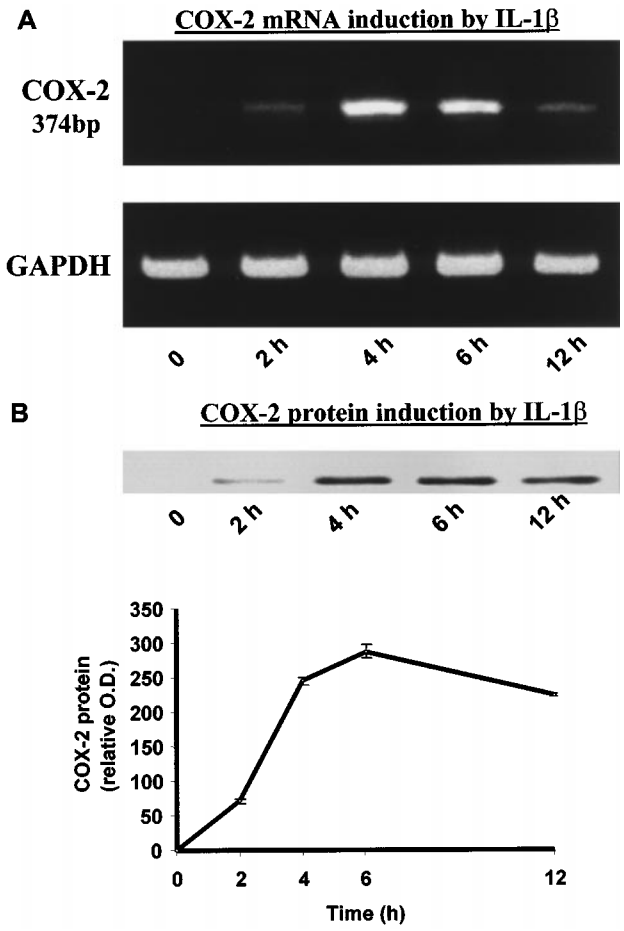

Figure 2 (A) Time-course of COX-2 mRNA expression in IL-1β (10 ng ml^{-1})-cultured astrocytes. Total RNA was extracted as described in Methods and levels of mRNA for COX-2 and GAPDH were determined by RT–PCR. Representative results of three independent experiments. (B) Time course of COX-2 protein expression in IL-1β (10 ng ml^{-1})-stimulated astrocytes. Cell lysates were used to determine COX-2 expression by Western blot analysis. Proteins were analysed by SDS-polyacrylamide gel electrophoresis and immunoblotting techniques using specific COX-2 antibody. Equal amount of proteins (20 μg) were loaded in all lanes. Bands were visualized by the ECL method. Representative results of three independent experiments. (C) Integrated band densities were obtained by scanning using a densitometer. Plotted means ± s.e.mean of three independents experiments.

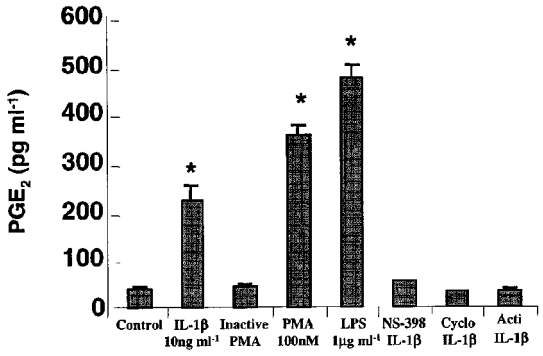

Figure 3 Interleukin-1β (10 ng ml^{-1}), PMA (100 nM) and LPS (1 μg ml^{-1}) stimulate the production of PGE_2 in murine astrocytes. Supernatants were collected after 24 h stimulation. Pre-treatment (60 min before) with the COX-2 inhibitor, NS-398 (2 μM), or the inhibitor of the synthesis protein, cycloheximide (Cyclo) (10 μg ml^{-1}) or the transcription inhibitor actinomycine-D (1 μg ml^{-1}) abrogates the stimulatory effect of IL-1β on PGE_2 secretion. The data are presented as the mean ± s.e.mean (vertical lines) of four independent determinations in triplicate. Statistical significance: $^*P<0.0001$ vs control; NS-398 + IL-1β, Cyclo + IL-1β and Acti + IL-1β.

tion is due to COX-2 activity. In addition, PGE_2 production was completely prevented by the protein synthesis inhibitor cycloheximide (10 μg ml^{-1}), as well as by the transcription inhibitor actinomycine D (1 μg ml^{-1}) confirming the requirement for the *de novo* protein synthesis.

