

LIPOLYSACCHARIDE is an inflammatory agent and interleukin-1 is a cytokine. Their pro-inflammatory effects may be mediated by prostanoids produced by inducible cyclooxygenase-2. The aim of this study was to determine the prostanoids produced by lipopolysaccharide and interleukin-1 stimulated enterocytes through the cyclooxygenase-1 and 2 pathways. Cultured enterocytes were stimulated with lipopolysaccharide or interleukin-1 β with and without cyclooxygenase inhibitors. Low concentrations of indomethacin and valerylsalicylic acid (VSA) were evaluated as cyclooxygenase-1 inhibitors and their effects compared with the effects of a specific cyclooxygenase-2 inhibitor, SC-58125. Prostaglandin E₂, 6-keto prostaglandin F_{1 α} , prostaglandin D₂ and leukotriene B₄ levels were determined by radioimmunoassay. Immunoblot analysis using isoform-specific antibodies showed that the inducible cyclooxygenase enzyme (COX-2) was expressed by 4h in LPS and IL-1 β treated cells while the constitutive COX-1 remained unaltered in its expression. Interleukin-1 β and lipopolysaccharide stimulated the formation of all prostanoids compared with untreated cells, but failed to stimulate leukotriene B₄. Indomethacin at 20 μ M concentration, and VSA inhibited lipopolysaccharide and interleukin 1 β stimulated prostaglandin E₂, but not 6-keto prostaglandin F_{1 α} formation. SC-58125 inhibited lipopolysaccharide and interleukin-1 β stimulated 6-keto prostaglandin F_{1 α} but not prostaglandin E₂ release. The specific cyclooxygenase-2 inhibitor also inhibited lipopolysaccharide produced prostaglandin D₂ but not interleukin-1 β stimulated prostaglandin D₂. While SC-58125 inhibited basal 6-keto prostaglandin-F_{1 α} formation it significantly increased basal prostaglandin E₂ and prostaglandin D₂ formation. As SC-58125 inhibited lipopolysaccharide and interleukin-1 β induced 6-keto prostaglandin F_{1 α} production but not prostaglandin E₂ production, it suggests that these agents stimulate prostacyclin production through a cyclooxygenase-2 mediated mechanism and prostaglandin E₂ production occurs through a cyclooxygenase-1 mediated mechanism. Prostaglandin D₂ production appeared to be variably produced by cyclooxygenase-1 or cyclooxygenase-2, depending on the stimulus.

Key words: Cytokines, Eicosanoids, Cyclooxygenase inhibitors

The role of cyclooxygenase-1 and cyclooxygenase-2 in lipopolysaccharide and interleukin-1 stimulated enterocyte prostanoid formation

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Introduction

Metabolism of arachidonic acid occurs by three major enzymatic pathways; cytochrome P450, lipooxygenase and cyclooxygenase.^{1,2} Prostanoid production in the cyclooxygenase system is the result of the activity of at least two prostaglandin H synthase (cyclooxygenase, COX) enzymes, COX-1 and COX-2.³ COX-1 is a constitutive enzyme, present in low levels, and

believed to produce continuously present cytoprotective prostanoids.^{4,5} COX-2 is inducible in response to a variety of stimulants including mitogens and inflammatory agents and is believed to produce pro-inflammatory prostanoids.^{6,7}

Limited information exists concerning the role specific eicosanoids play in inflammatory processes studied *in vivo* and why, *in vitro*, certain cell lines produce certain eicosanoids in response to various

pro-inflammatory stimuli.⁴ In studies evaluating the role of eicosanoids in intestinal inflammation, we examined the effect of *Clostridium difficile* toxin on the production of eicosanoids by a human colonic cancer cell line (Caco-2).⁸ The increased eicosanoid production stimulated by *Clostridium difficile* toxin was characterized by large increases in prostaglandin E₂ (PGE₂) and 6-ketoprostaglandin-F_{1α} (6KPGF_{1α}) production but not leukotriene B₄ (LTB₄). In a subsequent study in the same cell line,⁹ trinitrobenzene sulphonic acid (TNB), a hapten which produces experimental colitis, produced a response characterized by the release of 6KPG F_{1α} and LTB₄.

