Research Paper

The role of selective cyclooxygenase isoforms in human intestinal smooth muscle cell stimulated prostanoid formation and proliferation

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INTESTINAL smooth muscle plays a major role in the repair of injured intestine and contributes to the prostanoid pool during intestinal inflammatory states. Cyclooxygenase (COX), which catalyzes the conversion of arachidonic acid to prostanoids exists in two isoforms, COX-1 and COX-2. The purpose of this study was to determine the relative contributions of COX-1 and COX-2 in the production of prostanoids by human intestinal smooth muscle (HISM) cells when stimulated by interleukin-1β (IL-1β) and lipo-polsaccharide (LPS). Furthermore the effects of specific COX-1 and COX-2 inhibitors on the proliferation of smooth muscle cells was also evaluated. Confluent monolayer cultures of HISM cells were incubated with IL-1β or LPS for 0–24 h while control cells received medium alone. PGE$_2$ and PGI$_2$ as 6-keto-PGF1α and LTB$_4$ were measured by a specific radioimmunoassay. COX enzymes were evaluated by Western immunoblotting. Unstimulated and stimulated cells were exposed to the specific COX-1 inhibitor valerylsalicylic acid (VSA) and the COX-2 inhibitors NS-398 and SC-58125. The effects of serum on proliferation were then evaluated in the presence of each of the specific COX inhibitors by incorporation of ^{3}H-thymidine into DNA. IL-1β and LPS increased both PGE$_2$ and 6-keto-PGF1α in a dose dependent fashion with enhanced production detected two hours following exposure. Neither stimulus stimulated LTB$_4$ release. Immunoblot analysis using isoform-specific antibodies showed that both COX-1 and COX-2 were present constitutively. Furthermore, COX-1 was upregulated by each inflammatory stimulus. In a separate set of experiments cells were pretreated with either the selective COX-1 inhibitor VSA or the selective COX-2 inhibitors NS-398 or SC-58125 prior to treatment with IL-1β or LPS. The COX-1 and COX-2 inhibitors decreased both basal and IL-1β and LPS stimulated prostanoid release. Spontaneous DNA synthesis was present and serum consistently increased proliferation. ^{3}H-thymidine incorporation, stimulated by serum, was inhibited by both COX-1 and COX-2 inhibitors. This study suggests that the prostanoid response stimulated by proinflammatory agents of gut-derived smooth muscle cells appears to be mediated by both COX-1 and COX-2 enzymes. Proliferation of smooth muscles cells also appears to be influenced by both COX-1 and COX-2.

Key words: Human intestinal smooth muscle cells, IL-1β, LPS, Cyclooxygenase, Prostanoids, Mitogenesis

Introduction

Cyclooxygenase (COX), also known as prostaglandin H synthase, is a membrane bound, bifunctional enzyme that catalyzes the conversion of arachidonic acid to prostaglandin G$_2$ by its cyclooxygenase activity and prostaglandin G$_2$ to prostaglandin H$_2$ by peroxidase activity. It is the rate-limiting step in the biosynthesis of the biologically active and physio-logically important prostaglandins, thromboxanes and prostacyclin. COX exists in two distinct isoforms. COX-1 exists in most tissues and is involved in the physiological production of prostaglandins. COX-2 has been demonstrated to be expressed in response to many proinflammatory stimuli such as cytokines as well as growth factors.[1,2] Furthermore, COX-2 is thought to be the isoform responsible for the production of proinflammatory prostanoids in various

models of inflammation. The identification of constitutive and inducible COX enzymes has led to the hypothesis that COX-2 is primarily responsible for prostaglandins produced in inflammation and COX-1 is involved in normal homeostasis.[3,4]

