Selective inhibition of inducible cyclooxygenase 2 *in vivo* is antiinflammatory and nonulcerogenic

JAIME L. MASFERRER*, BEN S. ZWEIFEL, PAMELA T. MANNING, SCOTT D. HAUSER, KATHLEEN M. LEAHY, WALTER G. SMITH, PETER C. ISAKSON, AND KAREN SEIBERT

Searle Inflammatory Diseases Research, G. D. Searle, 800 North Lindbergh Street, St. Louis, MO 63167

ABSTRACT We have examined the role of cyclooxygenase 2 (COX-2) in a model of inflammation *in vivo*. Carrageenan administration to the subcutaneous rat air pouch induces a rapid inflammatory response characterized by high levels of prostaglandins (PGs) and leukotrienes in the fluid exudate. The time course of the induction of COX-2 mRNA and protein coincided with the production of PGs in the pouch tissue and cellular infiltrate. Carrageenan-induced COX-2 immunoreactivity was localized to macrophages obtained from the fluid exudate as well as to the inner surface layer of cells within the pouch lining. Dexamethasone inhibited both COX-2 expression and PG synthesis in the fluid exudate but failed to inhibit PG synthesis in the stomach. Furthermore, NS-398, a selective COX-2 inhibitor, and indomethacin, a nonselective COX-1/COX-2 inhibitor, blocked proinflammatory PG synthesis in the air pouch. In contrast, only indomethacin blocked gastric PG and, additionally, produced gastric lesions. These results suggest that inhibitors of COX-2 are potent antiinflammatory agents which do not produce the typical side effects (e.g., gastric ulcers) associated with the nonselective, COX-1-directed antiinflammatory drugs.

Nonsteroidal antiinflammatory drugs (NSAIDs) are used to treat acute and chronic inflammatory disorders such as rheumatoid arthritis. The antiinflammatory mechanism of NSAIDs is due to a reduction of prostaglandin (PG) synthesis by the direct inhibition of cyclooxygenase (COX; prostaglandin-endoperoxide synthase, EC 1.14.99.1) (1). Unfortunately, inhibition of PG production in organs such as stomach and kidney can result in gastric lesions, nephrotoxicity, and increased bleeding.

COX exists in two forms. COX-1 is in most tissues and is involved in the physiological production of PGs. COX-2 is cytokine-inducible and is expressed in inflammatory cells (2–9). The identification of constitutive and inducible COX enzymes led to the hypothesis that COX-2 is primarily responsible for PGs produced in inflammation and COX-1 for PGs involved in normal homeostasis (4–6, 10, 11).

The rat air pouch is a convenient model to study acute inflammation (12). It is formed by the subcutaneous injection of air over several days and is composed of a lining of cells that consists primarily of macrophages and fibroblasts. Injection of carrageenan into the fully formed air pouch produces an inflammatory granulomatous reaction characterized by a marked production of biochemical mediators in the fluid exudate, including PGs and leukotrienes, as well as a significant influx of polymorphonuclear leukocytes (PMNs) and macrophages (13). Using molecular and pharmacological reagents, we studied the role of COX-2 in this model of inflammation by specific examination of the induction of COX-2 mRNA and protein as well as the production of PGs in the pouch exudate. The results indicate that induction of

COX-2 is responsible for the production of PGs at the site of inflammation, whereas the normal synthesis of PGs in the stomach appears to depend on constitutive COX-1 activity.

METHODS

Air-Pouch Model of Inflammation. Male Lewis rats (175–200 g) were used. Air cavities were produced by subcutaneous injection of 20 ml of sterile air into the intrascapular area of the back. An additional 10 ml of air was injected into the cavity every 3 days to keep the space open. Seven days after the initial air injection, 2 ml of a 1% solution of carrageenan (Sigma) dissolved in saline was injected directly into the pouch to produce an inflammatory response (13). The volume of exudate was measured and the number of leukocytes present in the exudate was determined with a Coulter Counter. The differential cell count was determined by Wright–Giemsa staining. PGE$_2$ and 6-keto-PGF$_{1\alpha}$ were determined in the pouch exudates by specific ELISAs (Cayman Chemicals, Ann Arbor, MI).

