Regulation of Macrophage Cytokine Production by Prostaglandin E_2

DISTINCT ROLES OF CYCLOOXYGENASE-1 AND -2

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Prostaglandin E_2 (PGE_2) modulates a variety of physiological processes including the production of inflammatory cytokines. There are two cyclooxygenase (Cox) enzymes, Cox-1 and Cox-2, that are responsible for initiating PGE_2 synthesis. These isozymes catalyze identical biosynthetic reactions but are regulated by different mechanisms in the cell. This report examines differences in the roles of Cox-1 and Cox-2 in regulating cytokine synthesis in macrophages. We employed agents that selectively modulate the activity of each isozyme and measured their effects on synthesis of interleukin (IL)-6, IL-1, and tumor necrosis factor-α by peritoneal macrophages. Among these three cytokines, only IL-6 synthesis was stimulated by production of endogenous PGE_2. This effect was specifically linked to activation of Cox-2 and not Cox-1. The specificity derives, partly, from the timing of the production of PGE_2 following stimulation of each isozyme and from induction of ancillary signals that control the response to PGE_2. The experimental findings demonstrate that the effects of Cox-1 and Cox-2 activity on macrophage IL-6 synthesis are segregated. This provides a mechanism for IL-6 to be induced selectively during inflammation.

Prostaglandins are important mediators of a wide variety of physiological processes (reviewed in Ref. 1). There are two isozymes, Cox-1 and Cox-2, that initiate prostaglandin synthesis (2–5). Both enzymes utilize arachidonic acid as the predominant substrate, and both catalyze the same cyclooxygenase and peroxidase reactions that constitute the first two steps of eicosanoid metabolism (6). Although indistinguishable in their biosynthetic catalytic activities, the two isozymes appear to have different physiological functions. Cox-1 (prostaglandin synthase-1, EC 1.14.99.1) protein is expressed constitutively in most cell types and is thought to be responsible for regulating normal physiological functions such as gastric acid secretion and kidney function (7, 8). The cellular activity of Cox-1 is regulated primarily through substrate availability, e.g. from the release of free arachidonic acid from membrane phospholipids. Cox-2 is an inducible enzyme expressed in activated macrophages (5, 9–14), fibroblasts (10, 15–18), and several other cell types (19–23). In vivo expression of Cox-2 is seen in chronic inflammatory conditions such as arthritis (24, 25), experimental peritonitis (14), and in human colon cancer tissue (26, 27). Cox-2 expression is induced in vitro in response to stimuli such as LPS and growth factors (9, 11, 15, 16, 28). Due to small differences in the amino acid sequences near the cyclooxygenase catalytic site, Cox-1 and Cox-2 can be inhibited differentially by non-steroidal anti-inflammatory drugs (NSAIDs (29–31)). For example, aspirin and indomethacin inhibit both isozymes but second generation NSAIDs such as NS-398 inhibit Cox-2 preferentially (32). These drugs can be used to distinguish between the activities of Cox-1 and Cox-2 within the cell.

A substantial body of work indicates that PGE_2 modulates production of inflammatory cytokines (reviewed in Ref. 33). Different effects of PGE_2 are observed depending on the experimental system employed. Although there are discrepancies in the literature (34–38), addition of exogenous PGE_2 to macrophages prior to LPS stimulation generally down-regulates IL-6 and TNF-α synthesis while having no effect on IL-1β (35, 36). However, direct addition of PGE_2 to untreated macrophages induces low levels of both IL-6 (14) and TNF-α (39) synthesis. Since macrophages are a major source of PGE_2 during inflammation (40) and since they also have receptors for and respond to this eicosanoid (41), the PGE_2 generated by macrophages may regulate cytokine synthesis in an autocrine fashion (in contrast to paracrine regulation achieved through adding exogenous PGE_2 to cells). Previous studies have implicated a positive association between endogenous PGE_2 production and IL-6 synthesis in vitro (14, 42, 43), and animal models of chronic inflammation show that PGE_2 is a stimulator of IL-6 production in vivo (14, 25, 42, 44–46).