Working with the hypothesis that certain prostanoids were produced by COX-1 and others by COX-2, attempts were made to discern the relative contributions of COX-1 and COX-2 to prostanoid formation by intestinal epithelial cells stimulated by pro-inflammatory agents. Based on *in vitro* data that microsomal COX-1 enzyme is inhibited by low concentration indomethacin and COX-2 inhibition requires greater concentrations of indomethacin by a factor of five,^{10,11} we studied the relative contributions of COX-1 and COX-2 to prostanoid formation.⁸ Evaluation of Caco-2 cells stimulated by *Clostridium difficile* toxin demonstrated that low concentration indomethacin failed to inhibit *Clostridium difficile* toxin stimulated prostanoid formation while high concentration indomethacin did, consistent with the concept that *Clostridium difficile* stimulated prostanoid formation through a COX-2 mediated process.

We pursued the issue further by evaluating the role of COX-1 and COX-2 in TNB stimulated Caco-2 cells.⁹ Both low (20 μM) and high (100 μM) concentration indomethacin inhibited resting and TNB stimulated formation of all prostanoids. As low concentration indomethacin inhibited TNB stimulated prostanoid formation, we were unable to confirm the proposed role of COX-2 in mediating intestinal epithelial cell prostanoid formation in response to inflammatory stimuli utilizing this methodology. In a preliminary study a specific COX-2 inhibitor, NS398¹² inhibited enterocyte prostacyclin formation stimulated by proinflammatory agents, but did not significantly change the increase in PGE₂.¹³ In the present study, the specific COX-2 inhibitor, SC58125,¹⁴ was compared with both low dose indomethacin and valeryl-salicylic acid (VSA),¹⁵ a salicylate ester with considerable specificity toward COX-1, to attempt to determine the relative contributions of COX-1 and COX-2 to prostanoid formation by an epithelial cell line.

Lipopolysaccharide (LPS) is an inflammatory agent which induces interleukin-1 (IL-1)¹⁶ and prostanoid formation¹⁷ and alters intestinal permeability,¹⁸ and mitogenesis,¹⁹ and causes the production by intesti-

nal cells of a heat shock protein.²⁰ Interleukin-1, a pro-inflammatory cytokine which is capable of stimulating arachidonic acid metabolism²¹ has been implicated as an inflammatory mediator in the intestine.²² IL-1 has been shown to increase prostanoid synthesis by a COX-2 mediated mechanism.²¹ It is possible that this pro-inflammatory cytokine, released within intestinal tissues, could mediate prostanoid formation produced by pro-inflammatory agents. In this study, we evaluated the effects of LPS and IL-1 on COX-1 and COX-2 eicosanoid production in an enterocyte cell line.

Materials and Methods

Cell culture

The non-transformed rat enterocyte cell line, derived from distal ileum, IEC-18 (American Type Culture Collection, Rockville, MD) was utilized in all experiments employing culture techniques described previously.^{8,9} Briefly, the cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum containing gentamicin (0.1 mg/ml) and 1% MEM nonessential amino acids (Sigma, St Louis, MO). The cells were grown in a 37°C, humidified, 5% CO₂ incubator and maintained by weekly passage. The cells were plated on six-well tissue culture plates (Costar, Cambridge, MA) at 2 × 10⁵ cells per well and allowed to grow to 100% confluence. The cell morphology was evaluated by routine phase microscopy.

Materials and protocol

The cells were washed with KRB buffer and exposed for 4 h to LPS or IL-1β dissolved and diluted in 95% oxygenated KRB buffer. The lipopolysaccharide employed was *Escherichia coli* lipopolysaccharide, CO 111:B4 (Sigma, St Louis, MO). The human recombinant IL-1β was obtained from Merck Du Pont Laboratories, Glenolden, PA. Indomethacin (Sigma 20 μM), VSA (Cayman, Ann Arbor, MI, 50 μM) and SC-58125 (50 μg/ml) were dissolved in 5% sodium carbonate solution and diluted in KRB buffer immediately before use and added to the wells. The concentration of indomethacin was chosen to attempt to produce inhibition of COX-1 but not COX-2 enzyme activity.^{10,11} To evaluate the effect of the COX inhibitors, the cells were pre-treated with indomethacin, VSA or SC-58125 in 1 ml fresh tissue culture media for 1 h prior to washing the cells. Control cells and cells exposed to LPS or IL-1β were similarly treated with fresh media for 1 h. At the conclusion of the experiments the cells and buffer were collected and frozen at -80°C until the protein assays were performed.

