

Arachidonic Acid Is Preferentially Metabolized by Cyclooxygenase-2 to Prostacyclin and Prostaglandin E₂*

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The two cyclooxygenase isoforms, cyclooxygenase-1 and cyclooxygenase-2, both metabolize arachidonic acid to prostaglandin H₂, which is subsequently processed by downstream enzymes to the various prostanoids. In the present study, we asked if the two isoforms differ in the profile of prostanoids that ultimately arise from their action on arachidonic acid. Resident peritoneal macrophages contained only cyclooxygenase-1 and synthesized (from either endogenous or exogenous arachidonic acid) a balance of four major prostanoids: prostacyclin, thromboxane A₂, prostaglandin D₂, and 12-hydroxyheptadecatrienoic acid. Prostaglandin E₂ was a minor fifth product, although these cells efficiently converted exogenous prostaglandin H₂ to prostaglandin E₂. By contrast, induction of cyclooxygenase-2 with lipopolysaccharide resulted in the preferential production of prostacyclin and prostaglandin E₂. This shift in product profile was accentuated if cyclooxygenase-1 was permanently inactivated with aspirin before cyclooxygenase-2 induction. The conversion of exogenous prostaglandin H₂ to prostaglandin E₂ was only modestly increased by lipopolysaccharide treatment. Thus, cyclooxygenase-2 induction leads to a shift in arachidonic acid metabolism from the production of several prostanoids with diverse effects as mediated by cyclooxygenase-1 to the preferential synthesis of two prostanoids, prostacyclin and prostaglandin E₂, which evoke common effects at the cellular level.

Prostaglandins (PGs)¹ are a family of intercellular and intracellular messengers derived from arachidonic acid (AA). These mediators exert a wide range of effects on processes such as smooth muscle tone, vascular permeability, cellular prolif-

eration, and inflammatory/immune function. In many cases, different PGs will have opposing actions. For example, PGD₂ and thromboxane (TxA₂) cause smooth muscle contraction, whereas PGE₂ and prostacyclin (PGI₂) cause relaxation (reviewed in Refs. 1–4). Similarly, TxA₂ increases, but PGI₂ inhibits, platelet aggregation. The net effect evoked by PGs may, ultimately, depend on the balance of these opposing forces.

The initial step in the synthesis of PGs from AA is mediated by cyclooxygenase (COX, also known as prostaglandin H synthase or prostaglandin endoperoxide synthase), of which two isoforms are recognized (reviewed in Refs. 2–4). COX-1 is expressed constitutively in most cell types, and prostanoids derived from COX-1 are thought to be important in gastric and renal homeostasis. COX-2, on the other hand, is the product of an immediate early gene and is rapidly expressed only after exposure of cells to hormones, mitogenic stimuli, and inflammatory mediators, like bacterial lipopolysaccharide (LPS). The induction of COX-2, with the resultant production of prostanoids, can contribute to parturition, inflammation, pain, fever, and certain types of cancer. The ability of aspirin to permanently inactivate both COX isoforms indiscriminately explains both its analgesic and anti-inflammatory properties, through COX-2 inhibition, as well as its damaging effects on the gastric mucosa, through COX-1 inhibition.

Both COX isoforms convert AA to PGH₂, which is then acted upon by discrete PG synthases to give rise to the different PG species. The current model for the regulation of PG synthesis posits that the amount of each PG is determined by the availability of the substrate AA (through the action of phospholipases A₂) and by the total mass of COX protein present. According to this model, COX-2 induction results in more COX protein, more processing of AA to PGH₂, and more of each type of PG. The profile of different PGs produced by a given cell type is thought to be determined primarily by the relative amounts of the distal PG synthases present in that cell type. In simple cases, as when a single distal enzyme predominates, this model may be accurate. However, when several different PG synthases exist in a single cell, the opportunity exists for COX-1 and COX-2 to selectively deliver PGH₂ to different distal enzymes and thus produce different PGs. If so, then COX-2 induction and action could result in a shift in the profile of PGs, rather than simply an increase in total PG production. This could then change the integrated prostanoid signal that is delivered to target cells.

In the current study, we used intact rat peritoneal macrophages (PMs) to compare the products derived from either COX-1 or COX-2 action on AA. These primary cells were chosen because they contain several PG synthases and would thus allow detection of selective metabolism of PGH₂, if it existed. Freshly isolated PMs, which lack COX-2, provide an indication of the prostanoids that are derived from COX-1 action. By contrast, cells treated first with aspirin to permanently inacti-

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¹ The abbreviations used are: PG, prostaglandin; AA, arachidonic acid; PGI₂, prostacyclin; LT, leukotriene; COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; HHT, 12-hydroxyheptadecatrienoic acid; LPS, lipopolysaccharide; PM, peritoneal macrophage; Tx, thromboxane; PBS, phosphate-buffered saline; ASA, acetylsalicylic acid; TNF, tumor necrosis factor; IL, interleukin; HPLC, high pressure liquid chromatography; EIA, enzyme immunoassay.

vate COX-1 and then with LPS to induce COX-2 demonstrate those prostanoids that are derived from COX-2. Our results indicate that AA metabolized by COX-1 gives rise to balanced amounts of PGI₂, TxA₂, PGD₂, and 12-hydroxyheptadecatrienoic acid (HHT) as well as smaller amounts of PGE₂, whereas COX-2 preferentially metabolizes AA to PGI₂ and PGE₂. Because PGI₂ and PGE₂ can evoke similar effects on target cells and tissues, the result of COX-2 induction may be a dramatic shift in the balance of PG action.