Signal transduction pathways involved in IL-1β-increased prostaglandin production

Involvement of PKC and MAP kinases The ability of PMA to increase PGE_2 production in astrocytes, suggests that PKC may be involved in the action of IL-1β. To test this hypothesis, astrocytes were pre-treated with various kinase inhibitors at the adequate doses prior to stimulation with IL-1β (10 ng ml^{-1}). As shown in Figure 4, the compound, H7 (10 μM), or bisindolylmaleimide (2 μM), inhibitors of PKA and PKC suppressed PGE_2 accumulation by 52.4 and 51.8%, respectively. The more specific PKC inhibitor, calphostin C (1 μM), also blocked (64.5%) IL-1β-stimulated PGE_2 production. Since MAP kinase pathways have been shown to be activated by IL-1β treatment in a number of cells, we also used specific inhibitors of p38 and p42/44 (ERK1/2) MAP kinase cascade. As shown in Figure 4, PGE_2 production was inhibited by 77% in IL-1β-stimulated astrocytes exposed to the specific inhibitor of ERK kinase (MEK), PD 98059 at the dose of 15 μM, known to inhibit ERK phosphorylation (Alessi *et al.*, 1995). This dose of PD 98059 did not induce cell death during the 24 h treatment, as examined by the MTT reduction assay (data not shown). In addition, IL-1β-induced-PGE_2 release was blunted by 15 μM SB 203580 (79.4%), a specific inhibitor of p38 MAP kinase (Badger *et al.*, 1996), without affecting cellular viability. These results indicate the involvement of both, PKC and MAPKs in IL-1β-induced PGE_2 biosynthesis by astroglial cells.

To further confirm a role of PKC on PGE_2 biosynthesis, astrocyte cultures were depleted of PKC by overnight treatment with PMA (100 nM), then IL-1β (10 ng ml^{-1}) or PMA (10 nM) were added for 4 h and the amount of PGE_2 in the culture supernatants measured. As shown in Figure 5, PKC-depleted cells no longer responded to PMA or IL-1β,

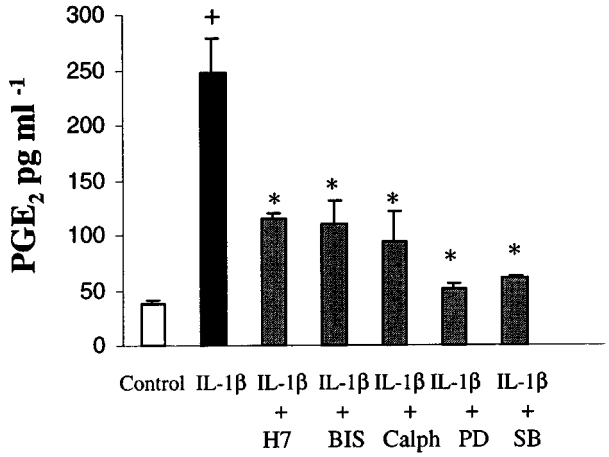

Figure 4 The protein kinase C inhibitors (10 μM H-7, 2 μM BIS and 1 μM Caphostin C) as well as MAPKinase inhibitors (15 μM PD 98059, 15 μM SB 203580) blocked IL-1β-induced PGE_2 production. Inhibitors were added 60 min before IL-1β (10 ng ml^{-1}) stimulation and supernatants were collected 24 h after for PGE_2 determination by enzyme-immunoassay. Values are the means ± s.e.mean (vertical bars) of four independent experiments in triplicate. Statistical significance: $^*P<0.0001$ vs IL-1β alone; $^+P<0.0001$ vs control.

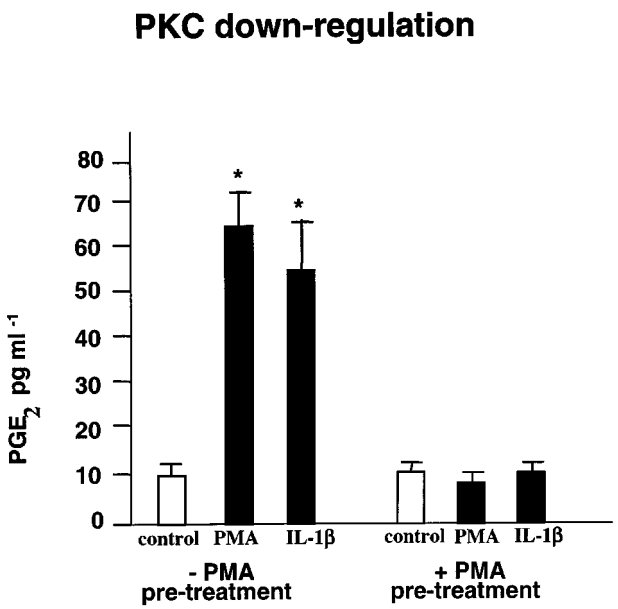

Figure 5 Depletion of PKC by overnight PMA pre-treatment prevents PGE_2 secretion by IL-1β or PMA. Astrocytes were treated with 100 nM PMA overnight and then were stimulated with IL-1β (10 ng ml^{-1}) or PMA (10 nM) for 4 h to collect supernatants for PGE_2 determination by enzyme-immunoassay. Results are means $\pm$s.e.mean of four independent experiments. $^{*}P<0.001$ vs control.

suggesting the requirement for a conventional PKC isoform. A potential candidate is cPKC-α, which is present in astrocytes at high level. Therefore, astrocyte cultures were treated with IL-1β for different periods of time and cell extracts were analysed for cPKC-β activation. Immunoblot analysis (Figure 6) showed that IL-1β (10 ng ml^{-1}) caused translocation of cPKC-α from cytosol to the membrane fraction. This effect occurred rapidly, after 5 min, was maximal after 30 min, and declined after 6 h treatment. As expected, TPA treatment also caused the translocation of PKC-α from cytosol to the membrane fraction (data not shown).

