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Repression of Inducible Nitric Oxide Synthase and Cyclooxygenase-2 by Prostaglandin E_2 and Other Cyclic AMP Stimulants in J774 Macrophages

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ABSTRACT. The enhanced nitric oxide (NO) and prostaglandin (PG) generation of activated macrophages is controlled by glucocorticoid-sensitive inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively. Negative feedback regulation of iNOS expression by the products of both pathways has been suggested, but their effects on COX-2 expression have not been examined. We have investigated the effect of E- and I-series prostaglandins that activate adenylate cyclase (AC), forskolin (a direct activator of AC), and other agents that influence the cyclicAMP/cyclicGMP systems on the ability of *E. coli* endotoxin (lipopolysaccharide, LPS) to induce iNOS and COX-2 in the murine macrophage cell line J774. After a 2-hr pretreatment before adding endotoxin, PGE_2, PGI_2, forskolin, IBMX (isobutylmethylxanthine, a cyclicAMP/cyclicGMP phosphodiesterase inhibitor), 8-bromo cyclicAMP, and arachidonic acid itself all inhibited the expression of both iNOS and COX-2 (as shown by Western blotting), and reduced NO release and COX activity, whereas $PGF_{2\alpha}$ and 8-bromo cyclic GMP were only weakly effective. The effects of PGE_2, PGI_2, and forskolin were enhanced by cotreatment with IBMX. The suppression of LPS-induced iNOS induction by PGE_2 was functionally significant, in that it protected against the mild cytotoxicity of the NO generated in response to endotoxin. These results provide the first direct evidence for the feedback regulatory suppression of COX-2 induction by a PG-driven cAMP-mediated process, and show that the modulation of iNOS and COX-2 induction shares common features. They also suggest that such modulation is normally held in check by high phosphodiesterase activity within these cells. BIOCHEM PHARMACOL 53;4:493–500, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. inducible nitric oxide synthase; cyclooxygenase-2; Western blot; lipopolysaccharide; cAMP; forskolin; prostaglandin E_2; phosphodiesterase inhibitors; cytotoxicity

Activated macrophages are capable of generating greatly increased amounts of nitric oxide (NO) and eicosanoids by means of enzymes whose expression is induced by such factors as cytokines and endotoxin, and that may be repressed by anti-inflammatory glucocorticoids. For example, LPS‡ causes expression of both iNOS and COX-2 isoforms and the consequent generation of large quantities of NO [1–7] and prostaglandins [6–12] in mouse macrophages and derived cell lines such as RAW264.7 or J774.

There is also evidence that the products of these pathways may exert feedback regulatory actions on the expression or activity of their biosynthetic enzymes in macrophages. Thus, NO may directly activate both constitutive and inducible COX isoforms to increase their ability to generate prostaglandins [6], although others have reported that NO can inhibit both COX induction and its activity in J774 macrophages [13]. Conversely, prostaglandin E_2 (PGE_2) and iloprost (a prostacyclin analogue) were found to partially inhibit NO generation in LPS-treated J774 cells, an action that was attributed to repression of induction of iNOS, although direct evidence for effects exerted at the level of expression of enzyme protein was not provided [14, 15]. However it is not yet known if the prostaglandin-cyclic AMP (cAMP) system can affect the expression of COX enzymes in macrophages or how any such effects relate to the induction of iNOS.

We have, therefore, investigated whether or not PGs that activate adenylate cyclase are capable of downregulating the expression of COX-2 and iNOS in J774 macrophage-like cells.

MATERIALS AND METHODS

Cell Culture; Measurement of Nitrite Released Into the Medium

J774 cells from the European Collection of Animal Cell Cultures, Porton Down, Wiltshire (U.K.), were plated at 5

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‡ *Abbreviations:* AC, adenylate cyclase; cAMP, cyclic AMP; cGMP, cyclic GMP; COX-2, inducible form of cyclo-oxygenase; DMEM, Dulbecco's modified Eagle's medium; IBMX, isobutylmethylxanthine; iNOS, inducible form of nitric oxide synthase; LPS, bacterial lipopolysaccharide; PGE_2, prostaglandin E_2; PMSF, phenylmethylsulphonyl fluoride.

