

Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain

(prostaglandin/nonsteroidal antiinflammatory drug/hyperalgesia)

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ABSTRACT Nonsteroidal antiinflammatory drugs (NSAIDs) are widely used for the treatment of inflammatory diseases, but significant side effects such as gastrointestinal erosion and renal damage limit their use. NSAIDs inhibit the enzyme cyclooxygenase (COX), which catalyzes the conversion of arachidonic acid to prostaglandins (PGs) and thromboxane. Two forms of COX have been identified—COX-1, which is constitutively expressed in most tissues and organs, and the inducible enzyme, COX-2, which has been localized primarily to inflammatory cells and tissues. In an animal model of acute inflammation (injection of carrageenan into the footpad), edema was produced that was associated with marked accumulation of COX-2 mRNA and thromboxane. A selective inhibitor of COX-2 (SC-58125) inhibited edema at the inflammatory site and was analgesic but had no effect on PG production in the stomach and did not cause gastric toxicity. These data suggest that selective inhibition of COX-2 may produce superior antiinflammatory drugs with substantial safety advantages over existing NSAIDs.

In the early 1970's nonsteroidal antiinflammatory drugs (NSAIDs) were found to prevent the production of prostaglandins (PGs) by inhibiting the enzyme cyclooxygenase (COX; EC 1.14.99.1), suggesting a biochemical mechanism of action for these drugs (1, 2). For many years it was thought that COX was a single enzyme present constitutively in most tissues and that inhibition of this enzyme led to decreased production of proinflammatory PGs (e.g., in inflamed tissue) as well as beneficial PGs produced in the stomach, kidney, and elsewhere. More recently, COX activity was found to be increased in certain inflammatory states and could be induced in cells by inflammatory cytokines (3–6). These data suggested the existence of two forms of COX, a constitutive enzyme present in tissues such as stomach and kidney and an inducible enzyme associated with inflammation. This hypothesis was supported by the isolation of a new form of COX (COX-2) encoded by a second cyclooxygenase gene (7–9).

The discovery of a second COX enzyme led to the hypothesis that toxicity associated with NSAID therapy in tissues such as stomach and kidney may be due to inhibition of the nonregulated or constitutive form of COX (COX-1), whereas the therapeutic benefit of NSAIDs derives from inhibition of the inducible COX-2 enzyme at the site of inflammation. In support of that hypothesis, the potent antiinflammatory steroid dexamethasone can selectively inhibit cytokine or endotoxin induction of COX-2 enzyme both *in vitro* and *in vivo*, while having no effect on expression of constitutive COX-1 (10–12). Although glucocorticoids are potent and efficacious antiinflammatory drugs, chronic administration often results in serious side effects unrelated to their ability to inhibit COX-2 expression, such as decreased

bone density, fluid and electrolyte imbalance, and "Cushing-like" symptoms. These untoward effects limit glucocorticoid use in chronic inflammatory disorders such as rheumatoid arthritis (13).

Studies were designed to evaluate the role of COX-2 *in vivo* at the site of inflammation. We report that in a model of inflammation useful in the characterization of NSAIDs, the carrageenan-injected rat paw, COX-2 was expressed locally in response to the proinflammatory stimulus and that the induction of COX-2 mRNA coincided with the synthesis of proinflammatory PGs and the development of edema and hyperalgesia. COX-1 mRNA was detectable in the normal rat paw, but its expression did not change following the onset of the inflammatory reaction. Furthermore, a selective inhibitor of COX-2 {SC-58125: 1-[(4-methylsulfonyl)phenyl]-3-trifluoromethyl-5-(4-fluorophenyl)pyrazole} blocked edema and hyperalgesia *in vivo* following an inflammatory insult, without causing gastric mucosal damage.

METHODS

Measurement of Edema, Hyperalgesia, and PG Production in the Carrageenan-Injected Rat Paw. Paw edema was induced by injecting 0.1 ml of 1% carrageenan saline solution into the hindpaw of a male Sprague–Dawley rat (200 g; Harlan Laboratories, Haslett, MI); the protocol was approved by the institutional Animal Care and Use Committee (14, 15). Prior to experiments, the rats were allowed free access to chow and water. Indomethacin or vehicle (0.5% methylcellulose in water), was administered orally 1 hr before the carrageenan injection, and paw volume was measured at intervals thereafter. Analgesic activity was measured in the inflammatory hyperalgesia model of Hargreaves *et al.* (16). Specifically, rats were administered drugs by oral gavage as described above, and then carrageenan was injected into the hindpaw. Hyperalgesia was measured as a measure of the antinociceptive response to thermal stimuli from a radiant heat source (high-intensity projector lamp bulb) positioned under a Plexiglas floor directly beneath the hindpaw. The withdrawal latency of the affected paw compared with the contralateral normal paw was determined to the nearest 0.1 sec with an electronic clock circuit and a microcomputer. Each point represents either the change in paw volume or withdrawal latency compared with control measurements taken prior to carrageenan injection ($n = 5$). At selected times after challenge, rats were euthanized by CO₂ inhalation and their hind feet were removed. The paw was immersed in liquid nitrogen for 30 sec, and the partially frozen pad was separated from the bone. The pad tissue was homogenized by Polytron (Brinkmann) in 2 ml of methanol followed by 2 ml of water. The sample was adjusted to 5% methanol with water and allowed

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Abbreviations: COX, cyclooxygenase; NSAID, nonsteroidal antiinflammatory drug; PG, prostaglandin; TxB₂, thromboxan B₂.