MAPKs activation in astrocyte cultures by IL-1β was assessed by Western blot analysis, using specific antibodies for the phosphorylated forms of p38 and ERK1/2. Figure 7 shows the time course of IL-1β activation of p38 MAPK. IL-1β (10 ng ml^{-1}) elicited a rapid (detectable at 5 min) Thr/Tyr phosphorylation of p38 MAPK that increased with time, reaching a maximum at 30 min – 1 h, followed by a gradual decline reaching basal levels by 5 h. Next, we examined whether the p42/44 MAPK pathway was also involved in IL-1β signalling in astrocytes. Figure 8 shows the time course activation of p42 and p44 MAPKs in response to IL-1β (10 ng ml^{-1}). IL-1β induced a rapid (5 min) and prolonged p42/44 MAPKs activation (over 5 h in the case of p44 MAPK), peaking at 30 min. Besides, IL-1β-induced phosphorylation of p42/44 MAPK was significantly inhibited by prior

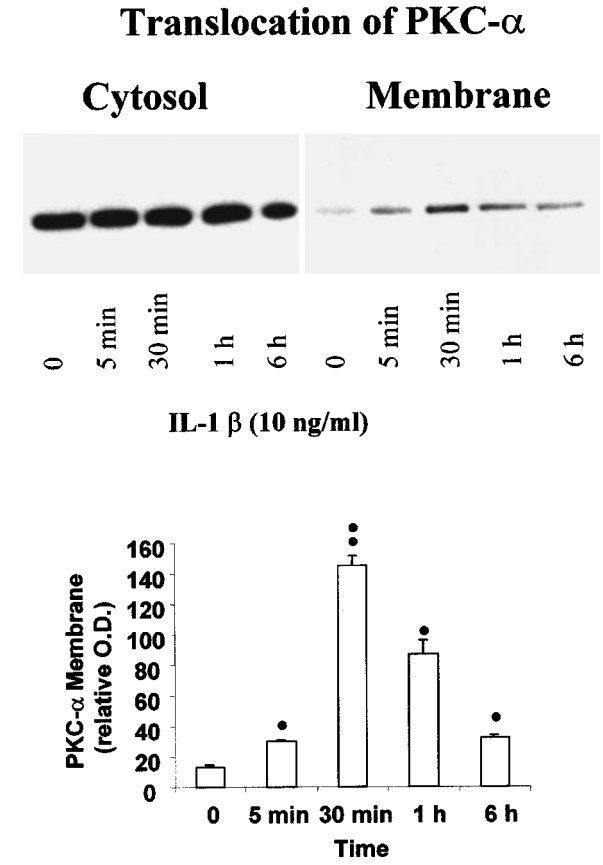

Figure 6 Subcellular distribution of cPKC-α in response to activation with IL-1β. Cultured astrocytes were stimulated with 10 ng ml^{-1} IL-1β, for 0, 5, 30 min, 1 h, or 6 h, and fractionated into soluble cytosolic or membrane fractions as described in Methods. Then, cytosolic and membrane fractions were immunoblotted with antibody against PKC-α. The experiments were repeated at least four times with similar results. Bands were visualized by the ECL method. Densitometric values were plotted, and values represent the means $\pm$s.e.mean of four experiments. $^{\bullet}P<0.01$; $^{\bullet\bullet}P<0.001$ vs basal.