× 10^5 cells/mL in 24-well plates in 1 mL DMEM containing 5% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) and cultured at 37°C in 5% CO_2. When confluent, the cells were pretreated with drugs for 2 hr, followed by 500 ng/mL *E. coli* lipopolysaccharide (serotype 026:B6, Sigma) for a further 16 hr. The medium was then removed and centrifuged at 3000 rpm for 5 min prior to assay of NOS activity, which was measured as the amount of NO_2^- generated. For this, 100 μL aliquots were added to 100 μL of Griess reagent and the OD_{550} values were measured in a microplate reader [13].

Analysis of Cyclooxygenase Activity: Radioimmunoassay and Radiochemical Methods

After preincubation and 16-hr culture, COX activity in adherent cells was assayed functionally by washing the cells 3 times in PBS and then adding 10^{-6}M arachidonic acid (AA) for a further 1-hr incubation in culture medium; this method was modified from [6]. The medium was retained and these samples were subjected to radioimmunoassay for PGE_2.

In additional experiments, COX activity was measured radiometrically [16]. For this, cells were placed at 5 × 10^5 cells/mL in 6-well plates in 2 mL DMEM and pretreated, cultured, and washed as above, but 0.75 μCi/mL [^{3}H]-arachidonic acid (Dupont, sp. act. 100 Ci/mmol) was then added for 1 hr. After acidifying and extracting the medium into ethyl acetate, the residue was applied to an aluminum-backed silica gel-coated TLC sheet (Merck, type 5554) and developed for 40 min in a solvent of ethyl acetate/formic acid [80:1, v/v]. The extent of conversion of AA to PGE_2 was assessed by comparing dpm in the relevant sections cut out from the chromatogram. A Panax radiochromatogram scanner was used to locate the positions of authentic radiolabelled arachidonic acid and prostaglandin E_2.

Western Blotting for iNOS and COX-2 Expression

Expression of iNOS and COX-2 proteins was measured by Western blotting [17]. After washing the cells with PBS, proteins were extracted into 0.9% NaCl, tris-HCl 20 mM, pH 7.6, Triton X-100 0.1%, PMSF 1 mM, leupeptin 0.01% with gentle shaking, centrifuged, mixed 1:1 with sample buffer (tris-HCl 20 mM, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.025% Bromophenol Blue) and boiled.

Electrophoresis was performed using 7.5% SDS-polyacrylamide gel (5 hr, 500V, 36 mA, 30 μg protein per track). Separated proteins were transferred to Biorad nitrocellulose membranes (2 hr, 40V), nonspecific IgGs were blocked with 8% fat-free milk powder and the blot incubated for 1 hr with primary antibody, viz. rabbit antisera to murine COX-2 (Cayman, 1:1000) or to murine iNOS (gift of Dr. V. Riveros-Moreno, 1:1000). The blot was incubated with secondary antibody (sheep antirabbit IgG linked to horseradish peroxidase conjugate, Sigma, 1:2000) for 1 hr and, finally, incubated with ECL reagent (Amersham) for 1 min and exposed to Hyperfilm-ECL (Amersham).

Cell Viability Assay

Cell viability was measured by adding 50 μL 5 mg/ml MTT (Thiazolyl Blue) to cells in 24-well plates and incubating for 1 hr at 37°C [17]. After removing the medium, 500 μL isopropanol was added to solubilize the blue-coloured tetrazolium and the plates were shaken for 5 min. Aliquots of 200 μL were transferred to 96-well plates and the OD_{550} values were read in a microplate reader. Viability was set as 100% in control cells.

Statistical Analysis

The statistical significance of differences between groups was assessed by Student's unpaired t-test.

RESULTS

Treatment of J774 macrophages for 16 hr with LPS caused a large increase in nitric oxide synthase activity (measured as nitrite accumulation in the medium) and enhanced COX activity (measured as PGE_2 generated when the washed cells were incubated with arachidonic acid, Table 1). These responses are due to the induction of iNOS and COX-2 enzyme proteins, and this was confirmed by immunoblotting (Fig. 1A, B, C: compare the first 2 lanes in each of the 3 blots).