Western blot analysis

Cells were seeded into 35 mm dishes (1.5×10^6 /dish) and grown to confluency for 2 days. The cultures were rinsed with serum-free medium and incubated with media alone or with IL-1 B or LPS for 8 h. At the conclusion of the experiments the cells were lysed with 1% Triton X-100, 120 mM sodium chloride, 25 mM HEPES (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 10 μ /ml leupeptin, 10 μ g/ml apoprotinin and 10 μ g/ml antipapain. After 30 min, the extracts were centrifuged at $12000 \times g$ for 10 min and the supernatants solubilized with SDS-PAGE buffer (68 mM Tris-HCl, pH 6.8, 5% B-mercaptoethanol, 2% SDS, and 10% glycerol). The samples were boiled for 5 min and equal amounts were applied to a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and then incubated overnight in Tris-buffered saline, 0.2% Tween-20, and 10% non-fat dry milk (BLOTTO). The membranes were incubated with a rabbit anti-serum generated against murine COX-2 (1:1000) or sheep COX-1 (1:1000). Following a 3-h incubation at 37°C, the membranes were washed with BLOTTO and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1000, Cappel) for 30 min at 37°C. Immunoreactive proteins were visualized using the enhanced chemiluminescence (ECL®; Amersham) method.

Eicosanoid determination

To determine the cell protein concentration per well the wells were thawed and washed with KRB buffer. The cells were freed by incubation with 1% collagenase solution for 20 min. The cells were scraped from the wells and centrifuged at $200 \times g$ for 20 min and washed with KRB. Protein was determined by the method of Bradford²³ on cell specimens which were solubilized with 0.1 N NaOH for 1 h at 37°C and then sonicated for 10 s. Bovine albumin was employed as the standard. PGE₂, 6-KPGF_{1 α} , PGD₂ and leukotriene B₄ (LTB₄) assays were performed on the buffer solutions in duplicate without separation by a competitive enzyme assay which utilizes an acetylcholinesterase tracer (Cayman, Ann Arbor, MI).^{8,9} The eicosanoid concentrations were determined by spectrophotometric analysis after addition of Ellman's reagent and comparison to a standard curve. The concentrations of eicosanoids were expressed as picograms per milligram cell protein.

Statistical analysis

The data is presented as mean \pm SEM. Statistical analysis was performed by analysis of variance. Differences between groups was determined by the least significant difference. As used throughout the manuscript 'significant' indicates $P < 0.05$.

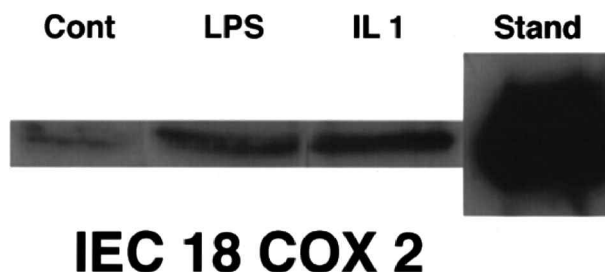


FIG. 1. LPS and IL- β stimulated COX-2 protein expression. IEC-18 cells were seeded into 35 mm dishes and grown for 2 days at which time they were incubated with LPS or IL-1 β . Control cells received medium alone during the incubation period. Extracts of the cells were prepared by SDS-PAGE, blotted onto nitrocellulose membranes, and probed with antibody specific for COX-2. COX-1 protein was very faintly visualized and no apparent increase was produced by LPS or IL-1 β (data not presented).

Results

Western blotting of resting cells (medium alone) faintly demonstrated detectable COX-1 and COX-2 protein. When cells were incubated with LPS or IL-1 β for periods of up to 24 h, there was no change in expression of COX-1 protein (data not presented). However, by 8 h of LPS or IL-1 β treatment, COX-2 expression was increased (Fig. 1). Prestained molecular weight markers were run on the same gels and the COX immunoreactive bands shown corresponded to proteins approximately 70 kDa in size consistent with COX enzymes.