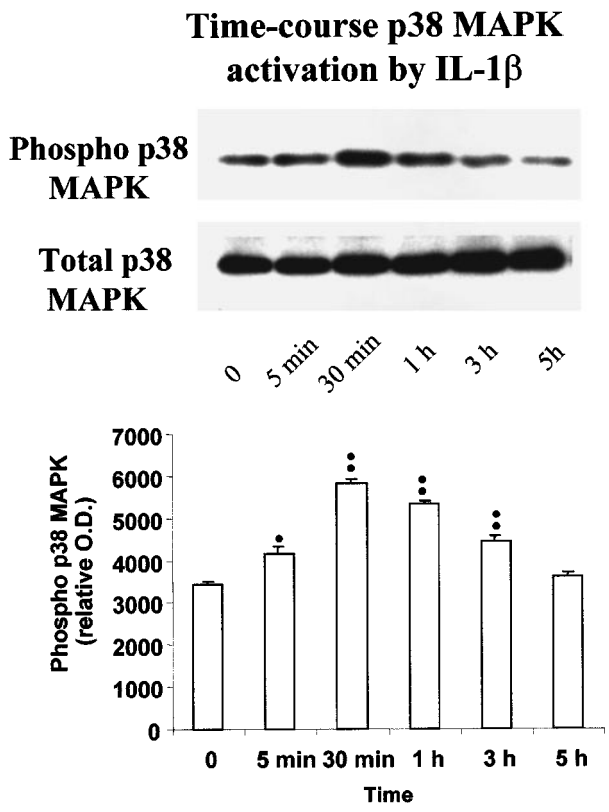

Figure 7 Time-dependent activation of p38 MAPK by IL-1β in astrocytes. Astrocyte cultures were treated with 10 ng ml^{-1} IL-1β for various time periods. Whole-cell lysates were prepared and subjected to immunoblot analysis using antibodies specific for the activated (phosphorylated) forms of p38 MAPK as described in Methods. Parallel blots were assayed using the antibodies recognizing the total p38 MAPK protein. The experiments were repeated at least four times with similar results. Bands were visualized by the ECL Method. Densitometric values were plotted, and values represent the means $\pm$s.e.mean of four experiments. $^{\bullet}P<0.01$; $^{\bullet\bullet}P<0.001$ vs basal.

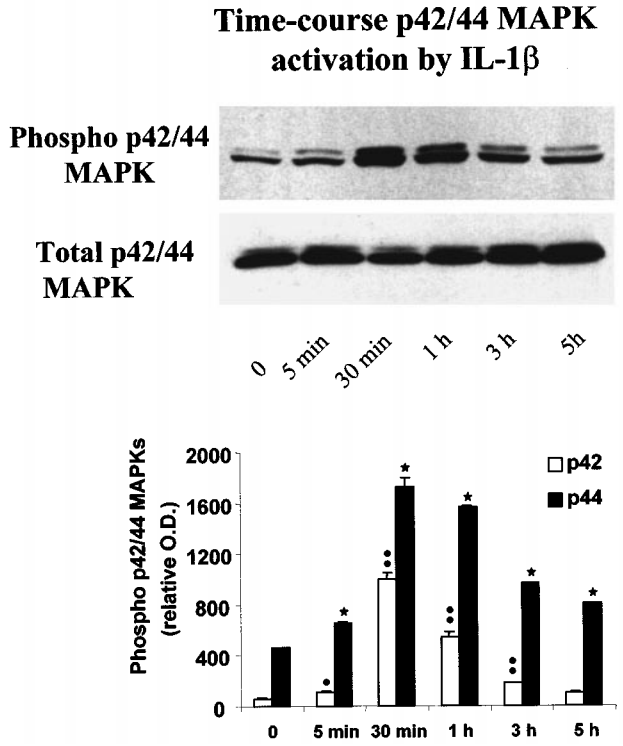

Figure 8 Time-dependent activation of p42/44 MAPKs by IL-1β in astrocytes. Astrocyte cultures were treated with 10 ng ml^{-1} IL-1β for various time periods. Whole-cell lysates were prepared and subjected to immunoblot analysis using antibodies specific for the activated (phosphorylated) forms of p42/44 MAPKs as described in Methods. Parallel blots were assayed using the antibodies recognizing the total p42/44 MAPK proteins. The experiments were repeated at least four times with similar results. Bands were visualized by the ECL method. Densitometric values were plotted, and values represent the means $\pm$s.e.mean of four experiments. $^{\bullet}P<0.05$; $^{\bullet\bullet}P<0.001$ vs corresponding basal. $^{*}P<0.001$ vs corresponding basal.

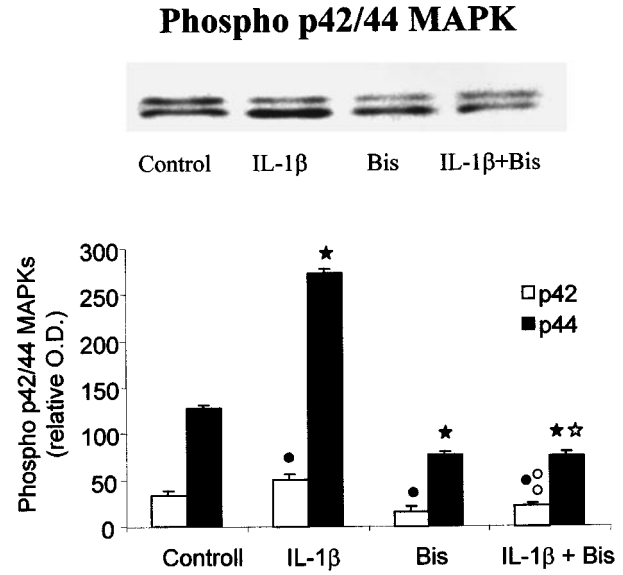

Figure 9 The inhibition of PKC activity by bisindolylmaleimide (2 μM) reduced the phosphorylation of p42/44 MAPK induced by IL-1β (10 ng ml^{-1}). Cells were treated with bisindolylmaleimide (Bis) 30 min prior to stimulation with IL-1β. Whole cell lysates were prepared and subjected to immunoblot analysis using a specific antibody for the phosphorylated form of p42/44 MAPK. Representative results of three independent experiments with similar results. Bands were visualized by the ECL method. Densitometric values were plotted, and values represent the means$\pm$s.e.mean of three experiments. p42 MAPK: $^{\bullet}P<0.05$ vs corresponding basal; $^{\circ\circ}P<0.001$ vs corresponding IL-1β. p44 MAPK: $^{*}P<0.001$ vs corresponding basal; $^{\star}P<0.001$ vs corresponding IL-1β.

treatment (30 min) with the PKC inhibitor, bisindolylmaleimide (Figure 9), suggesting that IL-1β induced p42/44 MAPK activation may be the downstream signal of PKC activation. Collectively, these results suggest that both, activation of PKC and then, the phosphorylation of p42/44 and p38 MAPKs are required for the production of PGE_2 by IL-1β in murine astrocytes.