EXPERIMENTAL PROCEDURES

Cells—Resident PMs were obtained by peritoneal lavage of specific pathogen-free, female Wistar rats (Charles River Laboratories, Portage, MI) as described previously (5). Lavaged cells were centrifuged at 600 × *g* for 5 min at 4 °C, resuspended, and subjected to hypotonic lysis to remove contaminating erythrocytes. Cells were then centrifuged again and resuspended at 0.5 × 10⁶ cells ml⁻¹ in M199 and either maintained in suspension in Teflon tubes ("freshly isolated cells") or plated on 24-well plates (for metabolic studies) or glass slides (for imaging). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air.

Cell Fractionation and Immunoblot Analysis—As described previously (6), cells were disrupted by sonication (10 bursts at 20% duty cycle) in ice-cold homogenizing buffer (50 mM Tris-HCl, 25 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, pH 7.4) and separated into soluble and pelletable fractions by centrifugation (100,000 × *g*, 30 min, 4 °C); and protein concentrations were determined by a modified Coomassie Blue dye binding assay (Pierce). Samples containing 10 μg of protein were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose membranes. Following blocking, membranes were probed with antibodies to COX-1 (rabbit polyclonal raised against sheep seminal vesicle COX (7)) and COX-2 (rabbit polyclonal raised against murine COX-2, from Cayman Chemical Co., Ann Arbor, MI; 1:10,000) and then peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000) with ECL detection (Amersham Pharmacia Biotech).

Indirect Immunofluorescent Microscopy—Cells were prepared for indirect immunofluorescent microscopy as described previously (8). Following mounting on glass slides, cells were fixed using methanol (-20 °C, 30 min), permeabilized with acetone (-20 °C, 3 min), air-dried, rehydrated with sterile PBS, and blocked for 30 min using 0.1% (w/v) bovine serum albumin in PBS containing 1% (v/v) nonimmune goat serum. Preparations were then probed with antibodies to either COX-1 or COX-2, each at 1:200 in PBS supplemented with 0.1% fatty acid-free bovine serum albumin, followed by rhodamine-conjugated goat anti-rabbit antibody (1:200, Sigma). Preparations were examined and photographed using a Zeiss Aristoplan microscope equipped for epifluorescence.

Cell Treatment and Stimulation—For overnight incubations, cells were adhered for 1 h, washed twice with sterile PBS, and cultured overnight in M199 containing 10% heat-inactivated newborn calf serum (Life Technologies, Inc.). In some experiments, aspirin (acetylsalicylic acid (ASA); 200 μM) was included during the 1 h adherence phase; this treatment did not affect cell adherence or survival, as determined by trypan blue staining and visualization of cells. During overnight incubation, some cells were cultured with LPS (1 μg/ml), TNF-α (100 ng/ml), IL-1 (5 ng/ml), or polymyxin B sulfate (10 μg/ml). For prelabeling of cellular lipids, 0.5 μCi of [³H]AA (specific activity, 76–100 Ci/mmol, NEN Life Science Products) was included in the incubation medium. Before cell stimulation, the unincorporated label was removed by washing three times with PBS. Cell stimulation was with 1 μM A23187 for 15 min at 37 °C. Inhibitors added during cell stimulation in designated experiments included NS-398 (3 μM) and indomethacin (1 μM). Metabolism of exogenous AA was evaluated by the addition of 0.5 μCi of [³H]AA (~5 pM) to unlabeled cells during stimulation with A23187; the addition of additional cold, carrier AA did not alter results. Metabolism of exogenous PGH₂ was evaluated by the addition of 0.1 μM [³H]PGH₂ (for HPLC analysis) or unlabeled PGH₂ (for enzyme immunoassay analysis) to unlabeled cells during stimulation with A23187.

Eicosanoid Analysis—Radiolabeled eicosanoids were assayed as described (5). Briefly, eicosanoids were extracted from culture medium using Sep-Pak C₁₈ cartridges (Waters Associates, Milford, MA), dried under nitrogen, resuspended in water:acetonitrile (2:1), and separated by reverse-phase HPLC using a Waters μBondapak C₁₈ column with a mobile phase of acetonitrile/water/trifluoroacetic acid. Radiolabeled products were identified by their coelution with authentic standards