Inflammatory bowel disease (IBD) is a chronic debilitating illness of the gastrointestinal tract that encompasses two primary forms of idiopathic intestinal inflammation. Ulcerative colitis is a disease that affects primarily the colon and rectum and involves mucosal (epithelial) inflammation, while Crohns disease affects the GI tract from mouth to anus and involves transmural inflammation, including the mucosal and muscle layers. As a result of the amplification of the inflammatory process, soluble mediators such as eicosanoids play a significant role in the pathogenesis and progression of IBD.[5] Production of eicosanoids are increased in gut mucosa in active IBD, whether assessed by *in vitro* culture of mucosal biopsies, *in vivo* rectal dialysis, or whole-gut lavage.[6,7] The production of prostaglandins declines in IBD patients treated with either corticosteroids or sulfasalazine. Although prostaglandin levels in mucosal specimens of IBD patients declines when they are treated with NSAIDs, there is no clinical improvement. In one study, selective COX-2 antagonists were shown to exacerbate colitis.[8]

Human intestinal smooth muscle cells play a major role in the repair of injured intestine. This process appears to be mediated by cytokines such as interleukin-1B which induces collagenase expression and inhibits collagen expression in human intestinal smooth muscle cells.[9,10] A common feature of Crohn's disease is a narrowing or stricturing of the bowel lumen. Stricture formation remains a clinically important complication of Crohn's disease. Previous studies have demonstrated that IL-1B enhances proliferation of smooth muscle cells suggesting that the cytokine produced during chronic inflammation may contribute to intestinal stricture formation.[11]

Selective COX inhibitors are currently available. In previous studies, utilizing specific COX-1 and COX-2 inhibitors, it has been found that selective COX-1 and/or COX-2 inhibitors variably inhibit cell prostanoid formation stimulated by cytokines dependent on the cell type and the stimulus.[12–14] Proliferation of both non-transformed epithelial cells and malignant intestinal epithelial cells was inhibited by specific COX-2, but not COX-1 inhibitors,[15] indicating a role for COX in mitogenesis as well as demonstrating the role of COX in prostanoid production by proinflammatory agents in various cells such as enterocytes,[12] colonocytes[14] and gallbladder cells.[13] Limited information exists regarding the activity of COX enzymes in human intestinal smooth muscle cells.[11] In the present study, the effect of selective COX-1 and COX-2 inhibitors on IL-1 and LPS stimulated prostanoid release by intestinal smooth muscle cells was evaluated. It was also intended to ascertain if COX enzymes play a role in the replication of human intestinal smooth muscle cells.

Methods

Cell isolation and culture

Human Intestinal Smooth Muscle cells (HISM, catalogue #1692-CRL) obtained from the American Type Culture Collection (Rockville, MD) were maintained at 37°C in an atmosphere of 5% CO_2 and 100% relative humidity. Cells were split at a ratio of 1:2 upon reaching confluence. Cells were detached using 0.5 g porcine trypsin and 0.2 g EDTA tetrasodium/l Hanks balanced salt solution (Sigma Chemical, St Louis-MO) and then plated into 24 well plates for experiments or into 175 cm^2 flasks (Costar, Cambridge, MA) for propagation. The medium was changed every five to seven days. The medium used was Dulbeccos modified Eagles supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotercin B (Sigma). Viability of the cells was verified by trypan blue exclusion and morphology was evaluated by phase contrast microscopy.

Materials and protocol

The cells were plated at a density of 1×10^6 cells/well and after 24 h were washed with KRB buffer and exposed for 0–24 h to LPS or IL-1β dissolved and diluted in 95% oxygenated KRB buffer. The lipopolysaccharide employed was *Eschericia coli* lipopolysaccharide, CO 111:B4 (Sigma, St Louis, MO) and the human recombinant IL-1β was obtained from Merck DuPont Laboratories (Glenolden, PA). VSA (Cayman, Ann Arbor, MI, 50 µM), SC-58125 (provided by Peter Isakson, Monsanto Searle, St Louis, MO, 50 µM) and NS-398 (Cayman, 50 µM) were dissolved in 5% sodium carbonate solution and diluted in KRB buffer immediately before use. The concentrations of proinflammatory agents and COX inhibitors chosen were based on previous dose response studies performed utilizing intestinal epithelial cells.[12,13] To evaluate the effect of the COX inhibitors the cells were pretreated with VSA, SC-58125, or NS-398 in 1 ml fresh tissue culture media for 1 h prior to washing. 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 assays were performed.