Discussion

The pro-inflammatory cytokine IL-1β enhanced PGE_2 production in mouse astrocyte cultures, in agreement with previous studies (Hartung *et al*., 1989; Katsuura *et al*., 1989; Gerbicke-Haerter, *et al*., 1991). Our study demonstrated that IL-1β-mediated PGE_2 release is a consequence of COX-2 induction, which is expressed by inflammatory agents and down-regulated by glucocorticoid hormones (O'Banion *et al*., 1992; Herschman, 1996). Thus, IL-1β treatment induced COX-2 mRNA expression as measured by RT–PCR, and COX-2 protein as shown by Western blot analysis; while the stimulatory action of IL-1β in PGE_2 release was abolished by the COX-2 selective inhibitor, NS-398, and by the glucocorticoid, dexamethasone. Furthermore, IL-1β-induced PGE_2 release was found to depend on the *de novo* transcription and protein synthesis since both actinomycine D and cycloheximide blocked the response.

However, the signalling pathways implicated in IL-1β-induced COX-2 expression and, thus, PGE_2 biosynthesis are not well understood. Cellular responses to IL-1β include a cascade of protein phosphorylation which transmits signals from cell surface to the nucleus and that ultimately regulates gene expression (O'Neill & Greene, 1998). The PKC family of serine/threonine kinases have been implicated in the signalling pathway of cell surface receptors, including those used by IL-1β in several cell types. Consistent with this, activation of PKC has been previously involved in IL-1-regulated astrocyte function (Aloisi *et al*., 1994; Norris *et al*., 1994; Ballestas & Benveniste, 1995). We observed a stimulatory effect of PMA on PGE_2 production, suggesting a positive role of PKC, in agreement with other studies (Hartung *et al*., 1989). Further evidence for the involvement of PKC was obtained using H-7 and bisindolylmaleimide, since these protein kinases inhibitors significantly reduced PGE_2 production in response to IL-1β. In addition, a more specific PKC inhibitor, calphostin C, at the dose of 1 μM almost completely blocked PGE_2 responses to IL-1β. Since calphostin C interacts with the regulatory domain of PKC and competes with the DAG phorbol ester binding site located in the C1 domain (Hug & Sarre, 1993; Hofman, 1997), our results suggest the possible involvement of a conventional PKC isoform in the signal transduction leading to the increased PGE_2 release by IL-1β. Consistent with this, down-regulation of PKC abolished the stimulatory effect of PMA, but also that of IL-1β. Moreover, Western blot analysis showed that IL-1β induced translocation of the conventional isoform, PKC-α, from cytosol to the membrane fraction indicating its activation. Nevertheless, because of lack of using selective inhibitors for PKC-α (Hofmann, 1997) in the present work, the possibility of involvement of other PKC isoenzymes in COX-2 expression after IL-1β treatment cannot be discounted.

Cellular prostaglandin production depends on cyclooxygenase activity as well as the availability of arachidonic acid substrate, which is controlled by enzymes such as phospholipase A_2 (PLA_2). Previous studies (Oka & Arita, 1991) have shown that enhanced PGE_2 production by IL-1β is

subsequent to PLA_2 activation in rat astrocytes. Furthermore, activation of PKC stimulates the release of arachidonic acid due to increased PLA_2 activity which also implicated MAPK activation (Nam *et al*., 1995). Thus, it is possible that enhanced PGE_2 release in PMA-stimulated astrocytes also involved PLA_2 activation.

Recent work has linked activation of the prostaglandin generative pathway with the MAPK pathway (Guan *et al*., 1998; Niiro *et al*., 1998). Moreover, one of the signalling mechanisms triggered by IL-1β includes the activation of MAPKs, a family of serine/threonine kinases activated by dual phosphorylation of Thr and Tyr within a Thr-X-Tyr motif (Kyriakis & Avruch, 1996; Guan *et al*., 1997). The kinase cascade involving MAPKs appears to have a fundamental role in integrating multiple intracellular signals activated by Tyr kinases, but also by PKC (Blumer & Johnson 1994). Therefore, we investigated whether IL-1β stimulation was able to activate the ERK1/2 and p38 subgroups of MAPKs and whether such activation could be related to the ability of IL-1β to activate PKC and induce the biosynthesis of PGE_2. Our results indicate for the first time that IL-1β activates ERK1/2 and p38 MAPK in astroglial cells. The activation occurs rapidly at 5 min and with maximum at 30 min, indicating that the activation of MAPK cascade is an early event in astrocytes in response to IL-1β stimulation. An important question was therefore whether IL-1β induced activation of MAPKs was related to PGE_2 production. To this end, treatment of astrocytes with the specific inhibitors of MEK and p38 MAPK (PD 98059 or SB 203580) dramatically decreased PGE_2 release by IL-1β, suggesting key roles for the kinases in this event. Support for this notion was provided by Guan *et al*. (1997) showing that IL-1β-mediates PGE_2 production and COX-2 expression with concomitant activation of p38 MAPK-mediated signalling pathways in human mesangial cells. Our study presents further evidence that the intracellular-signalling pathway activated by IL-1β involves p42/44 MAPK and requires PKC activation.