COX-2 Nuclease Protection Assay. Sections of pouch lining were cut and immediately frozen and pulverized under liquid nitrogen. Samples (5 μg) of total RNA (14) were hybridized with ^{32}P-labeled antisense RNA probe corresponding to rat COX-2 (gift of P. Worley, Johns Hopkins School of Medicine). The RNA hybrids were digested with RNases A and T1 at 37°C. All samples were analyzed in triplicate. The protected RNAs were separated by electrophoresis in 7.5 M urea/8% acrylamide sequencing gels. Gels were dried and exposed to film at room temperature.

Metabolic [^{35}S]Methionine Labeling of Cellular Pouch Exudates and Determination of COX-2 Synthesis by Immunoprecipitation. At various times after carrageenan administration, small pieces of pouch lining tissue were incubated in methionine-free Dulbecco's modified Eagle's medium (DMEM) containing [^{35}S]methionine (0.1 mCi per well; 10 mCi/ml, Amersham; 1 Ci = 37 GBq) for 4 hr. Cells were also collected, separated by centrifugation, and plated into 35-mm plastic culture dishes. After 30 min in methionine-free DMEM, the cells were incubated in the same medium containing [^{35}S]methionine as described above. Solubilization and immunoprecipitation of COX-1 and COX-2 were performed as described (4). COX-1 and COX-2 antibodies developed to amino acid sequences unique to murine COX-1 (peptide sequence; LLLPPTPSVLLADPGVPSPV) or murine COX-2 (peptide sequence, KTATINASASHSRLDDINPTV) were used for immunoprecipitation and staining purposes. The murine antibodies against COX-1 recognize rat COX-1; the murine antibodies for COX-2 recognize rat COX-2, but the antibodies are specific for each isoenzyme.

Abbreviations: COX, cyclooxygenase; NSAID, nonsteroidal inflammatory drug; PG, prostaglandin; PMN, polymorphonuclear leukocyte.
*To whom reprint requests should be addressed.

Immunohistochemical Localization of COX-2. Immunohistochemical staining for COX-2 was performed on cytospin preparations of cells (40,000 per slide) isolated from the pouch exudate and on 10-μm frozen sections of pouch lining tissues. Prior to preparing the cytospins, dead cells and debris were removed by centrifuging the cells at 300 × g for 10 min through a layer of fetal bovine serum. Additionally, the cells obtained from the carrageenan pouch fluid were enriched for mononuclear cells from ≈2% to 12% by separation using Ficoll/Hypaque. Cells and tissues were fixed with 1% paraformaldehyde, pH 7.2, for 5 min at room temperature, followed by 100% ethanol for 5 min at 4°C. Nonspecific binding was blocked with 0.5 M Tris-HCl, pH 7.4/1% (vol/vol) Triton X-100/and 3% (vol/vol) normal goat serum for 1 hr at room temperature. All subsequent incubations were carried out in this buffer. Cells and tissue sections were incubated 16 hr at 4°C with a 1:1000 dilution of either normal rabbit serum, the specific COX-2 polyclonal antiserum, or the COX-2 antiserum that had been preabsorbed for 1 hr with a 10-fold protein excess of recombinant murine COX-2. Endogenous peroxidase activity was then reduced with periodic acid (Zymed) for 45 sec at room temperature and sequentially incubated with biotinylated anti-rabbit IgG and avidin–biotin-peroxidase complex (ABC, Vector Laboratories) for 2 hr each. The reaction product was visualized by using 3,3'-diaminobenzidine intensified with nickel chloride for 6 min. Cells and tissue sections were counterstained with Mayer's hematoxylin, mounted, and photographed with either Hoffman modulation optics or brightfield microscopy.