Western blot analysis

Cells were seeded into 100 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-1β 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 phenyl-methylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml apoprotinin and 10 µg/ml antipapain. After 30 min the extracts were centrifuged at 12,000 *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% nonfat dry milk (BLOTTO). The membranes were incubated with a rabbit antiserum generated against murine COX-2 (1:1000) or sheep COX-1 (1:1000). Following a three-hour 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 3 min at 37°C. Immunoreactive proteins were visualized using the enhanced chemiluminescence (ECL®, Amersham) method.

Eicosanoid measurements

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 *g* for 20 min and washed with KRB. Protein was determined by the method of Bradford[16] 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. PGE$_2$, 6-KPGF$_{1\alpha}$ and leukotriene B$_4$ (LTB$_4$) 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). The eicosanoid concentrations were determined by spectrophotometric analysis after addition of Ellmans reagent and comparison to a standard curve. The concentrations of eicosanoids were expressed as picograms per milligram cell protein.

Proliferation studies

For the proliferation experiments 1×10^3 cells were seeded onto 96 well culture plates (Costar, Cambridge, MA) in 100 µl of Dulbelcco's minimum essential medium (DMEM, Sigma, St Louis, MO) with or without 10% fetal bovine serum (FBS, Sigma). After 24 h at approximately 70% confluency, the cells were treated with either the COX-1 inhibitor VSA (Cayman, Ann Arbor, MI), the COX-2 inhibitor SC-58125 (Monsanto/

Searle, St Louis, MO) or indomethacin (Sigma, St Louis, MO). After 24 h of exposure the cells were washed with phosphate-buffered saline (PBS, Sigma) and treated with the same types of media and agents, including 1.5 uCi/well ^{3}H-thymidine (ICN, Costa Mesa, CA) for 7 h. The cells were lysed with 100 ml of 0.1 M NaOH and 0.2% Triton X-100 (Sigma) and the lysates precipitated with 20% trichloroacetic acid (TCA, Sigma). The precipitates were then harvested in 5% TCA onto 240–1 glass-fiber filters (Cambridge, Watertown, MA). The amount of isotope incorporated into DNA was determined by liquid scintillation counting using a Beckman LS 100 instrument.

Statistical analysis

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

Results

Western analysis

Western blotting of resting cells (medium alone) demonstrated both constitutive COX-1 and COX-2 protein. When cells were incubated with LPS or IL-1β for periods of up to 24 h, there was no increase in expression of COX-2 protein. However, after 8 h of LPS or IL-1β treatment, COX-1 expression was increased (Fig. 1). Prestained molecular weight
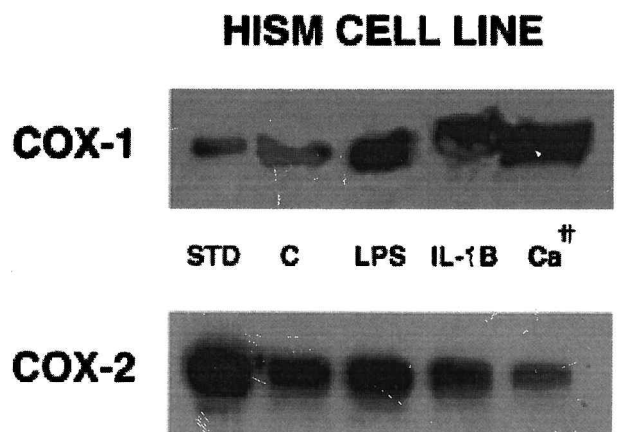

FIG. 1. Western immunoblotting of COX-1 protein and COX-2 protein in unstimulated HISM cells and in cells stimulated for 8h with LPS (100 µg/ml) and IL-1β (100 units/ml). Calcium ionophore (5 µM) was also evaluated. Unstimulated cells demonstrated constitutive COX-1 and COX-2 protein was increased by LPS or IL-1β. Unstimulated cells demonstrated constitutive COX-2 protein. Both LPS and IL-1β induced COX-2 protein formation.