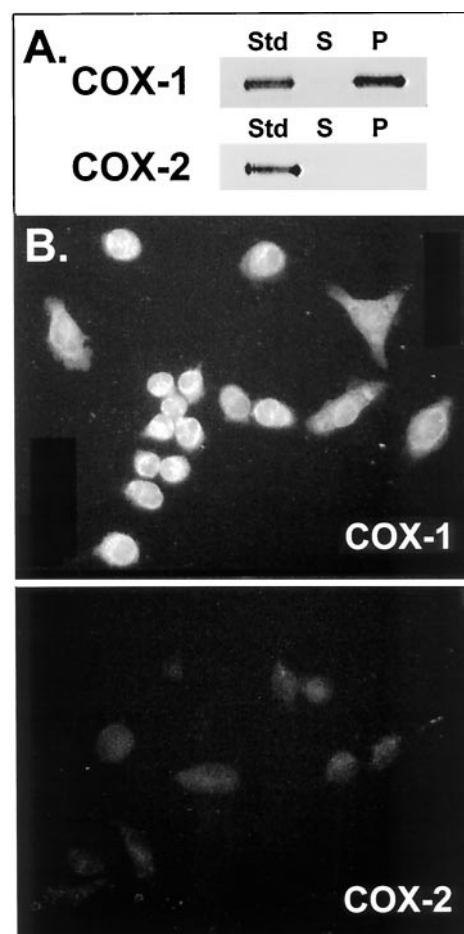


FIG. 1. Presence of COX-1, but not COX-2, protein in freshly isolated resident rat PMs. *A*, for immunoblot analysis, cells were disrupted, separated into soluble (*S*) and pelletable (*P*) fractions, and analyzed with authentic standard (*Std*). *B*, for immunofluorescent microscopy, cells were fixed, permeabilized, and stained for COX-1 or COX-2 as described under "Experimental Procedures." Results are representative of at least three independent experiments.

and quantitated by on-line radiodetection. Enzyme immunoassay (EIA) of conditioned media was performed according to the supplier's instructions (Cayman Chemicals).

Statistical Analysis—Statistical significance was evaluated by paired Student's *t* test using *p* < 0.05.

RESULTS

COX-1 Products—Freshly isolated PMs were evaluated for the presence of the two COX isoforms. By immunoblot analysis, fresh PMs contained abundant COX-1 in membrane-containing fractions but lacked detectable COX-2 (Fig. 1A). Similarly, COX-1, but not COX-2, was detectable in individual PMs by immunofluorescent microscopy (Fig. 1B). By this technique, COX-1 appeared to extensively decorate membranes throughout the cell, including perinuclear membranes and the endoplasmic reticulum.

To determine the profile of prostanoids synthesized by COX-1 alone, freshly isolated PMs were prelabeled with [³H]AA for 2 h, maintained in suspension in Teflon tubes, washed to remove the unincorporated label, and stimulated with the calcium ionophore A23187 (1 μM, 15 min, 37 °C) to release cellular AA from endogenous membrane phospholipids. By this method, several products were identified after HPLC separation with approximately 60% of the released AA converted to COX metabolites, including PGI₂ (detected as 6-keto-PGF_{1α}), TxA₂ (detected as TxB₂), PGE₂, PGD₂, and HHT and 30% metabolized to products of the 5-lipoxygenase pathway

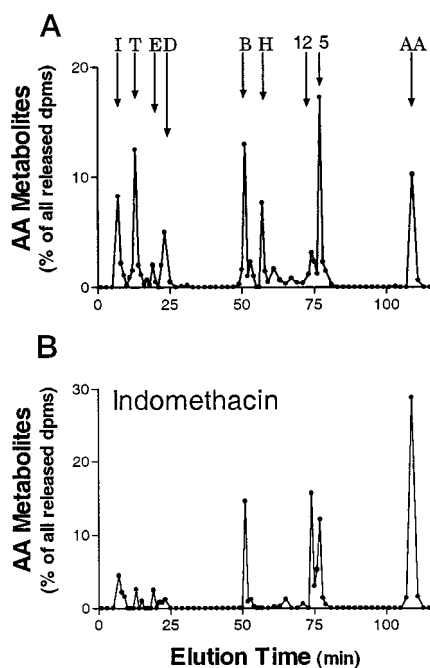


FIG. 2. Products from COX-1. Representative profiles of AA metabolites derived from fresh PMs are shown. Cells were prelabeled with [3 H]AA for 2 h, washed, and stimulated with $1 \mu\text{M}$ A23187 for 15 min at 37°C without (A) or with (B) $1 \mu\text{M}$ indomethacin. Radiolabeled metabolites in the conditioned media were separated by HPLC and identified by co-elution with unlabeled authentic standards. I, PGI $_2$; T, TxA $_2$; E, PGE $_2$; D, PGD $_2$; B, LTB $_4$; H, HHT; 12, 12-HETE; 5, 5-HETE. Results are representative of four independent experiments.

(Fig. 2A). This profile is similar to that reported previously (5). The production of prostanoids was inhibited by indomethacin ($1 \mu\text{M}$), a nonselective COX inhibitor, resulting in increases in free AA and 12-HETE but not the 5-lipoxygenase products, leukotriene (LT)B $_4$ and 5-HETE (Fig. 2B).

Pooled results from four independent experiments indicated that freshly isolated PMs generated a balance of four prostanoids from endogenous AA: PGI $_2$, TxA $_2$, PGD $_2$, and HHT (Table I). PGE $_2$ was a minor fifth product, accounting for less than 10% of all COX-1 products. Similar results were obtained when the AA was supplied exogenously; if trace levels of [3 H]AA were provided to fresh PMs in the absence of an agonist, then prostanoid ratios similar to those generated by prelabeled, A23187-stimulated cells were produced (Table I). Acute stimulation of cells with A23187 in the presence of exogenously supplied radiolabeled AA increased the synthesis of radiolabeled 5-lipoxygenase products but did not significantly change the ratios of the COX-1 products (data not shown).