Determination of PG levels in Pouch Exudate and Stomach. Animals were fasted for 16 hr. Indomethacin and NS-398 [N-(2-cyclohexyloxy-4-nitrophenyl)]-methanesulfonamide, provided by J. Rogier and J. Talley, Monsanto Corporate Research), dissolved in phosphate-buffered saline, pH 7.4/1% (vol/vol) Tween 80, were given by gavage (0.03–10 mg/kg) 1 hr before carrageenan injection. Dexamethasone (0.01–1.0 mg/kg) was given by gavage immediately prior to carrageenan. Animals were sacrificed 6 hr following carrageenan injections and pouch exudates were collected for PG determination. The stomachs of these animals were excised, opened, cleaned, and, after visual determination of gastric glandular mucosal damage, immediately frozen at −70°C. Stomachs were further processed by homogenization in 70% ethanol. After centrifugation, the supernatants were collected, dried under a stream of N_2, and resuspended in ELISA buffer for PG determination.

RESULTS

COX-2 Expression and Activity at the Inflammatory Site. Following the introduction of air into the intrascapular area of the back of rats, an air pouch forms that is composed of a newly formed lining of cells made up predominantly of macrophages and fibroblasts. This pouch produces a small amount of PGs constitutively, but upon stimulation (e.g., with carrageenan), prodigious amounts of PGs are released into the fluid exudate. These PGs can be blocked by clinically useful NSAIDs (15). The subcutaneous air pouch was utilized to examine the role of COX-2 in mediating the inflammatory response to injection of carrageenan. Vasodilation, edema, and a significant increase in cellular influx were noted (Table 1) within 6 hr after carrageenan injection, together with several inflammatory mediators, including PGs (Fig. 1).

Carrageenan induced a time-dependent increase in PGE_2 production in the pouch exudate fluid (Fig. 1A). Prostacyclin, measured as 6-keto-$PGF_{1\alpha}$, also increased from control levels of 0.6 ± 0.1 ng/ml to 377.2 ± 32.7 ng/ml at 6 hr. This was followed by a decline to 34.7 ± 4.8 ng/ml at 24 hr after carrageenan. PG synthesis in the pouch exudate from saline-treated animals did not change, remaining at basal levels of

Table 1. Leukocyte influx in acute inflammation after carrageenan treatment for 0, 6, or 24 hr

	% of cells		
Cell type	0 hr	6 hr	24 hr
Monocyte/macrophage	76	2	15
Lymphocyte	23	<1	<1
PMN	1	98	85

Cell no. was 0.5 ± 0.1, 18.8 ± 3.9, and 348 ± 75 × 10^6 at 0, 6, and 24 hr, respectively.

0.5 ± 0.1 ng of PGE_2 per ml. Quantitative mRNA and protein analysis of pouch tissue indicated that COX-2 expression paralleled the time course of carrageenan-induced PG production (Fig. 1A). COX-2 mRNA and protein were measurable 2 hr after carrageenan, were maximal at 6 hr, and remained elevated for up to 48 hr.

Administration of dexamethasone (1 mg/kg for 6 hr) to the carrageenan-treated rats inhibited COX-2 protein expression in the pouch lining and completely blocked the production of PGs in the pouch exudate (Fig. 1B). With a COX-1-selective antiserum, COX-1 immunoprecipitable protein was not detectable in the same pouch tissues (data not shown).

Cytospin preparations of the cellular exudate and frozen sections of pouch lining were analyzed immunohistochemically to identify the cell type that expressed COX-2. Exudates from sham-treated animals contained small numbers of cells, primarily monocytes/macrophages (Table 1). COX-2 immunoreactivity was not detected in these cells (Fig. 2A). In the carrageenan-treated rats, a significant increase in cellular infiltrate occurred (Table 1). PMNs accounted for >98% of the infiltrating cells 6 hr after carrageenan, but PMNs did not contain COX-2 immunoreactivity (Fig. 2B). To facilitate analysis of macrophage staining, fractions enriched for mononuclear cells (12% of the total) were prepared by Ficoll/Hypaque. Marked staining for COX-2 was observed in the monocytes/macrophages present in the fluid exudates (Fig. 2B). The specific COX-2 immunoreactivity in the macrophages was not observed when the COX-2 antibody was preabsorbed with an excess of mouse COX-2 (Fig. 2C). Similar to the results obtained by immunoprecipitation (Fig. 1B), macrophages obtained from animals treated with both carrageenan and dexamethasone did not express COX-2 by immunostaining (data not shown).