LPS and IL-1 β produced concentration related increases in PGE₂, 6KPGF_{1 α} and PGD₂ formation by IEC-18 cells. Representative data demonstrating the effect of IL-1 β on PGD₂ formation is presented in Fig. 2. IL-1 β (0.01, 0.1, 1.0, 10 and 100 units/ml)

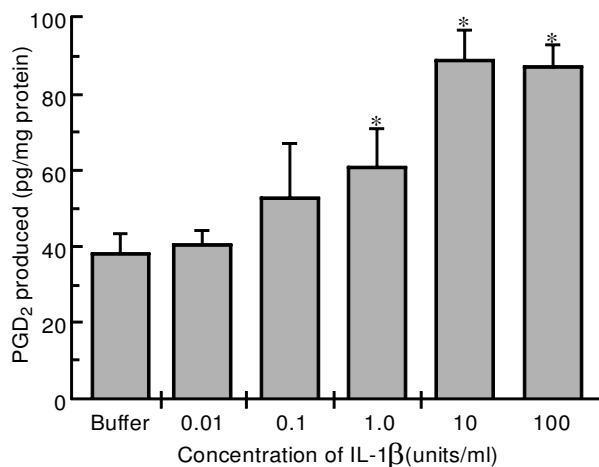


FIG. 2. The effect of IL-1 β on PGD₂ formation. Each bar represents the mean \pm SEM of six values obtained from duplicate measurements of samples from six wells. Asterisk indicates that the experimental value is significantly different from the control value.

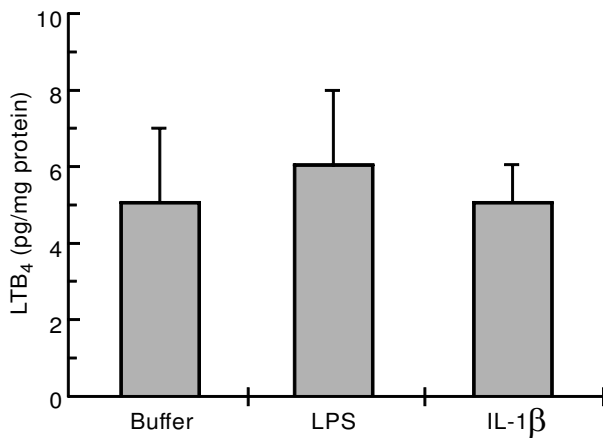


FIG. 3. The effect of LPS and IL-1β on LTB₄ production by IEC-18 cells. Neither LPS (100 μg/ml) or IL-1β (10 units/ml) stimulated LTB₄ formation compared with control values. Each bar represents the mean ± SEM of six values obtained from the mean of duplicate measurements from six wells containing 1 × 10⁵ confluent cells.

produced maximal responses in prostanoid formation at 10 units/ml. LPS (0.01, 0.1, 1.0, 10 and 100 μg/ml) produced significantly greater prostanoid levels when 100 μg/ml were administered than when 10 μg/ml was evaluated. Neither LPS or IL-1β in the concentrations and time intervals employed in this study significantly changed LTB₄ production by IEC-18 cells (Fig. 3). Also evaluation of LTB₄ levels in experiments associated with indomethacin, VSA or SC58125 administration with and without LPS or IL-1β was associated with no significant changes in LTB₄ formation (data not presented).

As seen in Fig. 4, LPS (100 μg/ml) and IL-1β (10 units/ml) stimulated PGE₂ production by IEC-18 cells compared with control values. VSA and indomethacin (20 μM) significantly decreased LPS and IL-1β stimulated PGE₂ production while the specific COX-2 inhibitor, SC-58125 (50 μg/ml) did not significantly alter LPS or IL-1β stimulated PGE₂ production and significantly increased basal PGE₂ production.

The pro-inflammatory agent LPS and the cytokine IL-1β both significantly increased prostacyclin formation as evidenced by increases in the stable metabolite of prostacyclin, 6-KPGF_{1α} (Fig. 5). Neither indomethacin (20 μM) or VSA significantly altered basal or stimulated 6KPGF_{1α} formation; however, the selective COX-2 inhibitor SC-58125 significantly decreased the formation of 6KPGF_{1α} in response to the pro-inflammatory agents. These results and the results of the evaluation of PGE₂ formation suggest that PGE₂ is produced by COX-1 and prostacyclin is produced by COX-2 in IEC-18 cells. The results also suggest that an inflammatory stimulus such as endotoxin and a cytokine such as IL-1β may stimulate the formation of a prostanoid (PGE₂) produced by the COX-1 pathway.