In conclusion, we propose that the phosphorylation of MAPKs might constitute the signalling pathway through which an activation of PKC by IL-1β leads to increased PGE_2 biosynthesis in astroglial cells. This pathway in astrocytes provides further evidence for the role of the MAPKs cascade in the mediation of prostaglandin biosynthesis and as a potential target for modulation of the inflammatory response.

The present studies were supported by grants from the Ministry of Education and Culture, (DGICYT: PM 98/014), and EC BIOMED 2 Program (CT 97-2492). We thank Le Minh Qujnh Uyen for her excellent technical assistance and Dr G. Almazán for critical reading of the manuscript and helpful advice. We also thank C. Torres for her technical assistance in cell cultures.

References

ALESSI, D.R. CUENDA, A. COHEN, P. DUDLEY, D. T. & SALTIEL, A.R. (1995). PD 98059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase, in vitro and in vivo. *J. Biol. Chem.,* **270,** 27489 – 27494.

ALOISI, F., ROSA, S, TESTA, U., BONSI, P., RUSSO, G., PESCHL, E C., & LEVI, G. (1994). Regulation of leukemia inhibitory factor synthesis in culture human astrocytes. *J. Immunol.,* **152,** 5022 – 5031.

BADGER, A.M., BRADBEER, J.N., VOTTA, B., LEE, J.C., ADAMS, J.L. & GRISWOLD, D.E. (1996). Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/ p38kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. *J. Pharmacol. Exp. Ther.,* **279,** 1453 – 1461.

BALLESTAS, M.E. & BENVENISTE E.N. (1995). Interleukin-1β and tumor necrosis factor α mediated regulation of ICAM-1 gene expression in astrocytes requires protein kinase C activity. *GLIA,* **14,** 267 – 278.

BAN, E.M., SARLIEVE, L.L. & HAOUR, F.G. (1993). Interleukin-1 binding sites on astrocytes. *Neuroscience,* **52,** 725 – 733.

BENVENISTE, E.N. (1992). Inflammatory cytokines within the central nervous system: sources, function and mechanism of action. *Am. J. Physiol.,* **263,** C1 – C16.

BERKENBOSCH, F., VAN OERS, J., DEL REY, A., TILDERS, F. & BESEDOVSKY, H. (1987). Corticotropin-releasing-factor producing neurons in the rat activated by interleukin-1. *Science,* **238,** 519 – 521.

BLUMER, K.J. & JOHNSON, G.L. (1994). Diversity in function and regulation of MAP kinase pathways. *Trends Biochem. Sci.,* **19,** 236 – 241.

BOLTON, C., PARKER, D., MCLEOD, J. & TURK, J.L. (1986). A longitudinal study of the prostaglandin content of central nervous system from guinea pigs with acute experimental allergic encephalomyelitis. *J. Neuroimmunol.,* **6,** 151 – 156.

BREDER, C.D. & SAPER, C.B. (1996). Expression of inducible cyclooxygenase mRNA in the mouse brain after systemic administration of bacterial lipopolyssacharide. *Brain Res.,* **713,** 64 – 69.

CANO, E. & MAHADEVAN, L.C. (1995). Parallel signal processing among mammalian MAPKs. *Trends Biochem. Sci.,* **20,** 117 – 122.

CAO, C.Y., MATSUMURA, K., YAMAGATA, K. & WATANABE, Y. (1996). Endothelial cells of the rat brain vasculature express cyclooxygenase-2 messenger RNA in response to systemic interleukin-1: a possible site of prostaglandin synthesis responsible for fever. *Brain Res.,* **733,** 263 – 272.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* **162,** 1556 – 1559.

CONQUER, J.A., KANDEL, R.A. & CRUZ, T.F. (1992). Interleukin-1 and phorbol 12-myristate 13-acetate induce collagenase and PGE_2 production through a protein kinase C independent mechanisms in chondrocytes. *Biochem Biophys. Acta,* **1134,** 1 – 10.

CROFFORD, L.J., WILDER, R.L., RISTIMÄKI, A.P., SANO, H,. REMMERS, E.F. EPPS, H.R. & HLA, T. (1994). Cyclooxygenase 1 and 2 expression in rheumatoid synovial tissues: effects of interleukin-1β, phorbol ester, and corticosteroids. *J. Clin. Invest.,* **93,** 1095 – 1099.