Pretreatment of the cells for 2 hr with various doses of PGE_2, prostacyclin (PGI_2), arachidonic acid (AA), forskolin (a direct activator of AC), or IBMX (a cyclicAMP/cyclicGMP phosphodiesterase inhibitor) all caused concentration-dependent reduction of NO generation (Table 1). The highest concentration of PGE_2 (10^{-4}M) abolished NO generation completely.

These treatments also reduced the LPS-induced expression of iNOS in a concentration-related manner, as shown in Fig. 1 (PGE_2 and forskolin in blot A; IBMX, AA, and PGI_2 in blot B). Iloprost at 10^{-6}M was also effective (Table 1, Fig. 1, blot B lane 9), but the stocks were not sufficient to test higher concentrations. $PGF_{2\alpha}$ was inactive, except at 10^{-4}M, which produced a small but significant reduction in NO generation without any evident effect on iNOS expression (Table 1, Fig. 1, blot A).

Coaddition of 10^{-5}M IBMX with various doses of either PGE_2, PGI_2, or forskolin caused a larger inhibition of NO generation than was observed with either alone (Table 1). Addition of the highest doses of PGE_2, PGI_2, forskolin, and IBMX 6 hr *after* LPS failed to blunt NO release (data not shown).

PGE_2, PGI_2, forskolin, and IBMX also caused dose-dependent suppression of LPS-induced COX-2 protein expression (Fig. 1), but $PGF_{2\alpha}$ had no effect. Arachidonic

TABLE 1. Effect of prostaglandins and other treatments on nitric oxide synthase and cyclooxygenase activity in J774 cells stimulated with lipopolysaccharide

	iNOS activity* *NO_2^- in medium after 16 hr, μM.		COX-2 activity† †PGE_2 generated from 10^{-6}M arachidonate, ng/mL.	
Treatment	**Alone**	**+ IBMX, 10^{-5}M**	**Alone**	**+ IBMX, 10^{-5}M**
Cells alone	0.78 ± 0.08		2.16 ± 0.18	
LPS 500 ng/mL	14.84 ± 0.31		7.01 ± 0.21	
+ PGE_2 10^{-8}M	13.48 ± 0.32		n.m. [see Fig 3]‡	
+ PGE_2 10^{-7}M	12.39 ± 0.18§	2.71 ± 0.08#	n.m. [see Fig 3]‡	
+ PGE_2 10^{-6}M	11.08 ± 0.34¶	2.03 ± 0.10#	n.m. [see Fig 3]‡	
+ PGE_2 10^{-5}M	3.36 ± 0.51¶	1.50 ± 0.07#	n.m. [see Fig 3]‡	
+ PGE_2 10^{-4}M	0.98 ± 0.11¶		n.m. [see Figs 2 and 3]‡	
+ PGI_2 10^{-6}M	10.96 ± 0.80‖	4.67 ± 0.14#	n.m.‡	
+ PGI_2 10^{-5}M	7.88 ± 0.31¶	4.03 ± 0.37#	n.m.‡	
+ PGI_2 10^{-4}M	5.54 ± 0.20¶	3.16 ± 0.16#	n.m. [See Fig 2]‡	
+ iloprost 10^{-6}M	9.82 ± 0.46¶	4.39 ± 0.88#	5.32 ± 0.24¶	4.56 ± 0.18#
+ $PGF_{2\alpha}$ 10^{-6}M	14.77 ± 0.64		n.m.‡	
+ $PGF_{2\alpha}$ 10^{-5}M	14.99 ± 0.57		n.m.‡	
+ $PGF_{2\alpha}$ 10^{-4}M	9.83 ± 0.86‖		n.m. [See Fig 2]‡	
+ forskolin 10^{-7}M	15.28 ± 0.55			
+ forskolin 10^{-6}M	12.67 ± 0.36§	6.42 ± 0.79#	4.45 ± 0.20¶	5.58 ± 0.54
+ forskolin 10^{-5}M	11.13 ± 0.41‖	3.96 ± 0.23#	3.80 ± 0.34¶	3.02 ± 0.10#
+ forskolin 10^{-4}M	9.23 ± 0.23¶	2.83 ± 0.75#	3.22 ± 0.32¶	1.92 ± 0.10#
+ dexamethasone 10^{-5}M	0.84 ± 0.21¶		1.98 ± 0.06¶	
+ IBMX 10^{-6}M	12.72 ± 0.74§		5.04 + 0.19¶	
+ IBMX 10^{-5}M	8.59 ± 0.82¶		4.62 + 0.12¶	
+ IBMX 10^{-4}M	5.27 ± 0.73¶		3.36 + 0.35¶	
+ AA 10^{-6}M	12.27 ± 0.22¶	6.79 ± 1.37#	6.71 ± 0.31	5.58 ± 0.54
+ AA 10^{-5}M	10.72 ± 0.17¶	5.86 ± 1.18#	5.89 ± 0.17¶	5.31 ± 0.41
+ AA 10^{-4}M	7.77 ± 0.45¶	5.53 ± 1.13#	3.54 ± 0.22¶	3.31 ± 0.15
+ 8Br-cAMP 10^{-4}M	1.11 ± 0.28¶		3.36 ± 0.38¶	
+ 8Br-cGMP 10^{-4}M	9.37 ± 0.37‖		5.28 ± 0.45‖	