Immunohistochemical analysis of the pouch lining of control animals showed no COX-2 staining (Fig. 2D), suggesting that COX-2 was not induced during the development of the pouch tissue lining. Carrageenan induced the expression of COX-2-like immunoreactivity in cells of the inflamed pouch lining (Fig. 2E). Based upon morphology and ED1-positive staining, COX-2 induction was localized primarily to fibroblasts and macrophages in the superficial layer of the inflamed pouch lining. COX-2 immunoreactivity in the carrageenan-induced pouch lining was substantially blocked by preabsorbing the antibody (Fig. 2F).

Dexamethasone Selectively Inhibits COX-2-Derived PG. The antiinflammatory glucocorticoids are known to affect PG synthesis by inhibiting the expression of COX-2 without affecting COX-1 synthesis or activity (4, 5, 16–18). Therefore, the effect of dexamethasone on PG production in the inflamed air pouch was compared to that occurring in the stomach. Dexamethasone caused a dose-dependent inhibition of exudate PG levels at a half-maximal inhibitory dose (ID_{50}) of 0.03 mg/kg (Fig. 3A). At the same time, a significant decrease in cellular infiltrate was observed (Fig. 3B). Although dexamethasone inhibited proinflammatory PG in the pouch, it did not inhibit stomach PG levels (Fig. 3A), nor were gastric lesions observed, consistent with its ability to selectively inhibit COX-2 while sparing COX-1.
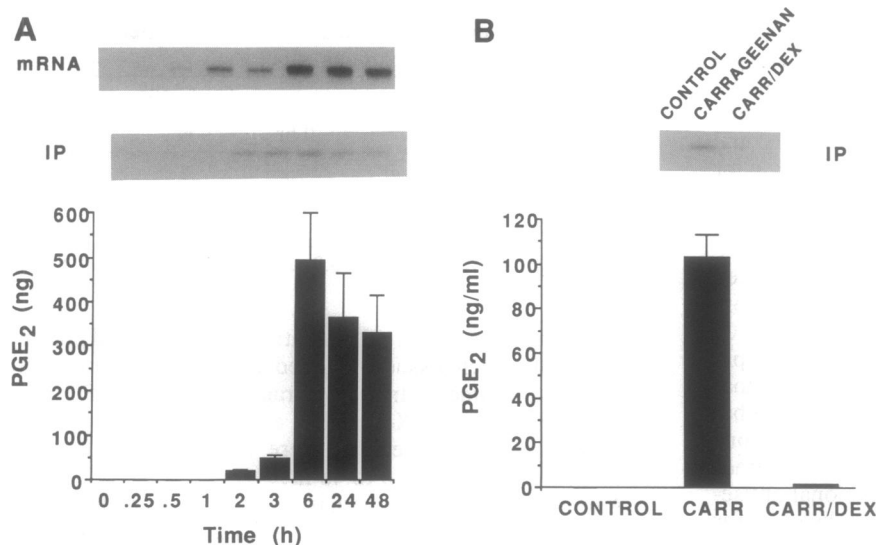
Differential Effect of Indomethacin and NS-398 on Inflamed Pouch PG Versus Stomach PG Production. Two NSAIDs, indomethacin and NS-398, which differ in their ability to inhibit baculovirus-expressed recombinant murine COX-1 and COX-2 activity *in vitro*, were used to examine PG production in the inflamed pouch versus the stomach. Indomethacin inhibits murine recombinant COX-1 (IC_{50} = 200 nM) and COX-2 (IC_{50} = 50 nM), whereas NS-398 selectively inhibits COX-2 (IC_{50} = 30 nM), with no effect on COX-1 activity at concentrations as high as 100 μM (unpublished data). Indomethacin, administered by gavage, reduced PG production in both the pouch exudate and the stomach with an ID_{50} of 0.3–0.5 mg/kg (Fig. 4*A*), with gastric lesions 6 hr after administration of a single dose (10 mg/kg). In contrast, the selective COX-2 inhibitor NS-398, at doses that completely inhibited PG synthesis in the pouch exudate (ID_{50} = 0.3 mg/kg), did not inhibit PG synthesis in the stomach (Fig.

