Selective Modulation of BV-2 Microglial Activation by Prostaglandin E₂

DIFFERENTIAL EFFECTS ON ENDOTOXIN-STIMULATED CYTOKINE INDUCTION*

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The influence of prostaglandins on glial functions and, more specifically, on glial activation is not well understood. We report here that prostaglandin E₂ (PGE₂), one of the major prostaglandins produced in the brain, acts as a potent and selective inhibitor of tumor necrosis factor α (TNF-α) production in lipopolysaccharide-stimulated primary microglia and the microglial cell line BV-2. The IC₅₀ for this effect is 1 nM, and 100 nM PGE₂ suppresses TNF-α production by >95%. More detailed studies of BV-2 cells show that PGE₂ also prevents the secretion of interleukin (IL)-1β, but does not significantly modify lipopolysaccharide-stimulated expression of cyclooxygenase-2, pro-IL-1β, or inducible nitric oxide synthase. PGE₂ appears to act primarily at the level of translation or protein stability, because TNF-α and IL-6 mRNA levels were only modestly decreased at high PGE₂ concentrations; concomitantly with this inhibition, PGE₂ up-regulated the levels of IL-1β mRNA. The effects of PGE₂ could be largely mimicked by 8-bromo-cAMP, suggesting that, as in other cell types, PGE₂ action is mediated at least in part by a rise in intracellular cyclic AMP. However, the protein kinase A inhibitor H89 only partially reversed the inhibition of TNF-α production by PGE₂, implying that the PGE₂ effect in BV-2 cells is mediated through both protein kinase A-dependent and -independent pathways.

One prominent feature of neurodegenerative diseases such as Alzheimer’s disease is the presence of activated microglial cells (1–3). Transition of microglia from the resting state to the activated one in response to a pathological stimulus is characterized in part by the production of various cytokines and the induction of inflammation-related enzymes such as inducible nitric oxide synthase (iNOS)¹ and cyclooxygenase-2 (COX-2) (4). Transient activation of microglia is most likely beneficial for brain-repairing processes; however, a chronic reactive state of microglia or an abnormally high proportion of activated microglia may become dangerous by increasing the inflammatory burden.

Recent data indicate that the prolonged treatment of individuals with nonsteroidal anti-inflammatory drugs decreases the probability of development of Alzheimer’s disease (5, 6). This in turn suggests that inhibition of COX-2 activity may be beneficial in the treatment of neurodegenerative conditions. Although the induction of COX-2 in glia (4, 7, 8) and neurons (9, 10) is well documented, the mechanisms underlying the effect of nonsteroidal anti-inflammatory drugs in the brain as well as the effects of increased prostaglandin production on glial functions are not well understood and need to be investigated at the molecular level. The goal of this study was to determine how prostaglandin E₂ (PGE₂), one of the major prostaglandins produced in the brain, affects the production of activation-related molecules in microglia. We studied the proteins produced both at early (TNF-α and IL-1β) and later (IL-6, iNOS, COX-2) stages of microglial activation in vitro, and we report here that PGE₂ acts as a potent and selective inhibitor of TNF-α and IL-6 production. The mechanism of this inhibition and the role of cAMP were also investigated.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**PGE₂ was purchased from Cayman Chemical (Ann Arbor, MI) and prepared from powder as ethanol solutions. Bacterial lipopolysaccharide (LPS) from Salmonella typhimurium (Sigma; l-2622) was resuspended in sterile phosphate-buffered saline at 10 mg/ml and stored at −20 °C. Mouse macrophage-colony stimulating factor (R&D Systems) was dissolved in phosphate-buffered saline containing 0.1% fatty acid-free bovine serum albumin (Sigma) to make a 20 µg/ml stock solution and stored in aliquots at −80 °C. H89 (Alexis) and 8-bromo-cAMP (Sigma) were prepared as 25 and 100 mM stock solutions, respectively, in sterile distilled water and stored in aliquots at −20 °C.