Results show mean ± SEM for 6 or more tests per treatment, performed on at least 2 separate batches of cells. ‡n.m. = not measured (due to interference with radioimmunoassay for PGE_2 despite washing of cells prior to addition of arachidonic acid). §$p < 0.05$; ‖$p < 0.01$; ¶$p < 0.001$; statistically significant inhibition with respect to LPS-treated cells; #$p < 0.05$, significantly greater inhibition than with each agent tested alone (Student's unpaired t test).

acid added at 10^{-4}M suppressed COX-2 expression (Fig. 1, blot B, lane 11).

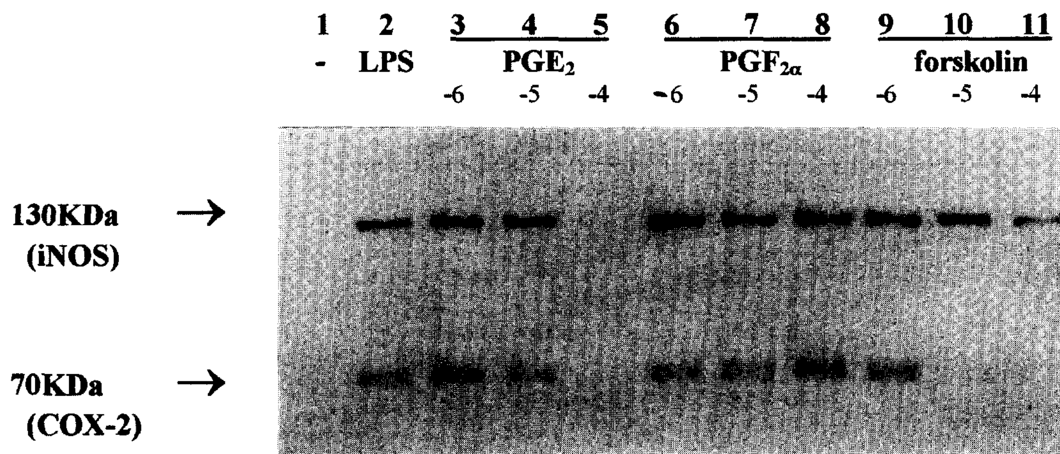
Functional evidence for COX-2 repression by the prostaglandins could not be obtained using the radioimmunoassay method (see footnote to Table 1) and a radiometric method was employed (see below); however, forskolin and IBMX both partially reduced the LPS-induced increase of COX activity toward the level observed in untreated cells. Added in combination, forskolin and IBMX completely abolished the induction of COX-2 protein (Table 1).

Preincubation with the stable lipophilic 8-bromo analogues of cyclic AMP and cyclic GMP also reduced LPS-induced iNOS and COX-2 enzyme activity and reduced the expression of their proteins, but the effect was much greater for the cAMP analogue (Table 1, Fig. 1, blot C).