4*B*), nor were gastric lesions observed when NS-398 was administered at 100-fold the efficacious dose (30 mg/kg).

The specificity of the *in vivo* inhibition of NS-398 on COX-2 activity was assessed by measuring the production of leukotriene B_4, a primary product of PMNs that have been recruited at the inflammatory site. NS-398 did not alter leukotriene B_4 levels at doses that completely blocked PGE_2 and prostacyclin synthesis in the pouch exudate (data not shown).

Antiinflammatory Effect of a Selective COX-2 Inhibitor. The ability of NS-398 to reverse the inflammatory response was evaluated by administering the drug (10 mg/kg) 6 hr after the carrageenan injection (Figs. 1*A* and 5 *A* and *C*). NS-398 visibly altered the inflammatory process, reducing the vasodilation and edema (Fig. 5 *B* and *D*). There was a 48% reduction in cellular infiltrate and a significant inhibition of PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis in the pouch exudate (carrageenan, 78.2 ± 37.4 and 9.3 ± 3.9 ng/ml; carrageenan/
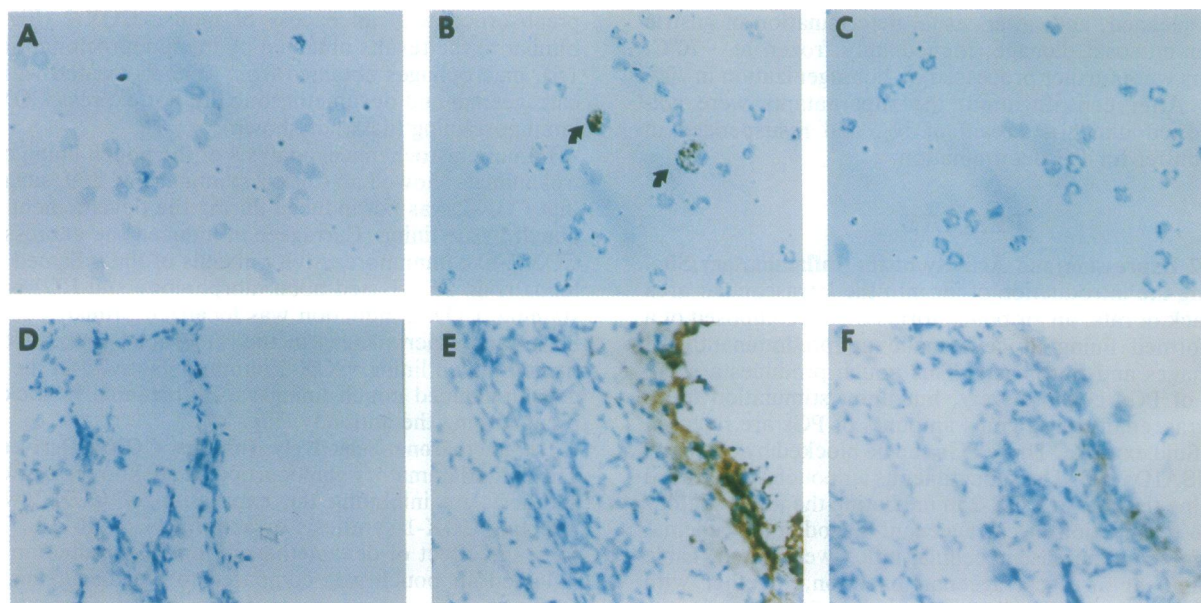