COX-2 Products—COX-2 induction in rat PMs was pronounced in response to LPS ($1 \mu\text{g}/\text{ml}$, 24 h), appearing as two bands of approximately 69 and 70 kDa on immunoblots (Fig. 3A). By indirect immunofluorescent microscopy, the degree of expression of COX-2 protein varied greatly among cells within a culture treated with LPS for 24 h (Fig. 3B). Some cells were largely negative for COX-2, some cells showed intermediate expression with COX-2 restricted to the nuclear envelope and perinuclear membranes, and others showed that COX-2 was concentrated on the nuclear envelope and was also abundant on the endoplasmic reticulum. By Nomarski optics, it appeared that cells staining greatest for COX-2 were spread and commonly binucleate, whereas those staining the least were rounded and mononucleate. In parallel experiments, all cells excluded trypan blue dye, indicating that the rounded cells were not dead. COX-2 protein was also induced to a lesser extent by TNF- α ($100 \text{ ng}/\text{ml}$), appearing as a single 69-kDa

band (Fig. 3A). There was no detectable induction by IL-1 ($5 \text{ ng}/\text{ml}$). COX-1 expression was unchanged following treatment with LPS, TNF- α , or IL-1 (Fig. 3A), and it was not altered by overnight incubation alone, ASA pretreatment, or the combination of ASA pretreatment and LPS treatment (Fig. 3C). Based on these results, subsequent experiments focused on LPS-induced COX-2.

According to current models, the result of elevated mass of COX protein via COX-2 induction should be a nonselective increase in the synthesis of all PGs that the given cell type can make. However, when PMs were treated with LPS for 24 h to induce COX-2, washed, and stimulated with A23187 ($1 \mu\text{M}$, 15 min, 37°C) to release AA from cell membranes, selective changes in PG production were readily apparent, as compared with that seen in freshly isolated cells. These PMs, which contained both active COX-1 and COX-2, synthesized the same products as freshly isolated PMs (Fig. 4A). However, following LPS treatment, the proportion of radiolabeled AA metabolized to PGE $_2$ was increased, whereas that converted to TxA $_2$, as well as the 5-lipoxygenase product 5-HETE, was reduced. One explanation for the altered PG profile following LPS treatment would be that COX-2 preferentially metabolizes AA to PGI $_2$ and PGE $_2$, whereas the other prostanoids were derived from COX-1 action. To address this, COX-1 was irreversibly inactivated in fresh PMs with ASA, the ASA was washed away, and COX-2 was induced with LPS for 24 h. The treatment with ASA ($200 \mu\text{M}$) alone for 1 h effectively eliminated COX activity, and continued culture (after washing to remove ASA) in LPS-free medium for 24 h did not result in significant new COX activity (Fig. 4B). The cells treated with ASA that were washed and given LPS produced predominantly PGI $_2$ and PGE $_2$, as well as LTB $_4$ (Fig. 4C). These cells, with only COX-2 active, had approximately 85% of all released AA converted to PGs, as compared with 60% in COX-1-containing fresh PMs. Interestingly, there were only minor changes in LTB $_4$ production. Finally, the production of PGs was inhibited by the selective COX-2 inhibitor NS-398; in cells pretreated with ASA and then given LPS to induce COX-2, PGE $_2$ synthesis was completely inhibited by $1 \mu\text{M}$ NS-398, as determined by EIA, with an IC $_{50}$ of $0.12 \mu\text{M}$ NS-398. In contrast, in freshly isolated PMs, 50% inhibition of PGE $_2$ synthesis required $8.3 \mu\text{M}$ NS-398 (approximately 70-fold more than the IC $_{50}$ of ASA-pretreated, LPS-treated cells); PGE $_2$ synthesis was inhibited only 75% by $30 \mu\text{M}$ NS-398, the highest dose tested.

Pooled data from three independent experiments indicated that cells given LPS overnight and thus with both active COX isoforms produced predominantly PGI $_2$, TxB $_2$, and PGE $_2$, with PGD $_2$ and HHT being less pronounced (Table II). This pattern was also generally seen when the radiolabeled AA was supplied exogenously. Pretreatment of PMs with ASA to inactivate COX-1 and thus leave COX-2 as the only active isoform following LPS treatment resulted in PGI $_2$ and PGE $_2$ being the major products of stimulated cells, with TxB $_2$ occasionally found in significant amounts (Table II). In contrast, PGD $_2$ and HHT were barely detectable. Similar results were obtained if radiolabeled AA was supplied exogenously and cells were not acutely stimulated. The two products, PGI $_2$ and PGE $_2$, constituted $85.8 \pm 14.8\%$ of all PGs released via COX-2, as compared with $26.2 \pm 13.9\%$ of all PGs released from cells containing only COX-1 (Table I). PMs incubated overnight in LPS-free medium with or without polymyxin B, which binds and inactivates LPS, generated the same profile of products as freshly isolated PMs, except that PGD $_2$ tended to be reduced and LTB $_4$ elevated with overnight culture (data not shown).

Distal Enzymes—A second possible explanation for the altered PG profile following LPS treatment would be that LPS

TABLE I
Products from COX-1 in freshly isolated PMs

Cells were either prelabeled with [³H]AA for 2 h, washed, and stimulated with A23187 (1 μM, 10 min, 37 °C) or given exogenous [³H]AA without A23187 (2 h, 37 °C). Radioactive metabolites in conditioned media were separated by HPLC; results indicate the disintegrations/min per product as a percentage of all prostanoid disintegrations/min. Values are the mean of four experiments; S.E. is in parentheses.