DINARELLO, C.A. (1996). Biologic basis for interleukin-1 disease. *Blood,* **87,** 2095 – 2147.

FAN, L., YOUNG, P.R., BARONE F., FEUERSTEIN, G.Z., SMITH, D. H. & MCINTOSH, K. (1995). Experimental brain injury induces expression of interleukin-1β mRNA in the rat brain. *Mol. Brain Res.,* **30,** 125 – 130.

FRETLAND, D.J. (1992). Potential role of prostaglandins and leukotrienes in multiple sclerosis and experimental allergic encephalomyelitis. *Prostaglandin s Leukot. Essent. Fatty Acids,* **45,** 149 – 257.

FUTAKI, N., TAKAHASHI S., YOKOYAMA, M., ARAI, I., IHIGUCHI, S., IZUKA,H. & OTOMO, S. (1993). Selective inhibition of NS-398 on prostanoid production in inflammed tissue in rat carrageenan-air-pouch inflammation. *J. Pharm. Pharmacol.,* **45,** 753 – 755.

GERBICKE-HAERTER, P.J., SCHOBERT, A. & HERRTING, G. (1991). Pertussis and cholera toxins inhibit prostaglandin synthesis in rat astrocyte cultures at distinct metabolic steps. *Biochem. Pharmacol.,* **42,** 1267 – 1271.

GIULIAN, D., BAKER, T.J., SHICH, L.H. & LACHMAN, L.B. (1986). Interleukin-1 of the central nervous system is produced by ameboid microglia. *J. Exp. Med.,* **164,** 594 – 604.

GRIFFIN, D.E., WESSELINGH, S.L. & MCARTHUR, J.C. (1994). Elevated central nervous system prostaglandins in human immunodeficiency virus-associated dementia. *Ann. Neurol.,* **35,** 592 – 597.

GUAN, Z., BAIER, L.D. & MORRISON, A.R. (1997). p38 Mitogen-activated protein kinase down-regulates nitric oxide and up-regulates prostaglandin E_2 biosynthesis stimulated by interleukin-1β. *J. Biol. Chem.,* **272,** 3296 – 3301.

GUAN, Z., BUCKMAN, S.Y., PENTLAND, A.P., TEMPLETON, D.J. & MORRISON, A.R. (1998). Induction of cyclooxygenase-2 by the activated MEKK1, SEK1/MKK4 p38 mitogen activated protein kinase pathway. *J. Biol. Chem.,* **273,** 12901 – 12908.

HAMBLETON, J., WEINSTEIN, S:L., LEM, L. & DEFRANCO, A.L. (1996). Activation of C-Jun N-terminal kinase in bacterial lipopolyssacharide-stimulated macrophages. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 2774 – 2778.

HANSEN, M.K. & KRUEGER, J.M. (1997). Subdiaphragmatic vagotomy blocks the sleep- and fever-promoting effects of interleukin-1β. *Am. J. Physiol.,* **273,** 1246 – 1253.

HARTUNG, H.P., SCHÄFFER, B., HEININGER, K. & TOYKA, K.V. (1989). Recombinant interleukin-1β stimulates eicosanoid production in rat primary culture astrocytes. *Brain Res.,* **489,** 113 – 119.

HERSCHMAN, H.R. (1996). Prostaglandin synthase 2. *Biochim. Biophis. Acta,* **1299,** 125 – 140.

HOFMANN, J. (1997). The potential for isoenzyme-selective modulation of protein kinase C. *FASEB J.,* **11,** 649 – 669.

HOPKINS, S.J. & ROTHWELL, N.J. (1995). Cytokines in the nervous system I. Expression and recognition. *Trends Neurosci.,* **18,** 83 – 88.

HUG, H. & SARRE, T.F. (1993). Protein kinase C isoenzymes: Divergence in signal transduction? *Biochem J.,* **291,** 329 – 343.

KATSUURA, G., GOTTSCHALL, P.E., DAHL, R. R., & ARIMURA, A. (1989) Interleukin-1β increases PGE_2 in rat astrocyte cultures: modulatory effects of neuropetides. *Endocrinology,* **124,** 3125 – 3127.

KYRIAKIS, J.M. & AVRUCH, J. (1996). Sounding the alarm: protein kinases cascades activated by stress and inflammation. *J. Biol. Chem.,* **271,** 24313 – 24316.

LACROIX, S. & RIVEST, S. (1998). Effect of acute systemic inflammatory response and cytokines on the transcription of genes encoding cyclooxygenase enzymes COX-1 and COX-2 in the rat brain. *J. Neurochem,* **70,** 452 – 466.

LAYE, S., PARNET, P., GOUJON, E., & DANTZER, R., (1994). Peripheral administration of lipopolyssacharide induces the expression of cytokine transcripts in the brain and pituitary of mice. *Mol. Brain. Res.,* **27,** 157 – 162.

LIN, L .L., WARTMANN, M., LIN, A.Y., KNOPF, J.L., SETH, A. & DAVIS, R. J. (1993). cPLA2 is phosphorylated and activated by MAP kinase. *Cell,* **72,** 269 – 278.