Table 1. The effect of cyclooxygenase 1 and 2 inhibitors on unstimulated prostaglandin E2 and prostacyclin (6-keto PGF$_{1\alpha}$) formation (ng/mg cell protein) by human intestinal smooth muscle cells

	Control	VSA	SC-58125	NS-398
PGE$_2$	46.4±6.3	7.9±1.2*	9.1±1.3*	4.4±1.6*
6KPGF$_{\alpha}$	2.7±0.3	0.7±0.02*	0.8±0.02*	1.1±0.2*

Each value represents the mean±SEM of six values obtained from the mean of duplicate measurements of prostanoids in the buffer solution from six tissue culture wells containing 1×10^6 HISM cells maintained in buffer solution, with or without COX inhibitors for 4 h. Asterisk indicates that the value is significantly different from the control value.

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. These results suggest that both COX-1 and COX-2 protein were both present constitutively and COX-1 was further upregulated by proinflammatory stimuli.

Basal prostanoid formation

When unstimulated HISM cells were evaluated, the cells produced both PGE$_2$ and PGI$_2$, the latter evaluated by measuring 6-keto-PGF1α (Table 1). The HISM cell line produced significantly more PGE$_2$ than 6-keto-PGF1α. In unstimulated HISM cells, VSA, SC-58125 and NS-398 inhibited both PGE$_2$ and 6-keto-

PGF1α formation, suggesting that both isoforms contribute to basal prostanoid levels (Table 1). Basal LTB$_4$ levels were unaffected by COX inhibition.

Stimulated prostanoid formation

The proinflammatory stimulant IL-1β produced concentration related increases in PGE$_2$ formation by HISM cells (Fig. 2). The increase in PGE$_2$ produced by IL-1β was also time dependent with the maximum production seen at 8 h (Fig. 3). IL-1β (100 units/ml) and LPS (100 μg/ml) stimulated PGE$_2$ production by HISM cells compared to control values (Tables 2 and 3). VSA, SC-58125 and NS-398 significantly decrease IL-1β and LPS stimulated PGE$_2$ production (Tables 2 and 3). LPS and IL-1β both also significantly increased prostacyclin formation as evidenced by increases in the stable metabolite of prostacyclin, 6-KPGF$_{1\alpha}$ (Tables 2 and 3). Similar to that seen with PGE$_2$, VSA, SC-58125 and NS-398 significantly decreased stimulated 6-KPGF$_{1\alpha}$ formation. These results suggest that both PGE$_2$ and prostacyclin formation have variable and not exclusive contributions by COX-1 and COX-2 in HISM cells. Neither LPS or IL-1β in the concentrations and time intervals employed in this study significantly changed LTB$_4$ production by HISM cells. Also evaluation of LTB$_4$ levels in experiments associated with VSA, SC-58125, or NS-398 administration with and without LPS or IL-1β was associated with no significant changes in LTB$_4$ formation (data not presented).
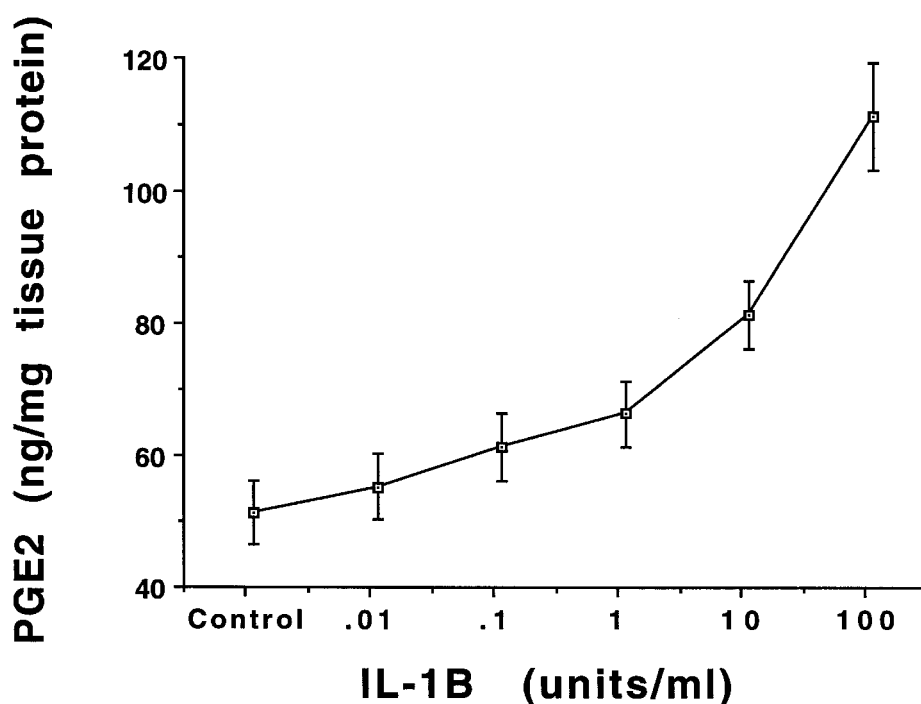