	Product				
	6-keto-PGF _{1α}	TxB ₂	PGE ₂	PGD ₂	HHT
Fresh PMs					
From endogenous AA	19.1 (8.7)	25.5 (6.1)	7.1 (5.1)	20.3 (10.3)	28.0 (8.4)
From exogenous AA	17.1 (6.4)	17.8 (5.3)	9.8 (4.1)	24.5 (8.4)	30.8 (7.6)

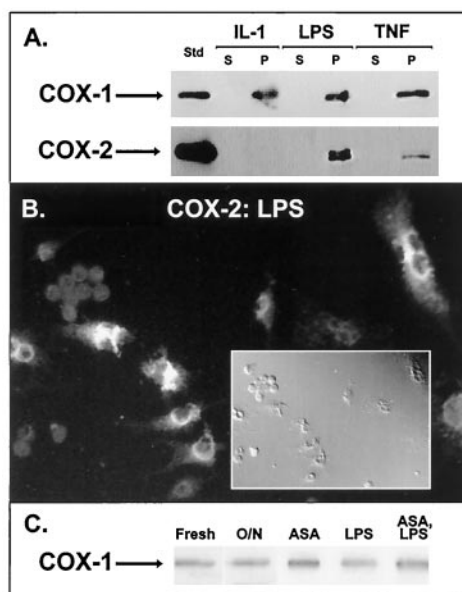


FIG. 3. Induction of COX-2 protein synthesis by LPS or TNF- α , but not IL-1, in PMs. Cells were treated with LPS (1 μg/ml), TNF- α (100 ng/ml), or IL-1 (5 ng/ml) for 24 h, separated into soluble (S) and pelletable (P) fractions, and compared by immunoblot analysis (A) with authentic standards (Std). Alternatively, cells were treated with LPS for 24 h, washed, fixed, and probed for COX-2 by immunofluorescent microscopy (B) as described under "Experimental Procedures." The inset presents the same field of cells as viewed by light microscopy using Nomarski optics. C, COX-1 protein expression in freshly isolated PMs (Fresh) or in PMs cultured overnight (O/N) with ASA pretreatment alone before overnight incubation, with LPS treatment, or with ASA pretreatment followed by washing and treatment with LPS overnight. Results are representative of at least three independent experiments.

selectively changed the amount or activity of all enzymes that act distal to the COX isozymes. Specifically, LPS might have had up-regulating effects on PGI₂ and PGE₂ synthases and down-regulatory effects on synthases giving TxA₂, PGD₂, and HHT. To address this possibility, we examined the effects of LPS on the processing of the COX product, PGH₂. Exogenous PGH₂ (0.1 μM) was efficiently metabolized to a variety of PGs by freshly isolated PMs (Fig. 5A). The most abundant product was PGE₂ at about 24% of all recovered product. This was surprising because freshly isolated PMs released little PGE₂ from either endogenous AA (following ionophore stimulation) or exogenous AA (Table I). A similar profile of products was obtained when PGH₂ was given to unfractionated cell lysates (data not shown). When PMs were first treated with LPS for 24 h, the proportion of PGH₂ converted to PGE₂ increased from 24 to approximately 30% of all recovered disintegrations/min (Fig. 5B), consistent with a slight increase in PGE₂ synthase activity. However, in three independent experiments using both whole cells and cell lysates, there were no significant differences between freshly isolated and LPS-treated PMs in the profiles of products generated from PGH₂.

The increase in PGE₂ synthase activity was further assessed

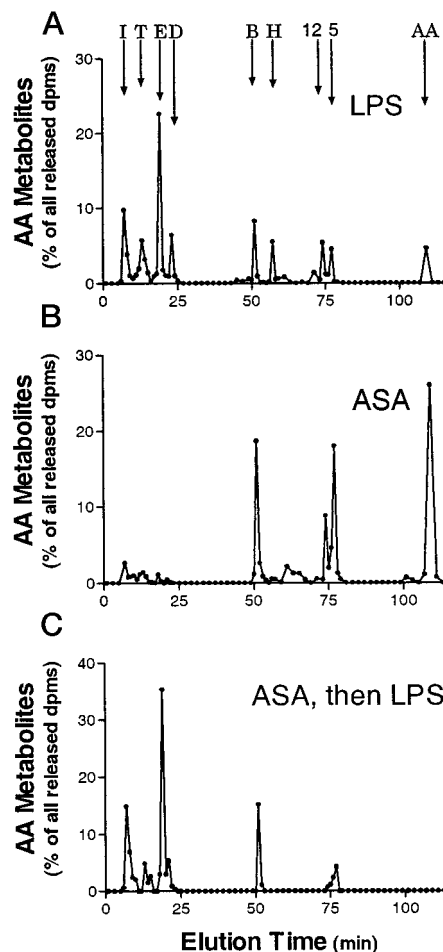


FIG. 4. Products from COX-2. Representative profiles of AA metabolites derived from PMs treated with LPS. PMs were cultured with: A, LPS (1 μg/ml) for 24 h to produce cells containing both active COX isoforms; B, ASA (200 μM) for 1 h, washed, and cultured in LPS-free medium for 24 h for cells with no active COX enzyme; or C, ASA for 1 h, then LPS for 24 h for active COX-2 only. In these experiments, all cells were prelabeled with [³H]AA during the overnight incubation, then washed, and stimulated with 1 μM A23187 for 15 min at 37 °C. Radio-labeled metabolites in the conditioned media were separated by HPLC and identified by co-elution with unlabeled authentic standards: I, PGI₂; T, TxA₂; E, PGE₂; D, PGD₂; B, LTB₄; H, HHT; 12, 12-HETE; 5, 5-HETE. Results are representative of three independent experiments.