**Cell Culture and Treatment—**Primary rat microglia were prepared and maintained as described elsewhere (11). The purity of microglial cultures was assessed using OX42 antibody (Serotec Inc., Raleigh, NC), and >95% of cells stained positively. After shake-off, cells were cultured for 2 days before treatment with LPS and prostaglandins. Immediately before treatment, serum-containing medium was removed, cells were washed twice with warm α-minimum Eagle’s medium, and then α-minimum Eagle’s medium containing N2 supplements (Life Technologies, Inc.) and 10 mg/ml macrophage-colony stimulating factor were added.

BV-2 (a murine microglial cell line generously provided by Dr. Michael McKinney; Mayo Clinic, Jacksonville, FL) cells were cultured in α-minimum Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were washed twice with warm α-minimum Eagle’s medium, and then α-minimum Eagle’s medium containing N2 supplements (Life Technologies, Inc.) and 10 mg/ml macrophage-colony stimulating factor were added.

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(Tousimis; Rockville, MD) for 10 min, permeabilized with 0.1% Triton X-100 for 5 min, and blocked with 10% normal donkey serum for 30 min. Slides were incubated for 1 h at room temperature with goat anti-rat IL-1β (R&D Systems) primary antibody at a 1:2000 dilution, washed, and then incubated for 30 min with fluorescein isothiocyanate-labeled donkey anti-goat secondary antibody (Jackson ImmunoResearch) at a 1:400 dilution in the presence of 10% normal donkey serum. Slides were mounted and viewed with a Zeiss Axiosplan microscope. No punctate staining or very weak punctate staining of microglial cells was observed when goat anti-rat TNF-α antibodies were used instead of goat anti-rat IL-1β. The lack of TNF-α staining is most likely because this cytokine is rapidly secreted from microglial cells, and the immunofluorescence method is not sensitive enough to detect the low levels of remaining intracellular and membrane-bound TNF-α protein. The absence of TNF-α staining provides a specificity control for the immunofluorescence method.

**Protein Kinase A Assays**—BV-2 cells were pre-treated with 5 μM H89 or control buffer for 30 min and then stimulated with 1 μM PGE₂ or vehicle for 3 min. Protein kinase A activity was determined in digitonin-permeabilized cells as described previously (12), except that cells were plated at 5 x 10⁵ cells/well in 48-well plates and grown for 2 days before assay.

**ELISA and Western Blotting**—TNF-α and IL-1β levels in cell conditioned media were determined using DuoSet TNF-α and IL-1β kits (Genzyme) according to the manufacturer’s instructions. Cell lysates were prepared, and Western blotting was performed as described previously (11) using the LumiGlo® chemiluminescence detection system (New England BioLabs). To determine IL-6 levels, bovine serum albumin was added to 300–400 μl of conditioned media to a final concentration of 0.2 mg/ml, and proteins were precipitated in 5% trichloroacetic acid. After a 5-min incubation on ice, precipitates were recovered by centrifugation for 5 min at 12,000 rpm at 4°C, and pellets were dissolved in 40 μl of 0.1 N Trisma base. Aliquots were saved for determination of protein concentration, and the levels of IL-6 in the samples were determined by Western blotting. IL-6, pro-IL-1β, iNOS, and COX-2 protein levels were determined by Western blotting using the following antibodies: goat anti-mouse IL-6 (R&D Systems), rabbit anti-mouse IL-1β (Genzyme), monoclonal anti-murine mac-NOS (Transduction Laboratories), and goat anti-human COX-2 (Biotechnology, Inc., Santa Cruz). All primary antibody dilutions were 1:1000. Secondary goat anti-rabbit, goat anti-mouse, and rabbit anti-goat antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch) were used at 1:5000 (goat anti-rabbit and rabbit anti-goat) and 1:2000 (goat anti-mouse) dilutions.