FIG. 2. Dose response evaluation of IL-1β stimulated PGE$_2$ formation in HISM cells 1×10^6 HISM cells were exposed to varying concentrations of IL-1β for 4 h. Each point represents the mean ± SEM of 6 values obtained from duplicate measurements of PGE$_2$ in buffer solution from six wells.
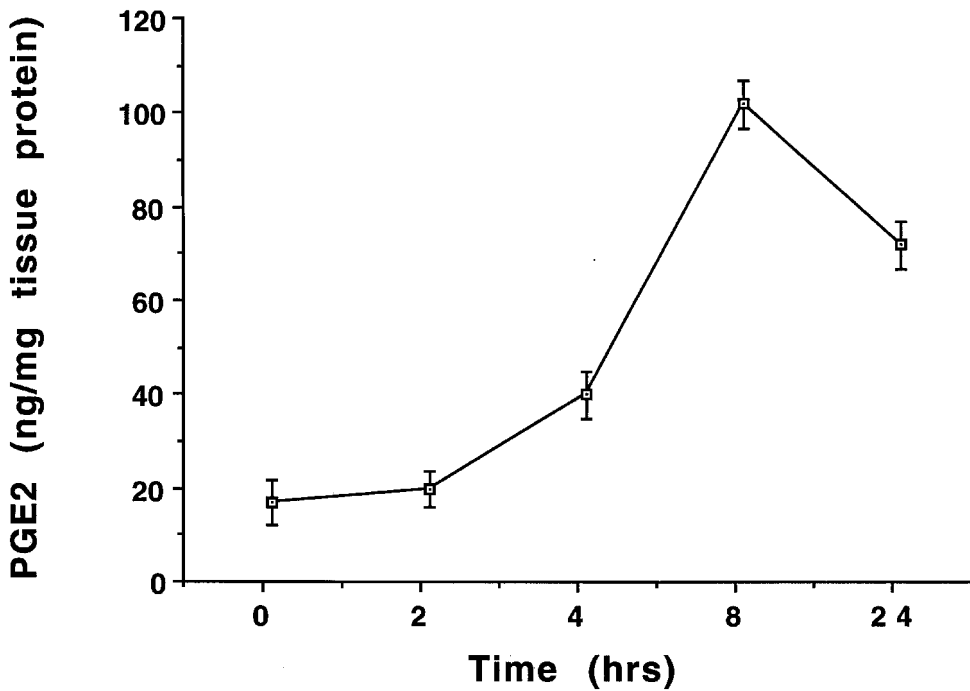
FIG. 3. Evaluation of IL-1 stimulated PGE$_2$ formation in HISM cells between 0–24 h. HISM cells were exposed to 10 units/ml IL-1β for the time periods indicated. Each point represents the mean±SEM of six values.