using EIAs to specifically measure PGE₂ production. Following ionophore stimulation to liberate endogenous AA, freshly isolated PMs made significant amounts of PGE₂, and PGE₂ production following acute stimulation increased approximately 2.5-fold when PMs were treated with LPS for 6 or 24 h (Fig. 6A). Exogenous PGH₂ was efficiently converted to PGE₂ by untreated, freshly isolated PMs, consistent with the HPLC analysis. After 6 or 24 h of LPS treatment, PGE₂ production from PGH₂ increased significantly. However, this increase, although statistically significant, was only by 30%. These effects were further extended to TNF- α , which also induced

TABLE II
 Products from LPS-treated PMs

Cells were adhered in media with or without ASA (200 μM) for 1 hr, washed, and incubated in media with LPS (1 μg/ml) for 24 h with [³H]AA. Cells were then washed and stimulated with A23187 (1 μM, 10 min, 37 °C). Radioactive metabolites in conditioned media were separated by HPLC; results indicate the disintegrations/min per product as a percentage of all prostanoid disintegrations/min. Values are the mean of three experiments; S.E. is in parentheses.

	Product				
	6-keto-PGF _{1α}	TxB ₂	PGE ₂	PGD ₂	HHT
PMs given LPS for 24 h					
From endogenous AA	32.9 (11.3)	21.9 (8.0)	30.4 (12)	4.7 (3.4)	10.1 (6.1)
From exogenous AA	31.5 (8.2)	8.21 (5.7)	36.7 (12)	7.4 (4.8)	16.2 (2.6)
PMs ASA pretreated, then given LPS for 24 h					
From endogenous AA	41.5 (9.9)	7.6 (6.1)	44.4 (11)	1.8 (2.5)	4.8 (6.7)
From exogenous AA	40.0 (2.7)	13.6 (7.0)	27.7 (8.0)	1.0 (1.3)	18.7 (9.2)

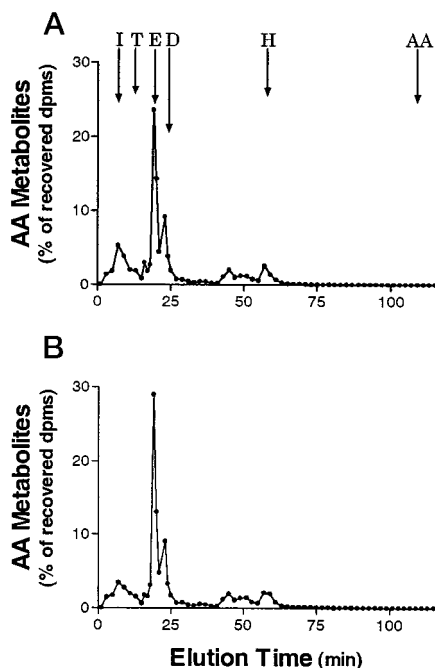


FIG. 5. Effect of LPS treatment on the conversion of PGH₂ to PGs. Freshly isolated PMs (A) or PMs incubated for 24 h with LPS (B) were washed and given [³H]PGH₂ for 10 min at 37 °C. Radiolabeled metabolites were separated by HPLC and identified by co-elution with unlabeled authentic standards: I, PGI₂; T, TxA₂; E, PGE₂; D, PGD₂; H, HHT. Results are representative of three independent experiments.

COX-2 synthesis in these cells (Fig. 3). The treatment of PMs with TNF-α for 24 h also increased PGE₂ synthesis from AA by 3-fold, whereas it increased PGE₂ production from PGH₂ again by only 30% (Fig. 6B). It should be noted that PGE₂ production from PGH₂, as presented in Fig. 6, is measured on a different scale than PGE₂ production from AA, with the former being 8 times the latter.

DISCUSSION

In this study, we found that freshly isolated PMs, which have COX-1 but not COX-2, make a balance of four major prostanoids from AA. A fifth prostanoid, PGE₂, is only a minor product, although these cells can readily convert PGH₂ to PGE₂. Similar results were obtained if PMs were maintained overnight in LPS-free medium. In stark contrast, when PMs were incubated with LPS to induce COX-2, the profile of PG products shifted to emphasize two PGs, PGI₂ and PGE₂. Pretreatment with ASA to inactivate COX-1, leaving COX-2 as the only active isoform, accentuated this change. These results were surprising because increasing COX mass through LPS treatment did not result in an across-the-board increase in production of all COX products. Instead, COX-2 induction caused a profile shift char-