In previous studies, we found that inflammatory agents that induce IL-6 synthesis in macrophages in vitro also induce expression of Cox-2 (14). Significantly, both Cox-2 mRNA expression and IL-6 protein were co-elevated in inflammatory exudates in vitro. Inhibition of Cox-2 activity by NS-398 in vitro inhibited IL-6 synthesis. These results suggested that Cox-2 activity might be responsible for modulating IL-6 production.

The question addressed in this report is whether stimulation of PGE_2 synthesis from Cox-1 has the same effect on macrophage cytokine synthesis as stimulation of PGE_2 production from Cox-2. Because Cox-1 protein is expressed constitutively, prostaglandin synthesis is activated within minutes after addition of an appropriate stimulus (e.g. free arachidonic acid). In contrast, synthesis of PGE_2 from Cox-2 is delayed by several hours due to the requirement for de novo mRNA and protein synthesis. The two isozymes are also localized in different membrane compartments within the cell (47). Thus, it is possible that the PGE_2 synthesized from the two cyclooxygenase enzymes may control different functions in the cells in which it is formed. We find that among the three pro-inflammatory...
cytokines examined here (IL-6, IL-1β, and TNF-α), only IL-6 production is stimulated by PGE₂ produced by macrophages. Moreover, the regulation of IL-6 is uniquely linked to Cox-2 activation; agents that stimulate Cox-1 fail to induce IL-6 synthesis.

EXPERIMENTAL PROCEDURES

Mice—BALB/c mice were purchased from Charles River Laboratories and fed Purina laboratory Chow and water ad libitum. Animals were cared for in accordance with the National Institutes of Health Animal Care Guidelines. Female mice (8–16 weeks old) received a single 0.5 ml intraperitoneal injection of pristane (2,6,10,14-tetramethylpentadecane).

Preparation of Peritoneal Macrophages—Peritoneal lavage samples were collected 2–4 months after pristane treatment (48). The peritoneal macrophage population was isolated as described previously (48). Briefly, neutrophils and macrophages were separated by density gradient centrifugation (49). Macrophages were purified further by plating at a concentration of 2.5 x 10⁶ cells/ml (1.25 x 10⁵ cells/well) in 24-well plastic tissue culture plates in DMEM containing antibiotics and allowing cells to adhere at 37 °C for 2–4 h. Nonadherent cells were removed by washing with DMEM. Cell numbers and viability were quantified by hemocytometry with trypan blue. Cell differentials were analyzed from Diff-Quick stained cyt centrifuge slides.

Macrophage Treatments in Vitro—Macrophages were stimulated with a bovine serum albumin (pAlb) preparation from Boehringer Mannheim (catalog number 100 069; lot 108303) that contains roughly 4% albumin polymers. This material was characterized previously and shown to stimulate macrophage IL-6 production both in vitro and in vivo (50). PGE₂ synthesis was also stimulated with exogenous arachidonic acid (Sigma), prepared freshly as a 400 μM stock in 50% ethanol. Macrophages were cultured in DMEM at 37 °C in a humidified atmosphere containing 5% CO₂ and stimulated by addition of either pAlb (50–200 μg/ml) or arachidonate (10 μM). Where indicated, indomethacin (1 μM) or NS-398 (1 μM) was added to the cell cultures 30 min before adding the stimulus. After various times of incubation, the culture medium was removed from each well and centrifuged. In experiments that contained no added protein, ultrapure bovine serum albumin (Boehringer Mannheim, catalog 238 031) was added to each macrophage supernatant at the time of collection to decrease the adsorption of cytokines to the walls of the collection tube. Supernatants were stored frozen at −20 °C until assayed for the presence of cytokines or PGE₂.

Purification of RNA and Northern Blot Analysis—Total RNA was purified from macrophages after various times of incubation using TRizol® (Life Technologies, Inc.) following the protocol recommended by the manufacturer. RNA samples (4 or 15 μg each) were run on 1% agarose, 0.7% formaldehyde gels containing ethidium bromide and transferred to nitrocellulose. 32P-Labeled IL-6, Cox-2, and actin cDNA probes were prepared using a random priming system (Life Technologies, Inc.) and [α-32P]dCTP (Amersham Corp.). Blots were hybridized overnight with probe (1 x 10⁶ cpm/μg) at 65 °C in Hybriol II solution (Onecon, Inc., Gaithersburg, MD) and then washed by standard procedures. Autoradiography was performed using Kodak XAR film. Films were scanned (Microtek Scanmaker) and analyzed using the Macintosh densitometry program NIH Image.