FIG. 2. Immunohistochemical staining of COX-2 in the carrageenan-induced rat air pouch. (*Upper*) COX-2 protein in cells from fluid exudates was obtained from control and carrageenan-treated rats. Cells were collected from the pouch exudates, centrifuged, fixed, and stained for COX-2 with a specific anti-peptide antibody. (*A*) Cells from a control pouch. (*B*) Cells from carrageenan-treated rats; positive staining was found in macrophages (identified morphologically and by a monocyte/macrophage-specific ED1-positive stain) but not in PMNs. (*C*) Blockade of carrageenan-induced COX-2 stain by COX-2 protein. (*Lower*) COX-2 in pouch lining from control and carrageenan-treated rats. Frozen sections from the pouch linings were fixed and stained as above. (*D*) Control pouch. (*E*) Carrageenan-treated pouch tissue with positive staining of COX-2 in superficial layer. (*F*) Carrageenan-treated tissue staining blocked with an excess of COX-2 protein.
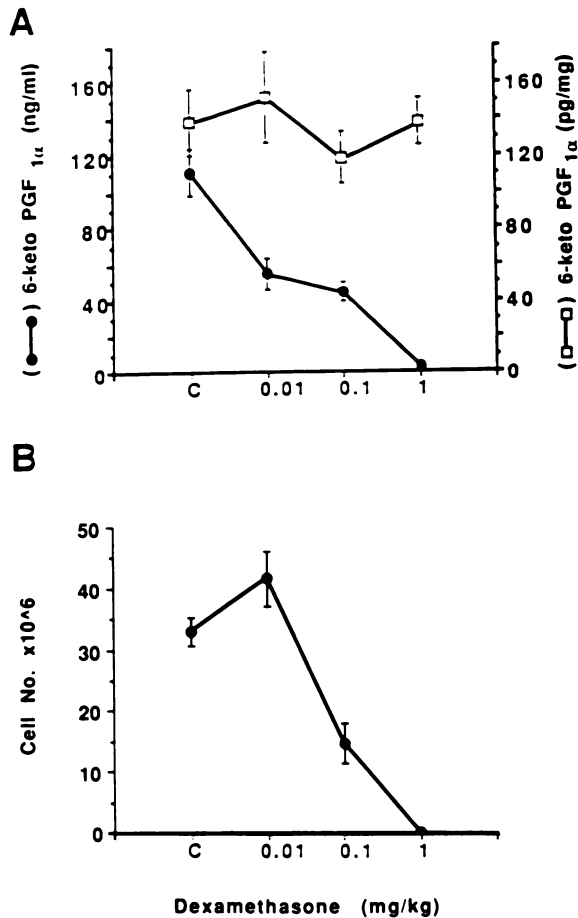
FIG. 3. Effect of dexamethasone on carrageenan-induced inflammation. Dexamethasone was administered by gavage to rats (0.01–1 mg/kg) immediately prior to carrageenan injections. (A) 6-Keto-$PGF_{1\alpha}$ was measured in the exudate fluids by ELISA (●). Stomachs were cleaned and frozen in liquid N_2, and PGs were extracted with 70% ethanol and measured as 6-keto-$PGF_{1\alpha}$ (□). (B) Total leukocyte number was determined by Coulter Counter. Each point represents mean ± SE (n = 6).

NS-398 = 2.6 ± 1.6 and 0.8 ± 0.3 ng/ml, respectively). Thus, the effect of NS-398 on PG formation in the pouch was not limited to prophylactic prechallenge administration but was also able to reverse the changes induced by carrageenan when administered therapeutically.

DISCUSSION

This study was designed to determine the role of COX-2 in the production of PGs in normal and inflamed tissues *in vivo*. The rat subcutaneous air-pouch model was utilized to produce an inflammatory response, characterized by release of eicosanoids into the inflammatory exudate following injection of the irritant carrageenan (12, 13). We observed a rapid induction of COX-2 mRNA and protein in the inflamed pouch lining and cells from fluid exudates. COX-2-like immunoreactivity was localized to macrophages and fibroblasts, but not to PMNs, suggesting that these cell types are the source of COX-2-derived PGs.