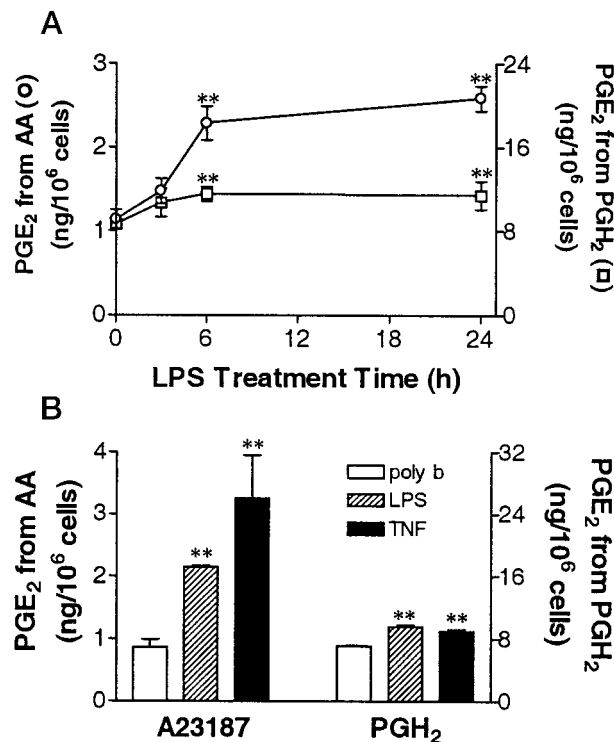


FIG. 6. PGE₂ production via COX-2 plus PGE₂ synthase or PGE₂ synthase alone. A, a time course of changes is shown. Freshly isolated PMs were cultured in media containing LPS (1 μg/ml) for the indicated times, washed, and stimulated with 1 μM A23187 to release endogenous AA (circles) or stimulated with exogenous PGH₂ (0.1 μM, squares) for 15 min at 37 °C. PGE₂ levels were measured in conditioned media by EIA. B, comparative effects of overnight culture with polymyxin B, LPS, or TNF-α are shown. PMs were cultured in media containing polymyxin B (open bars), LPS (slashed bars) or TNF-α (filled bars) for 24 h, washed, and stimulated with 1 μM A23187 to release endogenous AA or stimulated with exogenous PGH₂ (0.1 μM) for 15 min at 37 °C. PGE₂ levels were measured in conditioned media by EIA. Results are from one experiment in triplicate and are representative of three independent experiments. Bars show S.E.

acterized by preferential synthesis of PGI₂ and PGE₂ with reduced production of TxB₂, PGD₂, and HHT.

A shift in product profile may arise from a change in the amounts of each of the distal enzymes, which process the COX product, PGH₂. Indeed, Oh-ishi and colleagues (9, 10) have shown that PGE₂ synthase activity was induced by LPS in inflammatory PMs, whereas PGD₂ synthase and TxA₂ synthase activities were unaffected. In their studies, cell lysates from these cells lacked any detectable PGE₂ synthase activity; this would serve to make this enzymatic step rate-limiting in the synthesis of PGE₂. In this study, using resident PMs, we found significant PGE₂ synthase activity in both whole cells

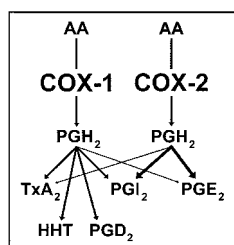


FIG. 7. Proposed model for differential processing of endogenously derived AA by COX-1 and COX-2 in resident rat PMs. The widths of lines leading to end products are intended to indicate the proportion of PGH₂ derived from each COX isoform that is metabolized to the given end product. Variations in AA delivery to the COX isoforms are not considered here.

and cell lysates before treatment with LPS as reported previously (6). Treatment with LPS resulted in a small (30%) but significant increase in PGE₂ synthase activity. This change certainly may contribute to the increase in PGE₂ production in LPS-treated cells. However, two results suggest that changes in distal enzymes were not sufficient to account for the change in PG profile. First, the changes in product profile from distal enzymes alone (Fig. 5) were not comparable to the changes in products derived from COX isoforms plus distal enzymes (Figs. 2 and 4). Second, the magnitude of increase in PGE₂ production from PGH₂ was much less than that from AA (Fig. 6). The difference in scale for PGE₂ synthesis from PGH₂ versus AA, noted in Fig. 6, argues that the activity of COX, not PGE₂ synthase, is rate-limiting in the production of PGE₂, and thus the small changes in PGE₂ synthase activity cannot account for the increased production of PGE₂ following LPS treatment. These results suggest that other factors contributed to the shift in product profile following COX-2 induction.

Previous researchers have suggested that the COX-1 and COX-2 metabolic pathways are at least partially independent from one another, particularly in terms of when and where the pathways are expressed (11–13). However, we propose that the COX-1 and COX-2 pathways differ in the profile of their products. A model of this relationship in PMs, based on the results of this study, is given in Fig. 7. According to this model, AA metabolism by COX-1 produces PGH₂ that is directed in a balanced way to enzymes that give rise to PGI₂, TxA₂, PGD₂, and HHT. In contrast, COX-2 metabolism is much more tightly coupled to the PGI₂- and PGE₂-synthesizing enzymes than to other downstream enzymes. Then the result of COX-2 induction by inflammatory mediators is not simply an increase in all PGs that a given cell type can produce. Instead, there is a shift in the balance of PGs toward the preferential production of PGI₂ and PGE₂.