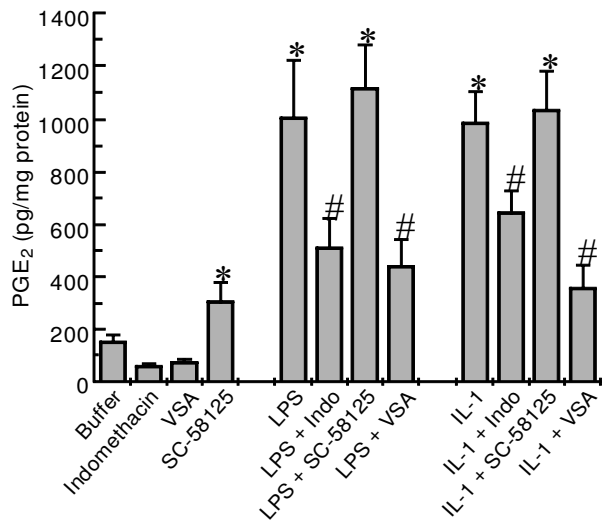


FIG. 4. The effect of indomethacin, VSA, a specific COX-1 inhibitor and SC-58125, a specific COX-2 inhibitor, on PGE₂ formation in response to LPS (100 μg/ml) and IL-1β (10 units/ml). Indomethacin (20 μM) and VSA inhibited the increase in PGE₂ formation produced by LPS and IL-1β; however SC-58125 (50 μg/ml) did not. Each bar represents the mean ± SEM of six values obtained from the mean of duplicate measurements from six wells of confluent ileal mucosal cells. One asterisk indicates that the stimulated value is significantly different (*P* < 0.05) from the resting value and # indicates that the inhibitor significantly altered the results produced by LPS or IL-1β.

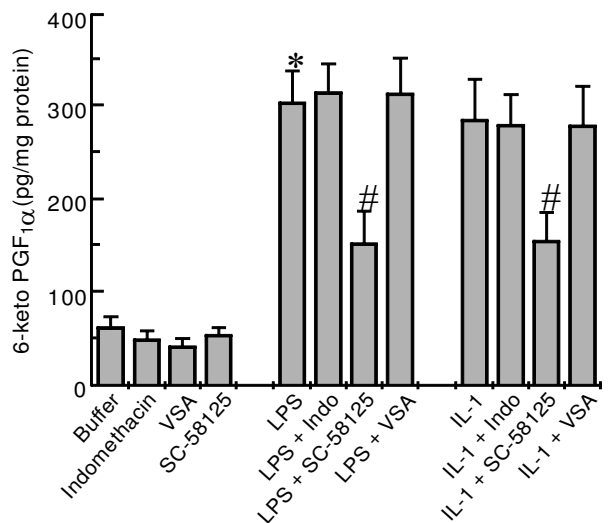


FIG. 5. The effect of indomethacin (20 μM), VSA (50 μM) and SC-58125 (50 μg/ml) on LPS and IL-1β stimulated 6KPGF_{1α} formation. SC 58125, the specific COX-2 inhibitor significantly decreased resting and stimulated 6KPGF_{1α} formation while indomethacin and VSA did not. Each bar represents the mean ± SEM of six values obtained from the mean of duplicate measurements from six wells of IEC-18 enterocytes. One asterisk indicates that the stimulated value is significantly different (*P* < 0.05) from the resting value and # indicates that the inhibitor significantly altered the results produced by LPS or IL-1β.