Production of inflammatory PGs in the pouch exudate fluid as a result of carrageenan induction coincided with the expression of COX-2 mRNA and protein. In further support of the role of COX-2 in carrageenan-induced inflammation, dexamethasone blocked COX-2 expression in the cells present in the pouch, resulting in a complete suppression of PG synthesis. Glucocorticoids selectively inhibit COX-2 expres-
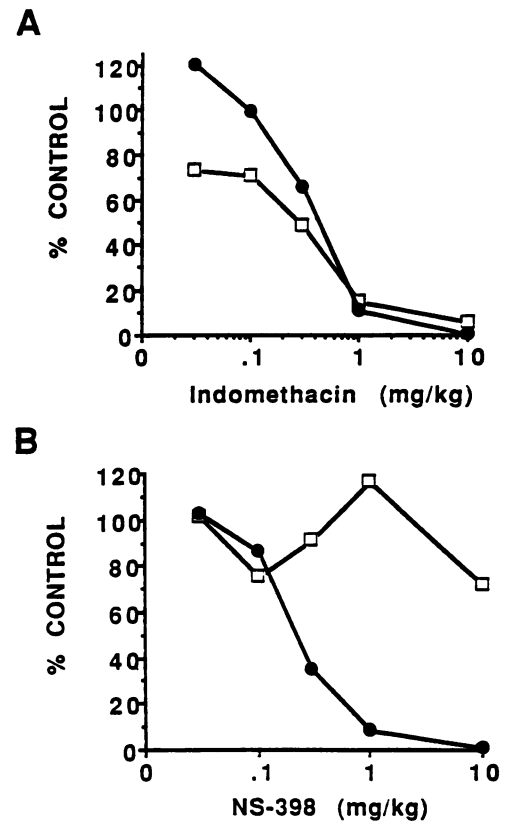

FIG. 4. Effect of indomethacin and NS-398 on pouch exudate and gastric PGs. Indomethacin (A) and NS-398 (B) were administered by gavage 1 hr prior to the carrageenan injection. PGs present in the pouch exudate (●) and extracted from the stomachs (□) of these animals were measured by ELISA. Control animals received only carrageenan and vehicle. Each point represents percent of control 6-keto-$PGF_{1\alpha}$ determinations (n = 6).

sion *in vitro* (16–18), and *in vivo* (4, 5, 10). However, dexamethasone did not inhibit PG production in the stomach, whereas a known inhibitor of COX-1 activity (indomethacin) substantially blocked stomach PGs. Thus, it appears that COX-2 activity is responsible for PG production in the inflamed pouch, whereas COX-1 activity is responsible for PG production in the stomach. Similar to the results found with dexamethasone, *in vivo* treatment with NS-398, a selective COX-2 inhibitor, blocked only the COX-2-derived pouch PGs without affecting stomach PG production and without producing stomach lesions. Futaki *et al.* (19, 20) have shown that NS-398 has antiinflammatory and analgesic properties without producing gastric ulcers.

Although not detectable by immunoprecipitation with specific COX-1 antiserum, there was minimal basal COX activity at the site. Injection of arachidonate directly into control pouches that did not express COX-2 produced small amounts of PGs (unpublished observation) that were not inhibited by dexamethasone. Previously, COX-1 message and protein have been detected in peritoneal macrophages, although this COX-1 expression was not inhibitable by glucocorticoids or adrenalectomy (ref. 5; unpublished data). The present results suggest that COX-1 activity does not contribute significantly to PG formation in this acute model of inflammation. This contrasts with the apparent function of COX-1 to produce physiologically important PGs in the stomach and other peripheral tissues.