**Preparation of TNF-α and IL-6 cDNA Probes, RNA Isolation, and Northern Blotting**—IL-16 cDNA probe was described previously (13). Partial cDNA fragments of TNF-α (692 base pairs; nucleotides 1–692; Ref. 14) and IL-6 (446 base pairs; nucleotides 134–581; Ref. 15) were prepared by reverse transcription-polymerase chain reaction (first-strand cDNA synthesis by SuperScript II, Life Technologies, Inc.) from rat astrocyte total RNA. We used the following polymerase chain reaction primers: (a) TNF-α (For), 5′-ATGACGACCAACGATTCCGCGCCG-3′; (b) TNF-α (Rev), 5′-CCAAAGTAGACCTGCCCGGACTC-3′ (Integrated DNA Technologies, Inc). Amplified products (AmpliTag, Perkin-Elmer) were TA-cloned into pCR2.1 (Invitrogen), and the insert sequences were verified by DNA sequence analysis (Dye Terminator Cycle Sequencing; ABI Prism, PE Applied Biosystems). The partial cDNA constructs were identified as pCRII-TNF-α (1–692) and pCRRII-IL-6 (134–581). The EcoRI digest fragments of these constructs were gel-purified and labeled with α-³²PdCTP (Redi-Prime; Amersham Pharmacia Biotech) for Northern blot analysis.

Total RNA was isolated from the cells, and Northern blots were performed as described previously (15). Equal loading of RNA was verified by stripping the membranes and reprobing with rat pTri-glyceraldehyde-3-phosphate dehydrogenase probe (Ambion, Austin, TX).

**Statistics**—Statistics have been calculated using Student’s t test with significance established at a level of p < 0.05.

**RESULTS**

**PGE₂ Suppresses TNF-α but not IL-1β Production in Primary Rat Microglia**—Primary microglia show a strong increase in TNF-α production at 3 h after stimulation with LPS, whereas pre-treatment with 1 μM PGE₂ significantly inhibited production of this cytokine (Fig. 1A). To examine the generality of this response, we tested whether the production of another pro-inflammatory cytokine, IL-1β, was affected by PGE₂. The levels of IL-1β were detected by immunostaining and Western blotting, based on previous findings that IL-1β in activated microglia, unlike TNF-α, remains primarily cell-associated (16). In contrast to TNF-α, levels of IL-1β were not affected by treatment with PGE₂ (Fig. 1B); on the contrary, PGE₂ even seemed to stimulate the production of IL-1β somewhat.

**PGE₂ Inhibits TNF-α and IL-6 Protein Production in LPS-stimulated BV-2 Cells**—The murine microglial cell line BV-2 was shown to reproduce many microglial responses in culture and has been used as a model microglial system in other studies (17, 18). Stimulation of BV-2 cells with LPS leads to a robust increase of TNF-α levels in the cell-conditioned media, which becomes evident by 3 h and reaches a maximum by 6 h (data not shown). Pre-treatment of BV-2 cells with PGE₂ inhibited LPS-induced TNF-α production in a dose-dependent manner, with an IC₅₀ of ~1 μM (Fig. 2A). Treatment of BV-2 cells with PGE₂ also prevented LPS-stimulated accumulation of another cytokine, IL-6, 18–24 h after the addition of LPS (Fig. 2B).

**PGE₂ Does Not Affect the Production of Other Inflammatory Response Proteins, Such as pro-IL-1β, iNOS, and COX-2 in BV-2 Cells**—We tested the influence of PGE₂ on the production of other activation-related cytokines or enzymes in BV-2 cells. Stimulation of BV-2 cells with LPS leads to a strong increase in the production of cell-associated pro-IL-1β, COX-2, and iNOS (Fig. 3). Although BV-2 cells produced high amounts of pro-IL-1β, processed IL-1β was not detected in conditioned medium by ELISA, even at later time points (data not shown). PGE₂ treatment did not inhibit LPS-induced pro-IL-1β or COX-2 and only weakly blocked (~50% inhibition) iNOS induction (Fig. 3). In addition, in the case of weak inhibition of iNOS, even high concentrations of PGE₂ (up to 10 μM) were unable to further suppress the production of this protein or the production of NO as measured by the accumulation of nitrite in the conditioned media (11). These data demonstrate that the action of PGE₂ in BV-2 microglia is highly selective, inhibiting TNF-α and IL-6 but not IL-1β, COX-2, or iNOS.