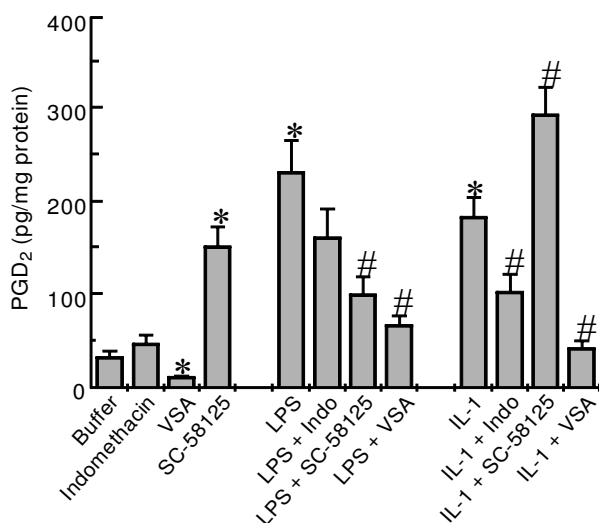


FIG. 6. The effect of indomethacin, VSA and SC-58125 on the formation of PGD₂ stimulated by LPS and IL-1 β . LPS stimulated PGD₂ formation was inhibited by SC-58125 and VSA but not by indomethacin while IL-1 β stimulated PGD₂ formation was inhibited by indomethacin and VSA but not SC-58125. When the COX-2 inhibitor was administered to resting or IL-1 β stimulated cells an exaggerated increased response in PGD₂ production occurred. Each bar represents the mean \pm SEM of six values obtained from six wells of confluent rat enterocytes. One asterisk indicates that the stimulated value is significantly different ($P < 0.05$) from the resting value and # indicates that the inhibitor significantly altered the results produced by LPS or IL-1 β .

PGD₂ is a prostanoid involved in the production of inflammation in various systems²⁴ and has been found to be a major prostanoid produced by intestinal epithelial cells in response to mitogenic stimuli.²⁵ Both LPS and IL-1 β increased PGD₂ production (Fig. 6). Indomethacin did not significantly alter LPS stimulated PGD₂ concentrations; however, it did significantly inhibit IL-1 β stimulated PGD₂ levels. The specific COX-2 inhibitor SC-58125 and the COX-1 inhibitor VSA decreased the PGD₂ response produced by LPS. The COX-2 inhibitor markedly augmented PGD₂ production by resting IEC-18 cells and by IL-1 β stimulated cells. The COX-1 inhibitor, VSA, decreased the PGD₂ response produced by LPS and IL-1 β . LPS appeared to increase PGD₂ formation through COX-1 and COX-2 metabolic pathways while IL-1 β stimulated PGD₂ formation produced by COX-1.

Discussion

The specific role that individual prostanoids play in the physiologic functions of mucosa or in the pathologic processes involving intestinal mucosa including inflammation is unknown. The general hypothesis presently directing many research efforts is that COX-1, a constitutive enzyme, produces basal cytoprotective prostanoids; and that COX-2, an indu-

cible enzyme responding to pro-inflammatory agents and mitogens, produces prostanoids involved in inflammation and replication of mucosal cells.^{2,5,7} The availability of specific COX-1 and COX-2 inhibitors, which have a high degree of specificity against pure enzyme preparations, should enable the determination of which enzyme produces which prostanoid in response to a specific stimulus.

In two previous studies employing enzyme preparations there was some dose specificity, with low concentrations of indomethacin inhibiting COX-1 while COX-2 inhibition required much higher concentrations.^{10,11} In another report low concentration indomethacin appeared to be an inhibitor of both COX-1 and COX-2.¹² In this study we chose to preincubate the cells with the inhibitors prior to exposing the cells to the pro-inflammatory agents. In other studies, COX-2, but not COX-1 containing J774.2 macrophages preincubated with indomethacin were resistant to indomethacin.²⁶ Additionally, other reports have demonstrated that even without preincubation, Chinese hamster ovary cells stably expressing COX-2 but not COX-1, were resistant to indomethacin.²⁷ The inhibition of PGE₂ production but not 6KPGF_{1 α} formation by 20 μ M indomethacin and VSA produced in the present study supports the premise that COX-1, but not COX-2 activity was inhibited by low dose indomethacin.