Preparation of Cell Lysates and Western Blot Immunnoassay—Cells were washed in phosphate-buffered saline, lysed in sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 1 mM DTPA) and heated at 100 °C for 12 min. Protein (5 x 10⁶ cells equivalent) was separated by SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Inc.). Membranes were blocked in 3% bovine serum albumin and then incubated with a polyclonal rabbit anti-murine Cox-2 antibody (Cayman Chemical Co., Ann Arbor, MI; catalog number 160106) or anti-Cox-1 antibody followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnologies; catalog number 4050-05). Immunopositive protein bands were visualized by chemiluminescence (Renaissance kit, NEN Life Science Products) and exposure of x-ray film.

Bioassay for IL-6—IL-6-dependent B9 hybridoma cells (51) were cultured in a serum-free medium (52) supplemented with 5% heat-inactivated fetal calf serum and IL-6. Prior to the assay, the B9 cells were washed to remove IL-6 and were then cultured in flat-bottom 96-well plates at 3000 cells/well. Samples to be assayed for IL-6 activity were added at serial 2-fold dilutions. Standard curves with recombinant murine IL-6 were run to control for interassay variation. After 3 days of culture at 37 °C, the number of viable cells was assayed using the colorimetric reagent 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 0.55 mg/ml (48, 53). The data were calculated using a four-parameter fit analysis. A unit of activity is defined as the dilution that gives half-maximal B9 cell growth in 3 days and corresponds to approximately 2 pg/ml homogeneous murine recombinant IL-6. Specificity of the assay was confirmed by blocking all activity with a polyclonal anti-IL-6 antibody. Results of the B9 bioassay were very similar to those obtained using an enzyme-linked immunosorbent assay specific for mouse IL-6 (Endogen, Cambridge, MA).

Enzyme-linked Immunosorbent Assays for IL-6, IL-1, and TNF-α—IL-6, IL-1, and TNF-α were measured using ELISA kits from Endogen (Woburn, MA).

Analysis of Prostaglandins—PGE₂ was assayed using a monoclonal antibody/enzyme immunosassay kit from Cayman Chemical. The linear range of the assay was from 10 to 1000 pg/ml.

Materials—Escherichia coli LPS 055:B5 was from Difco. Indomethacin, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, PGE₂, and arachidonate were from Sigma. NS-398 was purchased from Biomol (Plymouth Meeting, PA). Pristane was from Aldrich. A CDNA probe for murine IL-6 (0.65 kb) was a gift of Bob Brown (NCI, National Institutes of Health).

RESULTS

Induction of IL-6 and Cox-2 by LPS and pAlb—Pristane-elicted macrophages spontaneously secrete low levels of IL-6 and PGE₂ (48). Stimulation with either LPS or polymerized albumin (pAlb) promotes an increase in the expression of the Cox-2 and IL-6 genes (Fig. 1). We employ pAlb for our in vitro studies because it is pro-inflammatory in vivo and yet is less toxic than LPS to macrophages (50). The time course of expression of IL-6 and Cox-2 mRNAs after induction by pAlb is shown in Fig. 2. Elevated Cox-2 mRNA expression is first seen 30 min after addition of pAlb to the cells. By comparison, the elevation in IL-6 expression is not detected until 45 min after stimulation.