LLEDÖ, A., BORRELL, J. & GUAZA C. (1999). Dexamethasone regulation of interleukin-1 receptors in the hippocampus of Theiler's virus infected mice: effects on virus-mediated demyelination. *Eur. J. Pharmacol.,* **372,** 75 – 83.

MARQUETTE, C., VAN DAM, A.M., CECCALDI, P.E., WEBER, P., HAOUR, F., & TSIANG, T. (1996). Induction of immunoreactive interleukin-1β and tumor necrosis factor-α in the brains of rabies-virus-infected rats. *J. Neuroimmunol.,* **68,** 45 – 51.

MCCARTHY, K.D. & DE VELLIS, J. (1980). Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell Biol.,* **85,** 890 – 902.

MERRILL, J. & BENVENISTE, E.N. (1996). Cytokines in inflammatory brain lesions. *Trends Neurosci.,* **19,** 331 – 338.

MOLINA-HOLGADO, F., LLEDÓ, A. & GUAZA, C. (1995). Evidence for cyclooxygenase activation by nitric oxide in astrocytes. *GLIA,* **15,** 167 – 172.

NAM, M.J., THORE, C. & BUSIJA, D. (1995). Rapid induction of prostaglandin synthesis in piglet astroglial cells by interleukin-1-α. *Brain Res. Bull.,* **3,** 215 – 218.

NIIRO, H., OTSUKA, T., OGAMI, E., YAMAOKA, K., NAGANO, S., AKAHOSHI, M., NAKASHIMA, H., ARINOBU, Y., IZUHARA, K., & NIHO, Y. (1998). MAP kinase pathways as a route for regulatory mechanisms of IL-10 and IL-4 which inhibit COX-2 expression in human monocytes. *Biochem. Biophys. Res. Commun.,* **250,** 200 – 205.

NISHIZUKA, Y. (1986). Studies and perspectives of protein kinase C. *Science,* **233,** 305 – 312.

NISHIZUKA, Y. (1992). Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. *Science,* **258,** 607 – 614.

NOGAWA, S., ZHANG, F., ROSS, M.E., IADECOLA, C. (1997). Cyclo-oxygenase-2 gene expression in neurons contributes to ischemic brain damage. *J. Neurosci.,* **17,** 2746 – 2755.

NORRIS, J.G., TANG L.P., SPARACIO S.M., & BENVENISTE, E.N. (1994). Signal transduction pathways mediating IL-6 induction by IL-1β and tumor necrosis factor α *J. Immunol.,* **152,** 841 – 850.

O'BANION, M.K., MILLER, J.C., CHANG, J.W., KAPLAN, M.D., & COLEMAN, P.D. (1996). Interleukin-1β induces prostaglandin G/H synthase-2 (cyclooxygenase-2) in primary murine astrocyte cultures. *J. Neurochem.,* **66,** 2532 – 2540.

O'BANION, M.K., WINN, V.D. & YOUNG, D.A. (1992). cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 4888 – 4892.

OKA, S. & ARITA, H. (1991). Inflammatory factors stimulate expression of group II phospholipase A_2 in rat cultured astrocytes. *J .Biol. Chem.,* **266,** 9956 – 9960.

O'NEILL, L.A.J. & GREENE, C. (1998). Signal transduction pathways activated by the IL-1 receptor family: ancient signalling machinary in mammals, insect and plants. *J. Leukoc. Biol.,* **63,** 650 – 657.

PASINETTI, G.M. & AISEN, P.S. (1998). Cyclooxygenase-2 expression is increased in frontal cortex of Alzheimer's disease brain. *Neuroscience,* **87,** 319 – 324.

POULIOT, M., BAILLARGEON, J., LEE, J.C., CLELAND, L.G., & JAMES, M.J. (1997). Inhibition of prostaglandin endoperoxide synthase-2 expression in stimulated human monocytes by inhibitors of p38 mitogen-activated protein kinase. *J. Immunol.,* **158,** 4930 – 4937.

ROTHWELL, N.J. (1991). Functions and mechanisms of action of interleukin-1 in the brain. *Trends Pharmacol. Sci.,* **12,** 430 – 436.

SANGHERA, J.S., WEINSTEIN, S.L., ALUWALIA, M., GIRN, J., & PELECHJ, S.L. (1996). Activation of multiple proline-directed kinases by bacterial lipopolyssacharide in murine macrophages. *J Immunol.,* **156,** 4457 – 4465.

VANE, J.R., BAKHLE, Y.S., & BOTTING, R.M. (1998). Cyclooxygenases 1 and 2. *Annu. Rev Pharmacol. Toxicol.,* **38,** 97 – 120.

WILMER, W.A., TAN, L.C., DICKERSON, J.A., DANNE, M., & ROVIN, B.D. (1997). Interleukin-1β induction of mitogen-activated protein kinases in human mesangial cells. *J. Biol Chem.,* **272,** 10877 – 10881.

(Received March 14, 2000
Revised May 15, 2000
Accepted June 23, 2000)