Proliferation

The effects of COX-1 and COX-2 inhibitors on ^{3}H-thymidine incorporation in HISM cells are illustrated in Fig. 4. The concentrations of inhibitors employed were based on previous studies with these agents. There was low-level incorporation of ^{3}H-thymidine into DNA by HISM lines maintained in serum-starved circumstances. Serum produced a significantly increased rate of ^{3}H-thymidine incorporation in HISM cells. Both VSA and SC-58125 significantly decreased ^{3}H-thymidine incorporation in

Table 2. The effect of cyclooxygenase 1 and 2 inhibitors on IL-1β stimulated prostaglandin E2 and prostacyclin (6-keto PGF$_{1\alpha}$ formation (ng/mg cell protein) by human intestinal smooth muscle cells

	Control	IL-1	IL-1 (100 uM) plus 50 uM		
			VSA	SC-58125	NS-398
PGE$_2$	46.4±2.3	106.1±5.4*	27.2±1.3#	51.5±4.6#	8.3+1.2#
6KPGF	2.7±0.7	12.4+3.1*	3.1±0.2#	0.8±0.1#	0.9±0.2#

Each value represents the mean ±SEM of six values obtained from the mean of duplicate measurements of prostanoids in the buffer solution from six tissue culture wells containing 1×10^6 HISM cells. The cells were exposed to the cytokines with or without the COX inhibitors for four hours. Asterisk indicates that the stimulated value is significantly different from the control value. The # sign indicates that the COX inhibitor significantly altered the value stimulated by the cytokine.

Table 3. The effect of cyclooxygenase 1 and 2 inhibitors on LPS stimulated prostaglandin E2 and prostacyclin (6-keto PGF$_{1\alpha}$) formation (ng/mg cell protein) by human intestinal smooth muscle cells.

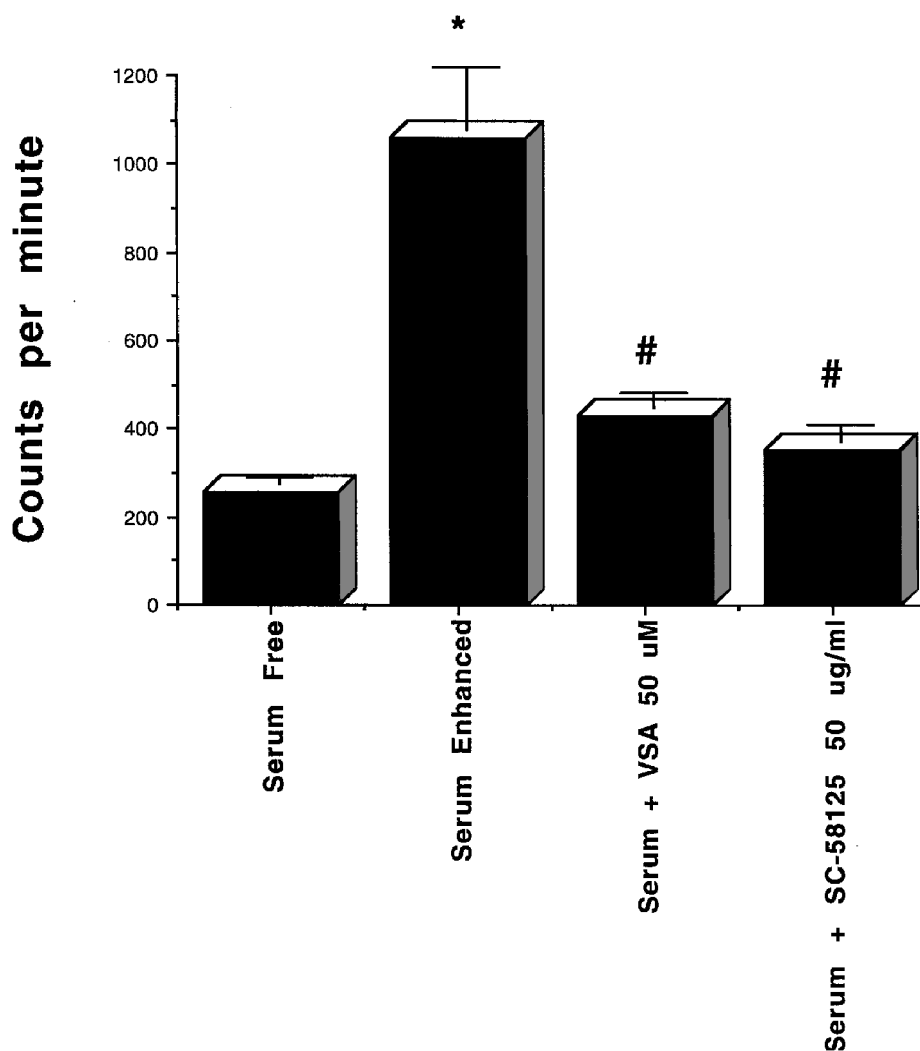|	Control	LPS	LPS (100 µg/m) plus 50 uM		
			VSA	SC-58125	NS-398
PGE$_2$	46.4±2.3	88.1±9.4*	22.4±2.4#	19.5±3.8#	6.1+2.0#
6KPGF	2.7±0.7	6.9+1.8*	0.5±0.06#	0.7±0.1#	0.9±0.3#