A simple explanation for the shift in product profile relates to the subcellular distribution of the metabolic enzymes. First, the two COX isoforms appear to differ in their subcellular distribution. COX-1 is dispersed throughout the intracellular membrane system, whereas COX-2 is preferentially accumulated on and around the nuclear envelope (14, 15). It should be noted that a recent study has questioned whether this difference is real or artifactual (16), but our results support distinct distributions for the two isoforms (Fig. 8, see also Figs. 1 and 3). Second, the different PG synthases appear to differ in their subcellular distribution, as has been recently reviewed (17). For example, the glutathione-requiring form of PGD₂ synthase is found in the cytoplasm of a variety of cell types (18, 19), whereas PGE₂ synthase activity has been localized to the endoplasmic reticulum and nuclear envelope (20). Finally, different pools of AA, liberated by distinct phospholipase A₂ isoforms, may feed into the different COX pathways (21–23). Thus, COX-1, because of its diffuse subcellular distribution,

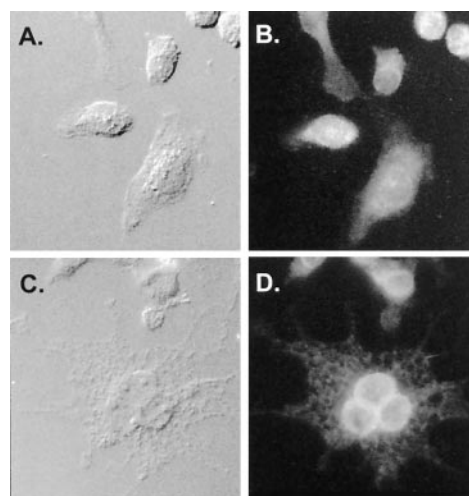


FIG. 8. Subcellular localization of COX-1 and COX-2 by indirect immunofluorescent microscopy. Rat PMs were adhered to glass slides and incubated for 24 h in media containing LPS (1 μg/ml) and then stained for either COX-1 (A, B) or COX-2 (C, D). Preparations were imaged with Nomarski optics (A, C) to visualize entire cells and by fluorescence to localize either COX-1 (B) or COX-2 (D). Results are representative of at least three independent experiments.

may deliver PGH₂ relatively nonselectively to downstream enzymes. On the other hand, COX-2 may monopolize AA liberated from the nuclear envelope, e.g. by the action of the 85-kDa phospholipase A₂ acting at that site (24–26), and preferentially deliver PGH₂ to distal enzymes located on and around the nuclear envelope, such as PGE₂ synthase and, perhaps, PGI₂ synthase.

The paired production of PGI₂ and PGE₂ is a common response to inflammatory mediators in a wide range of settings. Both PGI₂ and PGE₂ increased significantly in vascular endothelial cells in response to LPS or TNF-α (27), in ciliary epithelial cells in response to LPS (28), in gingival fibroblasts in response to IL-1 or TNF-α (29), and in myometrial cells in response to IL-1 or TNF-α (30). Similarly, PGI₂ and PGE₂ production by aortic endothelial cells was elevated in response to xenoreactive antibodies and complement (31). In *ex vivo* culture, the saphenous vein released PGI₂ and PGE₂ in response to IL-1 (32). In whole animal models, plasma levels of PGI₂ and PGE₂ were elevated when TNF-α was administered intravenously to rats (33) or sheep (34) and also when bleomycin was given intratracheally to hamsters (35). Because COX-2 induction is common to all these experimental systems, these results are consistent with the thesis that COX-2 preferentially metabolizes AA to PGI₂ and PGE₂.

What is the impact of the paired production of PGI₂ and PGE₂? Both PGs evoke a number of common cellular responses, including smooth muscle relaxation, decreased fibroblast and smooth muscle proliferation, diminished collagen deposition, and decreased leukocyte function. Although other signaling pathways are also used, both PGs evoke many of these effects through receptor-mediated elevation of cAMP (reviewed in Refs. 34 and 35). Also through cAMP signaling, both PGI₂ and PGE₂ inhibit activation-initiated responses of many leukocytes, including neutrophils, macrophages, mast cells, and some T lymphocytes (reviewed in Refs. 36 and 37). Thus, PGI₂ and PGE₂ inhibit cytokine production by Th1-type lymphocytes, resulting in decreased secretion of IL-2, interferon-γ, IL-8, IL-12, and TNF-α (38, 39). Signaling through cAMP has variable effects on Th2-type lymphocytes, and thus these PGs may either have no effect or actually increase the production of Th2-type cytokines, including IL-4, IL-5, IL-10, and IL-13 (40).

These results suggest that the induction of COX-2 may, by

shifting the profile of PG secretion, serve to actually diminish the inflammatory response in some tissues. Thus, in the absence of inflammatory mediators, COX-1 serves to generate a balance of PGs that may maintain a level of system homeostasis. Inflammatory mediators, like bacterial LPS, would evoke a number of responses, including the synthesis of Th1-type cytokines and COX-2. Our data, as well as the studies cited above, indicate that COX-2 metabolism of AA will tip the balance of secreted PGs to emphasize PGI₂ and PGE₂. Via cAMP signaling, these PGs would subsequently begin the resolution of the inflammatory response through the suppression of the synthesis of Th1-type cytokines and, at least indirectly, favor the development of a Th2-type profile of cytokines. According to this model, a defect in the ability to induce COX-2 expression would both suppress the synthesis of PGI₂ and PGE₂ and augment inflammation in some circumstances. Indeed, fibroblasts from patients with idiopathic pulmonary fibrosis have a severely impaired capacity to express COX-2, but not COX-1, and make much less PGE₂ than do control fibroblasts (41). The failure to induce COX-2 and produce prostanoids that can suppress inflammation, fibroblast proliferation, and collagen synthesis can thus contribute to disease progression.

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