In conclusion, the induction of COX-2 expression coincides with an increase in proinflammatory PG production *in vivo*. Both the potent antiinflammatory steroids which inhibit the expression of COX-2 and NS-398, a NSAID that selec-
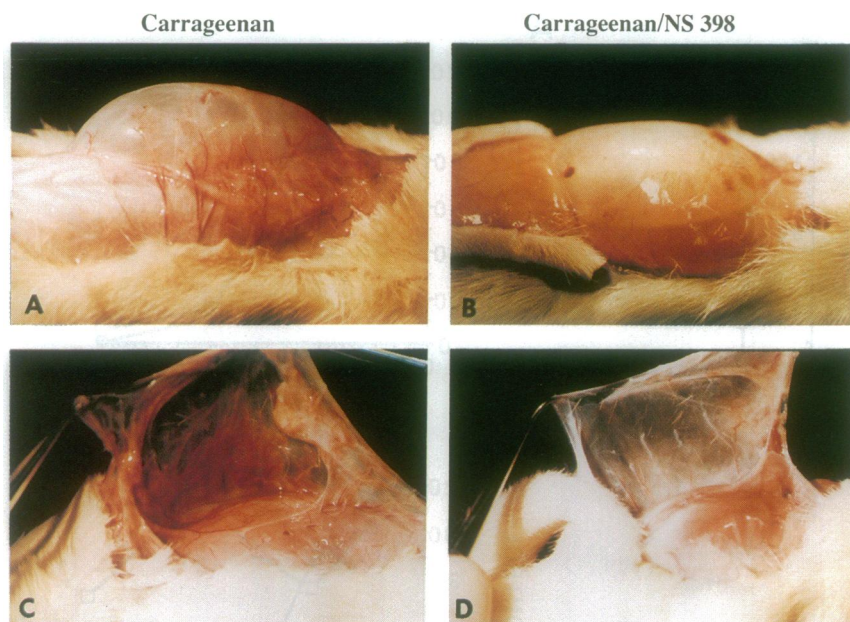
Carrageenan   Carrageenan/NS 398

FIG. 5. Anti-inflammatory effect of NS-398. NS-398 (10 mg/kg) was administered by gavage 6 hr after injection of carrageenan. Animals were sacrificed 24 hr following carrageenan treatment, and the pouch lining was exposed. (*A* and *C*) Inflammatory reaction in the sham-gavage animals injected with carrageenan. (*B* and *D*) Representative view of the antiinflammatory effects of NS-398 at 24 hr when administered 6 hr after carrageenan.

tively inhibits COX-2 enzymatic activity, block the synthesis of PGs in the inflammatory site. Normal production of PGs in the stomach appears to be dependent on COX-1 activity constitutively present in this tissue. The role of COX-2 in inflammation and the advantages of developing a COX-2-selective antiinflammatory agent are summarized in a model (Fig. 6). Under normal physiological conditions, tissue PG synthesis depends on the availability of arachidonate and the enzymatic activity of COX-1. Blockade of COX-1 by NSAIDs produces a reduction of tissue PG synthesis resulting in mechanism-based side effects such as the development of gastric lesions and renal toxicity (21–23). At the inflammatory site, cytokines and growth factors induce COX-2 in inflammatory cells, resulting in the production of PGs that promote edema formation and vascular changes. One of the antiinflammatory mechanisms of glucocorticoids appears to be suppression of the induction of COX-2. This action reduces inflammatory PG synthesis without affecting normal tissue PG levels that result from COX-1 activity. Unfortunately, side effects of steroids independent of their ability to inhibit COX-2 expression limit their use. NSAIDs with a mechanism of action such as NS-398 inhibit COX-2 activity selectively, decreasing inflammation-associated PGs without affecting COX-1 in the stomach. Such selective inhibitors may be efficacious antiinflammatory drugs devoid of the side effects associated with the inhibition of COX-1 and thus provide a significant improvement to current therapy.
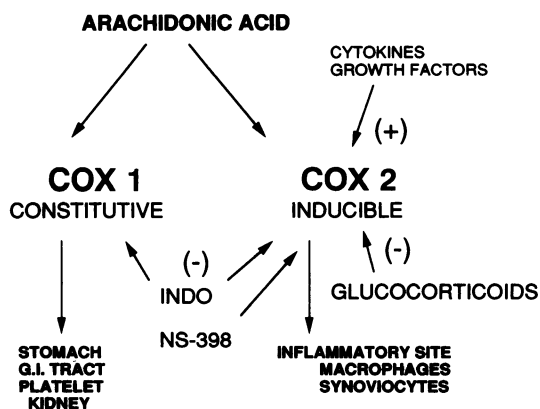

FIG. 6. Model for regulation of PG synthesis in normal and inflamed states. INDO, indomethacin; G.I., gastrointestinal.

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