Symbols as for Table 2.

FIG. 4. The effect of specific COX inhibitors on serum enhanced ^{3}H thymidine incorporation by HISM cells. The * indicates that serum significantly increased ^{3}H thymidine incorporation compared to serum free cells. The # indicates that the COX inhibitor significantly decreased ^{3}H thymidine incorporation compared to serum enhanced cells.

HISM cells grown in 10% FBS suggesting that both COX-1 and COX-2 enzymes may contribute to human smooth muscle cell proliferation.

Discussion

This study examined the induction by IL-1 and LPS of COX protein and the effect of selective COX antagonists on prostanoid formation and on cell proliferation in a human gut derived smooth muscle cell line. This cell line appears to be a useful model for studying the production of prostanoids by smooth muscle cells during inflammatory states. Previous studies from our laboratory employing gastrointestinal epithelial cells have shown that trinitrobenzene sulfonic acid (TNB), a hapten which produces experimental colitis, led to significant increases in PGE_2 and 6-keto $PGF1\alpha$ release, but not leukotriene release; responses which were inhibited by indomethacin.[17] In the present study, IL-1β and LPS stimulate prostanoid production in HISM cells which was inhibited by selective COX inhibitors. To investigate the mechanism by which IL-1β and LPS enhance the production of PGE_2 and 6-keto $PGF_{1\alpha}$ in the HISM cell, we analyzed the expression of both COX-1 and COX-2 protein following treatment. We found that IL-1β and LPS resulted in the rapid and transient induction of both the COX-1 and COX-2 isoform.

Other investigators have examined the induction of COX isoforms in smooth muscle cells other than those that are gut derived. Increased levels of several pro-inflammatory cytokines including IL-1 and TNF have been found in bronchoalveolar lavage fluid from symptomatic asthmatic patients. The effects of IL-1β, TNF and IFN on the induction of COX isoforms in cultured human airway smooth muscle cells demonstrated that only IL-1β produced a time and concentration dependent enhancement of PGE_2 and 6-keto

PGF$_{1\alpha}$ and a corresponding increase in COX-2, but not COX-1 activity. Pretreatment with conventional NSAIDs and selective COX-2 inhibitors completely blocked IL-1β induced PGE$_2$ release and both glucocorticoids and protein synthesis inhibitors suppressed IL-1β induced COX-2 induction. This suggests that airway smooth muscle may be an important source of prostaglandins in human airways and that COX-2 may play an important role in the regulation of the inflammatory process in asthma.[18] A similar study demonstrated that COX-2 expression and PGE$_2$ formation were inhibited by an IL-1 receptor antagonist.[19] Both studies suggest that IL-1β produced PGE$_2$ formation in human bronchial smooth muscle cells that is mediated by *de novo* expression of COX-2 enzyme.