**Influence of PGE₂ on TNF-α, IL-6, and IL-1β mRNA Levels**—We have investigated at what level the inhibition of TNF-α and IL-6 may occur in BV-2 cells. Stimulation with LPS leads to ~20-fold increase in TNF-α mRNA compared with control; pre-treatment with 1 μM PGE₂ results in a 40% decrease in LPS-induced TNF-α mRNA levels (Fig. 4). Although a small decrease in TNF-α mRNA was also observed in cells
Cells lysates were prepared at 6 h (pro-IL-1β) for 30 min and then stimulated with 80 ng/ml LPS or control buffer. TNF-α levels in conditioned media were determined by ELISA. Values correspond to the mean ± S.E. of four independent experiments, each done in duplicate.

Fig. 2. PGE2 inhibition of TNF-α and IL-6 production in BV-2 cells. A, cells were pre-treated with 1, 10, 100, or 1000 nM PGE2 or the control buffer and then treated for 6 h with 80 ng/ml LPS or control buffer. TNF-α levels in conditioned media were determined by ELISA. Values correspond to the mean ± S.E. of four independent experiments, each done in duplicate. B, cells were pre-treated with 1, 10, 100, or 1000 nM PGE2 or the control buffer and then treated for 18 h with 80 ng/ml LPS. IL-6 protein levels in conditioned medium were determined by Western blotting. Similar results were observed in three independent experiments. IL-6 has been reported to produce bands of ~23 and 27–30 kDa during polyacrylamide gel electrophoresis (42).

While inhibiting TNF-α and IL-6 mRNA accumulation, PGE2 treatment increased the production of IL-1β mRNA (Fig. 4), demonstrating that PGE2 has a differential effect on the mRNA accumulation of these cytokines. IL-1β mRNA levels were ~2.5-fold higher in cells treated with LPS in the presence of 1 μM PGE2 in comparison with cells treated with LPS alone (Fig. 4).

Role of cAMP and PKA in Inhibition of TNF-α Production by PGE2—The effect of PGE2 on cells can be modulated by four subtypes of prostaglandin receptors, EP1–EP4, with EP2 and EP4 receptors positively coupled to adenylate cyclase (19). A recent study (20) has shown that in microglial cells, the effects of PGE2 were mediated by the EP2 subtype, thus implicating the importance of a rise in intracellular cAMP for the activity of PGE2 in microglia. In agreement with these data, we found that the activity of PGE2 can be largely mimicked by a cAMP analog, 8-bromo-cAMP. Indeed, pre-treatment of BV-2 cells with 8-bromo-cAMP for 30 min completely abolished the LPS-induced accumulation of TNF-α and IL-6 proteins, whereas the production of pro-IL-1β was not affected, and iNOS was only weakly inhibited (Fig. 5). Unlike PGE2, however, 8-bromo-cAMP significantly inhibited COX-2 production (Fig. 5).

We also investigated whether PKA mediates PGE2 effects in BV-2 cells. Pre-treatment with the PKA-selective inhibitor H89 (21) only partially reversed the PGE2 inhibition of TNF-α (Fig. 6A). At the same time, this concentration of H89 almost completely inhibited PGE2-induced activation of PKA (Fig. 6B). These data suggest that although activation of PKA by PGE2 may represent one of the important steps in the action of PGE2 in microglia, PKA-independent pathways are also likely to be involved.