Effects of Cox-2 Inhibition on IL-6, IL-1, and TNF-α Production by Macrophages—PGE₂ is the predominant eicosanoid produced by pAlb- and LPS-stimulated macrophages and hence is the focus of these studies. We have hypothesized that induction of macrophage IL-6 mRNA expression is controlled, in part, by PGE₂ derived from Cox-2 (14). This conclusion was drawn from the finding that induction of IL-6 mRNA expres-
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Fig. 2. Time course of induction of Cox-2 and IL-6 mRNAs. Peritoneal macrophages were prepared as described in the legend to Fig. 1. At time 0, the cells were stimulated by addition of 200 μg/ml pAlb. Total RNA was isolated at the times indicated. The figure shows a representative Northern blot probed simultaneously for Cox-2 (1.7-kb probe, \(1 \times 10^6\) cpm/μg) and IL-6 (0.7-kb probe, \(1 \times 10^6\) cpm/μg). A longer exposure of the \(^{32}P\)-labeled blot did not change the timing of appearance of the bands. Identical results were obtained in two separate experiments.

Stimulation of Cox-1 Activity Does Not Result in Increased IL-6 Production.—To test further whether Cox-1 might be involved in pAlb-stimulated synthesis of PGE2 and IL-6, we looked for Cox-1 protein induction by pAlb. Cox-2 protein is induced approximately 300-fold in 5 h by stimulation with pAlb (Fig. 4A). The levels of Cox-1 protein are low but remain constant over the same time period (Fig. 4B). Since Cox-1 activity is generally regulated through modulation of substrate availability and not through gene induction (55), the absence of a change in Cox-1 protein levels does not preclude a change in Cox-1 activity. To test whether pAlb stimulates any Cox-1 activity, macrophages were pretreated with aspirin, washed, and then stimulated for 6 h with pAlb. Aspirin irreversibly inhibits cyclooxygenases by acetylating a serine residue near the cyclooxygenase active site (6). Since Cox-2 is not present at the time of the aspirin pretreatment, the drug only inactivates pre-existing Cox-1. As shown in Fig. 5, pAlb-induced PGE2 synthesis was lowered by roughly 16% following pretreatment with aspirin, and IL-6 synthesis was decreased by approximately 20%. This small effect may have derived from incomplete elimination of aspirin from the cells by the washing procedure. In contrast, when aspirin was present throughout the incubation period with pAlb, PGE2 production was 98% inhibited (consistent with the ability of aspirin to inhibit the activities of both Cox-1 and Cox-2), and IL-6 synthesis was reduced by 56%. Taken together, the data presented thus far suggest that pAlb stimulates IL-6 synthesis by inducing Cox-2 activity and that Cox-1 is not involved in this particular process.

Because pAlb stimulates PGE2 synthesis by activating Cox-2 selectively, the above results do not reveal whether Cox-1 activity would induce IL-6 synthesis if the enzyme became activated. To examine this possibility, we tested the effect of arachidonic acid on PGE2 and IL-6 synthesis. Arachidonate is the substrate for cyclooxygenases, and it stimulates PGE2 synthesis by Cox-1 in macrophages (56). Treatment of cells with 10 μM arachidonic acid did not induce any measurable IL-6 production even though substantial PGE2 was generated (Fig. 6).

In control experiments, we verified that addition of exogenous arachidonic acid to these cells stimulates PGE2 synthesis from Cox-1 and not Cox-2. First, we examined the time course of PGE2 synthesis by this agent. As shown in Fig. 7, arachidonic acid stimulated rapid secretion of PGE2 (within 30 min) compared with pAlb (2–3 h). This time course of arachidonate-induced prostaglandin synthesis is similar to that documented previously by others (57). The production of PGE2 from arachidonate-stimulated macrophages occurred in the absence of detectable Cox-2 protein, determined by Western blot immunosay of cell extracts collected at each of the time points shown in Fig. 7 (data not shown). Moreover, pretreatment of the cells with aspirin for 30 min prior to addition of arachidonate completely inhibited PGE2 production (Fig. 8). Since there was no Cox-2 protein present in the cells during the preincubation period, the inhibition had to derive from an effect on Cox-1. This result can be contrasted to the effect of aspirin pretreatment on pAlb-stimulated PGE2 production (see Fig. 5). Thus, we conclude that arachidonic acid stimulates PGE2 synthesis from Cox-1 and that this activity is not sufficient to induce IL-6.