As expected, dexamethasone prevented the activity and expression of both enzymes (Table 1, Fig. 1: blot B, lane 10).

Although the evidence described this far shows that pretreatment with PGE_2 and some other prostaglandins downregulates the expression of COX-2 protein, it was not possible to assay this directly using the standard protocol in which arachidonic acid is added to washed cells. This is because some of the prostaglandin must remain within or adherent to the cells, even after washing them several times, thus interfering with any prostaglandin formed after adding exogenous arachidonic acid for the COX activity (see Methods and legend to Table 1). Instead, it was necessary to employ a radiochemical method. For this, the cells were washed after the 2-hr pretreatment and 16-hr incubation with LPS, and then exposed for 1 hr to ^{3}H-AA. The resulting PGE_2-like material was measured after TLC separation from substrate. Figure 2 (upper part) shows that, after pretreatment with either PGE_2 or IBMX at 10^{-4}M, COX activity was substantially and significantly reduced, whereas $PGF_{2\alpha}$ was less effective. The combination of PGE_2 and

A



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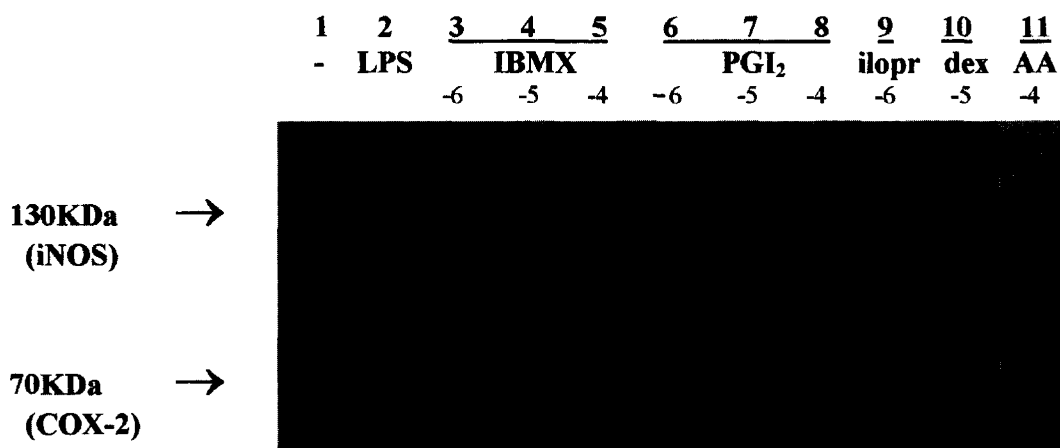

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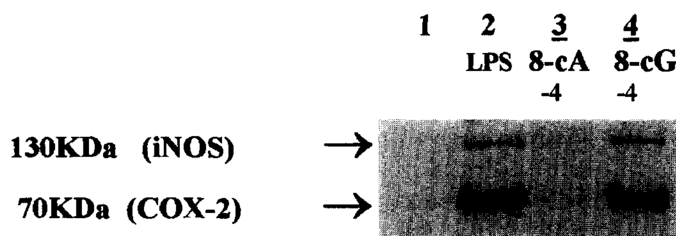

FIG. 1. Induction and repression of iNOS and COX-2 in J774 cells: effects of prostaglandins and other treatments. Each blot shows control untreated cells (marked -) and cells treated for 16 hr with 500 ng/mL LPS. All other lanes show cells treated with 500 ng/mL LPS together with 2-hr pretreatments as shown at the top of each lane. (A) Top blot: Lanes 3–5, Pretreated with PGE_2 at 10^{-6}M, 10^{-5}M, and 10^{-4}M, respectively; Lanes 6–8, $PGF_{2\alpha}$; Lanes 9–12, forskolin. (B) Middle blot: Lanes 3–5, Pretreated with IBMX at 10^{-6}M, 10^{-5}M, and 10^{-4}M, respectively; Lanes 6–8, PGI_2 (prostacyclin); Lane 9, iloprost 10^{-6}M; Lane 10, dexamethasone, 10^{-5}M; Lane 11, arachidonic acid, 10^{-4}M. (C) Bottom blot: Lane 3, 8-bromo cyclic AMP; Lane 4, 8-bromo cyclic GMP, both 10^{-4}M. The positions and molecular weights of iNOS and COX-2 were validated by reference to molecular weight markers (Rainbow™, Amersham). These blots are representative of similar results obtained 3 or more times.