The metabolism of arachidonic acid and production of eicosanoids plays a vital role in the function of the central nervous system, especially the cerebrovascular function. Smooth muscle cells isolated from murine cerebral microvessels produced primarily PGE$_2$ and PGI$_2$ in response to exogenous arachidonic acid and calcium ionophore. Serum deprivation of smooth muscle cells caused an 80–90% diminution in both PGE$_2$ and PGI$_2$, which was restored with the reintroduction of serum within 6 h. COX-1 and COX-2 mRNA was detectable in smooth muscle cells grown in the presence of serum, but COX-2 mRNA was not present in serum deprived cells. Readdition of serum induced a massive increase in COX-2 mRNA with only a small increase in COX-1 mRNA.[20] Cultured rat aortic smooth muscle cells exhibited a 45-fold increase in COX-2 mRNA levels after 2 h exposure to serum. This was associated with increased COX-2 protein and a 3-fold increase in PGE$_2$.[21] COX-1 protein is present in freshly isolated myometrial cells and is not affected by treatment with IL-1. Constitutive COX-2 is also present and is unregulated by IL-1β treatment. Both basal and IL-1β stimulated increase in COX-2 were reduced by both a protein synthesis inhibitor and an inhibitor of transcription.[22]

The possible relationship of COX enzymes, prostanoids, specific COX-1 and COX-2 inhibitors and standard NSAIDs to decreasing the rate of development of gastrointestinal cancer is currently an active area of investigation. Previous work from our laboratory has demonstrated that in both nonmalignant- and malignant-derived murine intestinal epithelial cell lines there was evidence that the proliferation of cells, stimulated by serum, was influenced by COX-2, but not COX-1 enzyme.[15] These results confirm previous *in vitro* studies demonstrating the inhibition of cell proliferation by specific COX-2 inhibitors.[23–26] These experimental results support a role for COX-2 in intestinal epithelial cell mitogenesis and provided ground work for similar studies with smooth muscle cells. Unlike that seen with intestinal epithelial cells, proliferation of HISM cells appears to

have variable contributions from both COX-1 and COX-2.

Crohn's disease is an idiopathic, chronic inflammation of the gastrointestinal tract that causes narrowing and stricturing of the small and large intestine. Although the mechanisms by which chronic inflammation promotes stricture formation remain ill defined, it does appear to be associated histologically with a hyperplasia of smooth muscle cells and an increased deposition of collagen within the bowel wall. The proinflammatory cytokines IL-1β caused a significant dose-dependent increase in intestinal smooth muscle cell proliferation.[27] This suggests that proinflammatory cytokines produced during chronic inflammation may enhance the proliferation of smooth muscle cells and may contribute to the narrowing and stricturing observed in Crohn's disease. Smooth muscle cells in the intestinal wall play a significant role in the healing of the injured intestine and in the fibrosis that complicates Crohn's disease.[27] IL-1β is mitogenic for human intestinal smooth muscle cells and is associated with a concomitant down regulation of collagen synthesis and secretion and an augmentation of collegenase expression.[28] Corticosteroids repress the induction of collagenase expression and the inhibition of collagen secretion by IL-1β in HISM cells. We feel that based on our preliminary work, selective COX inhibition may be a potential avenue for addressing the problem of stricture formation such that changes in prostanoid milleu or even smooth muscle cell mitogenesis may either delay or ameliorate progression of intestinal Crohn's disease.

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