Addition of Exogenous PGE2 to pAlb-stimulated Macrophages Can Substitute for Cox-2 Activity When Added at Appropriate Times.—How can stimulation of PGE2 production from Cox-2 result in increased IL-6 production while PGE2 derived from Cox-1 has no effect? One explanation could be that agents such as pAlb that induce Cox-2 gene expression also activate ancillary signaling pathways that are required for elaboration of the PGE2 effect. Absent activation of these pathways, the PGE2 generated by the cells is ineffective at stimulating IL-6 synthesis. To test this hypothesis, macrophages were stimulated with pAlb in the presence of NS-398 which completely inhibited PGE2 synthesis from Cox-2. Exogenous PGE2 was added either 30 min prior to or 4 h after treatment of the cells with pAlb and NS-398. As shown in Fig. 9, exogenous PGE2 can stimulate IL-6 synthesis and can overcome the inhibition caused by NS-398 but only when added to the cells at the delayed time point, at which time Cox-2 and Cox-2-derived PGE2 levels are normally elevated (see Figs. 4 and 6). When PGE2 is added to macrophages prior to stimulation with pAlb, IL-6 levels remain at or below the NS-398-inhibited level. This is not a generalized effect because IL-1 synthesis is insensitive to the presence of PGE2, regardless of the time it is added.

DISCUSSION

The findings in these studies can be summarized as follows: Inflammatory agents that stimulate IL-6 expression by macrophages do so in part by inducing Cox-2 gene expression and Cox-2-derived PGE2 synthesis. Of the three pro-inflammatory cytokines examined here (IL-6, IL-1, and TNF-α), only IL-6 is induced by Cox-2-derived PGE2. Stimulation of PGE2 production by Cox-1 is not sufficient to induce IL-6 synthesis. Our results suggest that appropriate control of IL-6 synthesis is dependent upon such segregation of macrophage Cox-1 and Cox-2 activities. In terms of understanding physiological outcomes, it seems appropriate that Cox-1 activity does not stim-
ulate IL-6 synthesis since Cox-1 is expressed constitutively in most cells. If IL-6 could be stimulated by Cox-1 activity, IL-6 synthesis would be constantly turned on in those tissues in which Cox-1 is active. Instead, the dependence on Cox-2 synthesis is compatible with IL-6 being induced only when inflammatory stimuli are present. Collagenase synthesis and matrix metalloprotein expression represent other examples in which there is a specific association between Cox-2 activity and activation of inflammation-associated genes (58, 59).

The difference in outcome derived from activating Cox-1 or Cox-2 may lie in part in the time course of production of PGE2 by each isozyme. That is, PGE2 synthesis from Cox-1 begins within minutes after addition of an appropriate stimulus and is complete within 1 h. In contrast, PGE2 synthesis derived from induction of Cox-2 commences after a delay of more than an hour following stimulation and continues to accumulate for hours thereafter. The delayed production of PGE2 by Cox-2 may be necessary to generate the IL-6 response. Experiments using exogenous PGE2 demonstrate this point. If the PGE2 is added early, at roughly the same time as pAlb, then IL-6 synthesis is slightly decreased. However, if the addition of...
PGE₂ is delayed to 4 h after pAlb stimulation, then IL-6 synthesis is greatly augmented. The delayed time frame coincides with the time when pAlb normally induces maximal Cox-2 synthesis and activity. Thus, addition of PGE₂ at this time may mimic Cox-2 activity. It should be noted that the concentration of exogenous PGE₂ added to the macrophages is roughly equivalent to the amount generated during an overnight incubation of the cells with pAlb or LPS.