**DISCUSSION**

This study addresses the question of how PGE2, one of the major prostaglandins produced in the brain, influences microglial activation. Here we report for the first time that in LPS-stimulated BV-2 microglial cells, PGE2 selectively suppresses the production of TNF-α and IL-6, whereas it has little or no effect on the accumulation of other activation-related proteins, such as pro-IL-1β, iNOS, or COX-2. These results demonstrate the potential for modulation of specific signal transduction pathways in activated glia and provide a precedent for future attempts to develop selective therapeutics for neurodegenera-
Suppression of TNF-α production by PGE₂ in peripheral macrophages is a well-described process; PGE₂ acts primarily by suppressing TNF-α transcription (22). Based on our data, it appears that transcriptional suppression is not the mechanism in microglia, or at least in the BV-2 microglial cell line. Despite the complete inhibition of TNF-α and IL-6 protein production, PGE₂ at high concentrations decreased the TNF-α and IL-6 mRNA levels by only 40–50%. These data suggest that PGE₂, in addition to the modest effect on TNF-α and IL-6 mRNA accumulation at high concentrations, acts principally by regulating protein accumulation at the level of translation or protein stability. The production of TNF-α in LPS-stimulated macrophages and microglia has been shown to be regulated both at the level of transcription and translation, with p38, extracellular signal regulated kinase 1/2, and c-Jun NH₂-terminal kinase being implicated in the translational control (23, 24). Interestingly, the suppression of IL-6 accumulation by PGE₂ in BV-2 cells was significant only at later time points, when the viability of LPS-treated cells is impaired (11), thus suggesting that the action of PGE₂ on IL-6 production may be mediated indirectly through a cytoprotective effect rather than a direct regulation of IL-6 protein synthesis.

As in other cell systems, PGE₂ appears to act by increasing intracellular cAMP levels, because the cAMP-mimicking agent, 8-bromo-cAMP, has the same effect as PGE₂ on TNF-α and IL-6 production. PGE₂ also rapidly activates PKA in BV-2 cells, and the selective inhibitor of protein kinase A, H89, partially reverses the inhibitory action of PGE₂ on TNF-α production. Nevertheless, the modest effect of PKA inhibition on the TNF-α levels suggests that additional pathways are likely to be involved. Recently, two new guanine-nucleotide-exchange factors that bind cAMP directly were described (25, 26). The binding of cAMP to these factors leads to the activation of the small GTPase Rap1 that is abundantly expressed in the brain (26). As in other cell systems, Rap1 activity; however, additional studies are necessary to evaluate this and other possibilities.

PGE₂ has been reported to inhibit iNOS (27) and IL-1β (28) production in LPS-stimulated rat microglia. In agreement with the former study (27) that found ~40% inhibition of iNOS, we also observed partial (~30–50%) inhibition of NO production (11) and iNOS protein (this study) by PGE₂ in BV-2 cells.
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We have previously shown that a cyclopentaneone derivative of prostaglandin \(D_2\), 15-deoxy-D\(_{12,14}\)-PGJ\(_2\), acts as an inhibitor of iNOS transcription in activated glia (11). Our additional studies\(^2\) indicate that cyclopentaneone prostaglandins also inhibit the production of TNF-\(\alpha\), COX-2, IL-6, and IL-1\(\beta\). Taken together, these data suggest that there is a remarkable specificity and complexity in the effects of prostaglandins that matches the complexity of responses of glia to activating stimuli and that prostaglandins may play the role of both fine-tuning agents (PGE\(_2\)) and general inhibitors of glial activation (cyclopentaneone prostaglandins).

TNF-\(\alpha\) and IL-6 appear to play a dual role in brain injury and neurodegeneration that can include both neurotrophic and neurotoxic effects. For example, increased levels of TNF-\(\alpha\) and IL-6 are observed after acute brain insult, such as stroke, in mice demonstrating diminished microglial activation and exacerbation of cell death (38, 41). It is tempting to speculate that PGE\(_2\) in some circum-

\(^2\) T. V. Petrova, manuscript in preparation.