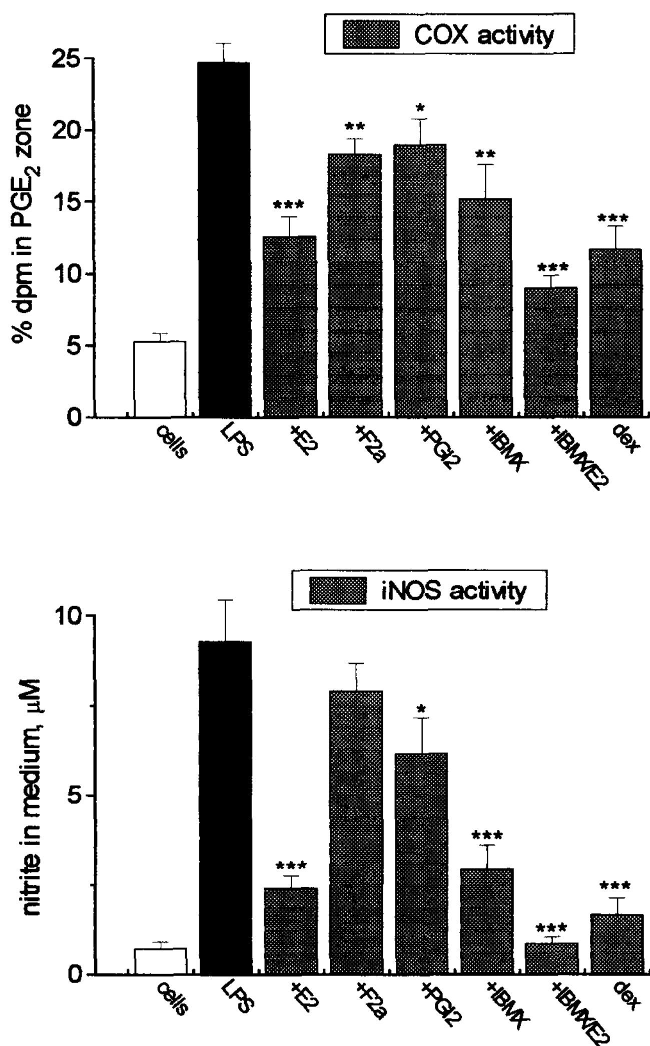

Fig. 2. Repression of LPS-induced COX-2 and iNOS activity in J774 cells. Cells were pretreated for 2 hr with PGE_2, $PGF_{2\alpha}$, PGI_2 and IBMX (all at 10^{-4}M), dexamethasone (10^{-5}M), indomethacin (10^{-6}M), or with vehicle control, then 500 ng/mL LPS was added for a further 16-hr incubation. After removing the medium for nitrite assay, the cells were washed and treated with 2.28 µg/ml ^{3}H-labelled arachidonate (0.75 µCi/mL) for 1 hr. After extraction, the conversion to PGE_2 was measured by TLC as described in Methods. Values show mean ± SEM for 6–12 determinations from 3–6 separate experiments; Statistically significant inhibition with respect to LPS-treated cells, *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

IBMX had a larger inhibitory effect than either added alone, and this was greater than that observed for dexamethasone. The prostaglandin/IBMX pretreatments also reduced iNOS activity (Fig. 2, lower part), in line with the data given in Table 1. The effect of PGE_2 pretreatment to reduce the activity of COX-2 after induction by LPS was dose-related (Fig. 3).