COX-1 and COX-2 produce similar products (PGG₁ and PGH₂)⁷ but exist in separate cellular compartments.²⁸ In a previous study employing pure COX-1 or COX-2 enzyme, both enzymes produced PGE₂.¹² When COS-1 cells were transfected with COX-1 or COX-2 containing expression vector, both transformed cell lines produced comparable basal levels of PGE₂, PGD₂ and PGF_{2 α} from arachidonic acid.¹⁰ The effect of specific COX-1 and COX-2 inhibitors on resting prostanoid production in the present study also suggests that unstimulated prostanoid formation was produced primarily by COX-1 activity. Inhibition of COX-2 enzyme activity by SC-58125 significantly increased basal PGE₂ and PGD₂ formation. This result suggests that COX-2 inhibition in resting IEC-18 cells drives arachidonic acid into COX-1 produced PGE₂. It is unclear whether this is the result of a low level COX-2 protein continuously present or due to low level induction of COX-2 produced by cell manipulation and culture techniques. Utilizing Western blotting the COX-2 protein was identified in resting cells. In previous studies evaluating isolated human gall bladder mucosal cells, COX-2 protein expression was markedly increased by lysophosphatidylcholine; however, resting gall bladder mucosal cells faintly expressed COX-2 protein.²⁹

It has been an important consideration in eicosanoid metabolism that cyclooxygenase inhibition directs arachidonic acid into the lipooxygenase pathway.³⁰ This phenomenon may be occurring in the

present study with COX-2 inhibition directing arachidonic acid metabolism into the COX-1 metabolic pathway associated with enhanced PGE₂ and PGD₂ formation. Alternatively it is possible that COX-2 produced prostacyclin produces a negative feedback mechanism, inhibiting COX-1 from producing PGE₂ and PGD₂. Future studies will need to be performed evaluating the effect of specific COX-1 inhibitors¹⁵ and exogenous prostacyclin on the response of resting IEC-18 cells to SC-58125.

An important finding in the present study suggests that both LPS and IL-1 β stimulate PGE₂ formation through a COX-1 mediated process. COX-1 produced products were increased in response to stimulation by inflammatory agents. It is unlikely that the inflammatory agents increased constitutive COX-1 protein formation, as it was not evidently induced as evaluated by Western immunoblotting and as it has generally not been inducible by cytokines;² however, there does seem to be stimulation of COX-1 enzyme activity.

These results suggest that inflammatory or mitogenic stimuli which induce COX-2 but not COX-1 expression do not necessarily produce prostanoids exclusively through COX-2 activity. Prostacyclin formation in response to inflammatory stimuli appeared to be primarily associated with COX-2 activity. The relationship that resulted in comparable responses regarding PGE₂ and prostacyclin formation being produced by LPS and IL-1 β was not present when the PGD₂ response was evaluated. LPS stimulated PGD₂ appeared to be produced by COX-1 and COX-2 while the IL-1 β response appeared to be mediated by COX-1. SC-58125 again produced an exaggerated response with increased production of PGD₂ in association with the purported COX-1 stimulant, IL-1 β .

Recently, overexpression of COX-2 has been associated with epithelial malignancy.³¹ In studies of human colorectal cancer, COX-2 is increased in approximately 90% of the cancers and 40% of premalignant colorectal adenomas, but it is not expressed in non-tumour colon tissue.³² Cyclooxygenase inhibitors that block the activity of COX-2 are associated with a decreased incidence of colon cancer.³¹ Sulindac a COX inhibitor produced polyp regression in patients with familial polyposis.³³ The availability of specific COX-2 inhibitors may result in improved effectiveness of these drugs in altering carcinogenesis, while decreasing their side effects.

The results produced in this study suggest that increased production of a specific prostanoid in response to a pro-inflammatory agent or a cytokine may not be due to stimulation of inducible COX-2. Delineating which enzymatic pathway produces which prostanoids in response to various inflammatory, mitogenic or cytoprotective stimuli may improve our understanding of the role of prostanoids in health and disease.

Other recent studies have found that in the same cell line, different stimuli produced different prostanoids.³⁴ The mechanism by which differential prostanoid or leukotriene formation occurs is not known. It is possible that a specific stimulus may alter the activity of one of the rate controlling enzymes in arachidonic acid formation, secretory 14 kDa phospholipase A₂ or cytoplasmic 85 kDa phospholipase A₂ resulting in the generation of prostanoids through a specific synthetic pathway which generates one prostanoid rather than another.³⁵ As suggested in the present study, it is possible that a cytokine stimulates COX-1 or COX-2 to generate specific prostanoid species. Erdbrugger *et al.*³⁴ proposed that cells contain multiple compartments³⁶ that differentially express the phospholipase, COX, isomerase and synthase enzymes responsible for producing a specific prostanoid and also that these compartments may present receptors for specific stimuli. Further research will be required to determine how and why cells make one prostanoid rather than another.

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