The results also raise the possibility that induction of IL-6 synthesis by inflammatory agents such as pAlb requires co-induction of ancillary signals that control the response to PGE₂. In previous experiments, we (14) showed that addition of exogenous PGE₂ to untreated macrophages stimulates IL-6 synthesis. However, the degree of induction was relatively low, increasing the levels of IL-6 generated from roughly 20 to 80 pg/ml (14). Here we show that if PGE₂ is added to cells that have been pretreated with pAlb in the presence of an inhibitor of endogenous prostaglandin synthesis, then the exogenous PGE₂ stimulates the cells to produce an additional 45,000 pg/ml IL-6 (see Fig. 9), fully restoring the level of IL-6 synthesis to that which was achieved in the absence of the cyclooxygenase inhibitor. The augmented level of IL-6 induced by adding exogenous PGE₂ to pAlb-treated cells compared with untreated cells shows that pAlb primes the cells to respond to the PGE₂. Since agents that stimulate Cox-1 activity result in the production of endogenous PGE₂, the increased PGE₂ synthesis due to activation of Cox-1 fails to induce IL-6 synthesis. A similar mechanism for induction of human monocyte responsiveness to cAMP has been proposed by Corcoran et al. (60). Thus, agents that induce IL-6 synthesis may have two distinguishing characteristics: induction of delayed PGE₂ synthesis due to induction of Cox-2 and not Cox-1, and activation of secondary signals that control the cellular response to PGE₂. A hypothetical scheme depicting the regulation of IL-6 synthesis in macrophages by PGE₂ is shown in Scheme 1. An alternative explanation for the different outcomes achieved by stimulation of Cox-1 and Cox-2 is that PGE₂...
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produced by the two isozymes is channeled differently in the cell. Cox-1 is found primarily in the endoplasmic reticulum, whereas Cox-2 is also active in the nuclear envelope (47). This raises the possibility that some of the PGE2 generated by Cox-2 acts directly on nuclear gene expression without exiting the cell. This mechanism need not be invoked to explain our results since exogenous PGE2 was able to substitute for Cox-2-derived PGE2. However, it will be of interest to determine whether the PGE2 generated from Cox-2 needs to exit the cell to stimulate IL-6 synthesis.

Our data indicate that pAlb stimulates PGE2 production primarily from Cox-2 even though Cox-1 is present in the cell. This implies that the two isozymes respond to different pools of arachidonic acid, consistent with the experimental results of Reddy and Herschman (56) showing that Cox-1 cannot access arachidonic acid released by LPS. The same appears to be true for pAlb since aspirin pretreatment had little effect on the level of PGE2 produced in response to this agent, and NS-398 was equally as effective in inhibiting pAlb-induced PGE2 synthesis as indomethacin (not shown). In addition, Reddy and Herschman (56) have suggested that exogenous arachidonic acid is utilized only by Cox-1 in the cell. Our results are consistent with this conclusion since PGE2 production induced by arachidonate is rapid and is completely inhibited by pretreatment of the cells with aspirin. However, our experiments do not address the question of whether exogenous arachidonic acid could be utilized by Cox-2 since, under our experimental conditions, Cox-2 is not present at the time of addition of the arachidonate. Nonetheless, the results support the conclusion that Cox-1 and Cox-2 are functionally segregated by differences in the availability of arachidonic acid to each isozyme (56) and that pAlb stimulates PGE2 production only from Cox-2 because it generates arachidonic acid which is not accessible to Cox-1.

IL-1 and TNF-α synthesis are also induced by treatment of macrophages with inflammatory agents such as LPS and pAlb, but our results indicate that they are regulated by separate mechanisms that are not dependent upon co-induction of PGE2 synthesis. Other researchers have also found that drugs that inhibit cyclooxygenases either have no effect or cause an increase in TNF-α and IL-1 production, suggesting that autocrine PGE2 synthesis does not affect these cytokines in the same way as it affects IL-6 (38, 42, 61–64, and this report). These findings may explain recent results with in vivo models showing that NSAIDs that inhibit Cox-2 modulate IL-6 but not TNF-α levels (14, 25, 44).

Overall, the results demonstrate that among the three pro-inflammatory cytokines examined here, IL-6 is unique in being stimulated by Cox-2-derived PGE2. Thus, chronic overexpression of Cox-2 such as occurs in rheumatoid arthritis (65–67) is expected to be accompanied by chronic high levels of IL-6 and the symptoms derived from these abnormally high IL-6 levels (65). Second generation NSAIDs aimed specifically at inhibiting Cox-2 activity may be useful in treating chronic inflammatory conditions in which IL-6 is abnormally elevated.
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