Because NO is known to exert cytotoxic and enzyme inhibitory actions in vitro, it was of interest to evaluate whether or not PGE_2 might protect against LPS-induced damage to the J774 macrophages. Figure 4 shows that 16-hr

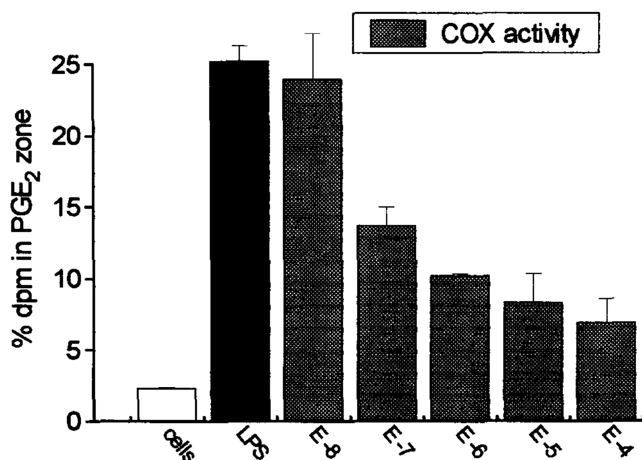

FIG. 3. Dose-dependent repression of LPS-induced COX-2 by PGE_2. Cells were pretreated for 2 hr with various doses of PGE_2, then incubated with LPS and assayed for COX activity as in Fig. 2. Values show mean value and range for duplicate determinations in a single experiment.

culture with LPS reduces cell viability by about 30%, and that PGE_2 exerts a dose-related protective effect against this mild cytotoxicity, restoring the cells to near complete viability at 10^{-4}M. Treatment of the J774 cells with the same doses of PGE_2 alone did not affect their viability (data not shown).

DISCUSSION

These data show that high concentrations of E- and I-type prostaglandins and other treatments that are likely to elevate cAMP in J774 macrophages dose-dependently suppress the LPS-induced induction of the iNOS and COX-2 enzymes. This is the first direct evidence for the feedback

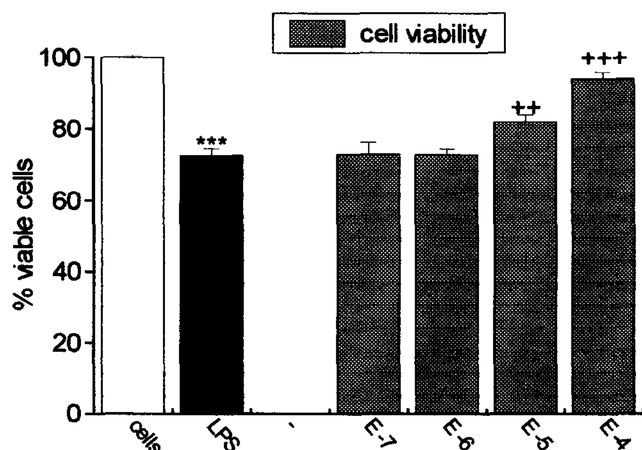

FIG. 4. LPS induces a mild cytotoxicity of J774 cells that is protected in dose-dependent fashion by PGE_2. Cells in a 96-well microtitre plate were pretreated for 2 hr with various doses of PGE_2, then incubated with 500 ng/mL LPS for 16 hr and assayed for cytotoxicity using the MTT assay. Values show mean value ± SEM for 4 tests in a single experiment.

regulatory suppression of COX-2 expression via a PG-driven cAMP-related mechanism, and confirmation of previous reports that iNOS expression can be prevented in this way [14, 15, 18–20]. Direct evidence of reduced content of both enzymes was obtained by Western blotting, but the mechanism whereby cAMP/protein kinase A activation causes this effect (at transcriptional or posttranslational levels, or both) has not been established. This requires investigation of how the treatments affect the rate of gene transcription and the stability of the mRNA for the two enzymes. However, the latter possibility (i.e. that there is more rapid degradation of mRNA or of the proteins themselves) does not appear to be very likely because the treatments were ineffective if applied after the LPS at a time when NO and PGE_2 generation were increasing.

Intense interest in the pathophysiological roles of nitric oxide has prompted many recent studies to determine how iNOS enzyme activity is controlled. Among these are several showing that iNOS induction in macrophages and other cells can be reduced or prevented pharmacologically (e.g. by glucocorticoids, cytokines such as IL-10 and IL-13, growth factors such as TGFβ, PGDF and IGF-1, tyrosine kinase inhibitors, polyamines, and the phospholipase C inhibitor D609 [21–31]), as well as by large amounts of NO generated intrinsically or released from NO donors [32–37]. As mentioned above, there are also previous studies showing that PGE_2 or elevated cAMP can negatively modulate iNOS induction, both in macrophages and in mesangial cells [14, 15, 18–20], in line with the present report. However, others have found that cAMP-elevating agents can, under certain conditions, themselves cause induction of iNOS, albeit weakly, or synergistically enhance cytokine-induced iNOS expression in both macrophages and mesangial cells [15, 38–41]. These conflicting reports show that the interactions of the cAMP system with cellular activation and genomic regulation of inducible enzymes are complex and that simplistic generalisations are inadvisable.

In contrast to the relative abundance of work on iNOS, COX-2 regulation in macrophages has received less attention. However, it is well-established that glucocorticosteroids inhibit COX-2 expression induced in monocytes and macrophages as well as in other cells (e.g. [8–10, 42–45], Fig. 1). The present work shows clearly that COX-2 expression can also be functionally downregulated (at least in part) by a cAMP-responsive mechanism that can be activated by PGs that stimulate adenylate cyclase and by forskolin (a direct adenylate cyclase activator) and a cAMP analogue, or by enhancing cyclic nucleotide levels using the nonselective phosphodiesterase inhibitor IBMX. Unfortunately, the adenylate cyclase inhibitor MDL-12330 [46] proved to be too toxic to the J774 cells to enable further pharmacological evaluation of the role of adenylate cyclase in these responses.

However, the situation is complex, because genetic studies of the promoter region of the human COX-2 gene have shown that the cAMP-sensitive element is needed for full COX-2 expression in U937 monocytic cells [47], and this may be the case also for the mouse gene. Moreover, in our experiments, PGE_2 may not only be acting via a cAMP-elevating mechanism. There are at least 4 different EP receptors involving various transduction mechanisms, including G_s-mediated elevation of cAMP (EP_2, EP_4) and $G_{q/11}$-coupled activation of phosphoinositide metabolism (EP_1, EP_3) [48]. Our work does not establish which of these receptors are operative in J774 cells. In fact, we cannot exclude contributions (albeit rather weak) from the cGMP system, because both $PGF_{2\alpha}$ and 8Br-cGMP caused small reductions in COX-2 activity and expression (see Table 1 and Fig. 1, respectively), but the cAMP-related mechanism is obviously more important.

The concept that prostaglandins acting via cAMP elevation can downregulate macrophage functions is well established [49–51], reviewed in [52, 53]. For example, PGE_2 suppresses mouse macrophage TNFα gene expression [54]. However, macrophage sensitivity to the prostaglandin rapidly wanes due to desensitization [55]: it is not known whether the 2-hr preincubation period used for the experiments here had already reduced sensitivity to the prostaglandins. If so, this might explain why somewhat higher concentrations were required to downregulate iNOS than observed by Marotta et al. [14], although they did not investigate higher doses and inhibition was only partial, whereas here it was complete.

It was interesting that pretreatment of the cells with arachidonic acid itself also caused downregulation of the iNOS and COX-2 proteins and that this was enhanced by IBMX. This indicates that the arachidonic acid-prostaglandin system might be capable of exerting an intrinsic modulatory role in intact cells. It also shows that, although the concentrations of added prostaglandins required for suppression of the enzyme inductions were high, adequate concentrations can be obtained if the eicosanoids are generated at the target site, rather than added exogenously.

Finally, it is obvious that cyclic nucleotide phosphodiesterases play an important role in keeping these cAMP regulatory mechanisms in check. This is in line with other studies that have shown that, for optimal inhibitory effects of cAMP-mediated processes in macrophages, coaddition of prostaglandins and phosphodiesterase inhibitors is desirable and may represent a valuable therapeutic option [56, 57]. It would be useful to establish how these enzymes are themselves regulated in macrophages, so as to gain further insight into the mechanisms governing the regulation of important immunoregulatory enzymes whose expression is influenced by cyclic AMP.

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