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to sit for 20 min in an ice bath. The bulk of precipitated material was collected by centrifugation at $2000 \times g$ for 25 min at 4°C . The resulting supernatant was applied to a C_{18} Sep-Pak cartridge (Waters) which was preconditioned with 20 ml of methanol followed by 20 ml of water; after the homogenates were loaded, the columns were sequentially washed with water, 10% methanol, and petroleum ether (10 ml of each). Thromboxane B_2 (TxB_2) was eluted with 5 ml of methyl formate and evaporated under a stream of nitrogen. Samples were reconstituted in ELISA buffer and assayed for TxB_2 as a convenient metabolite for the measurement of PGs *in vivo* by ELISA (Cayman Chemicals, Ann Arbor, MI).

In Vivo Biochemical Efficacy of a Selective COX-2 Inhibitor. An air cavity was produced by subcutaneous injection of 20 ml of sterile air into the intrascapular area of the back of a male rat (17). 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 dissolved in saline was injected directly into the pouch. Animals were fasted for 17 hr and then pretreated with indomethacin or SC-58125 (0.001–10 mg/kg, orally) 1 hr prior to administration of carrageenan. After 6 hr, animals were sacrificed, and the pouch exudates were collected for prostaglandin determination. Additionally, the stomachs of these animals were excised, examined for visual signs of gastric glandular damage, and then processed for tissue prostaglandin production as described above.

Determination of COX mRNA Levels in Rat Tissues. Normal rat tissues and inflamed paws were removed and immediately frozen and pulverized under liquid nitrogen; RNA was isolated by the guanidinium isothiocyanate method (18). The rat COX-1 probe was constructed by subcloning a 220-bp fragment from the coding region of rat COX-1 (19) into the polylinker of pGEM-7Z (Promega). The rat COX-2 probe was constructed by subcloning a 150-bp fragment from the COX-2 cDNA into the baculovirus expression vector (20) (the cDNA was a gift of Paul Worley, Johns Hopkins School of Medicine). The resulting plasmids were linearized and used as templates for transcription of the antisense RNA probe with SP6 RNA polymerase and [α - ^{32}P]CTP (New England Nuclear). COX-1 and COX-2 RNA levels in the paws were determined by nuclease protection assay with an Ambion NPA II kit (Austin, TX). Five-microgram samples of total RNA were hybridized to 3×10^5 cpm of probe overnight at 45°C . The RNA hybrids were digested with RNases A and T_1 at 37°C . All samples were analyzed in triplicate. For quantitative purposes, the RNA samples were also analyzed for expression of the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene with a commercially available probe (Ambion). The protected RNAs were fractionated by electrophoresis in 7.5 M urea/8% polyacrylamide sequencing gels. Gels were dried and exposed to film at room temperature. Relative expression of COX-2 was determined by densitometry in three experiments as compared to expression of GAPDH \pm SEM.

Gastric Toxicity. Animals were fasted for 16 hr prior to administration by gavage of either indomethacin or SC-58125 (0.03–30 mg/kg). Five hours after administration of the drugs, the animals were killed by CO_2 , and the stomachs were removed, excised along the greater curvature, and inspected for evidence of gastric glandular mucosal damage with a stereomicroscope. The ED_{50} was defined as the dose at which 50% of the animals showed evidence of gastric damage by visual inspection.

Intestinal Toxicity. Fed rats were dosed once with a suspension of vehicle (0.5% methylcellulose in water) or drug (indomethacin, 1–30 mg/kg; or SC-58125, 400 or 600 mg/kg, intragastrically). After 72 hr, the animals were euthanized by CO_2 asphyxiation, the abdominal cavity of each was opened, and the presence or absence of adhesions was noted by an

investigator “blinded as to treatment.” The data are expressed as ED_{50} , defined as dose resulting in 50% of animals with lesions ($n = 7$).

In Vitro COX Enzyme Assay. The coding regions of mouse COX-1 and COX-2 were subcloned in the baculovirus expression vector pVL1393 (Invitrogen); recombinant baculoviruses were isolated by transfecting 4 μg of either plasmid DNA with 200 ng of linearized baculovirus DNA by the calcium phosphate procedure (21) and transfected into Sf9 insect cells. Expression of COX protein was determined by assessing PG synthetic capability in homogenates from cells incubated 3 days with COX-1 or COX-2 baculovirus. Cells expressing COX-1 or COX-2 were homogenized (22) and incubated with arachidonic acid (10 μM); COX activity was determined by monitoring PG production; no COX activity was detected in mock-infected Sf9 cells. Indomethacin and SC-58125 (0.001–10 μM) were preincubated with homogenates (2–10 μg of protein) for 10 min prior to addition of arachidonic acid. PGE_2 formed during a 10 min incubation was detected by ELISA (Cayman Chemicals).

RESULTS AND DISCUSSION

To examine the role of COX-2 *in vivo*, experiments were designed to evaluate both the presence and function of COX-1 and COX-2 at the site of inflammation. An animal model that has been pharmacologically useful in the charac-

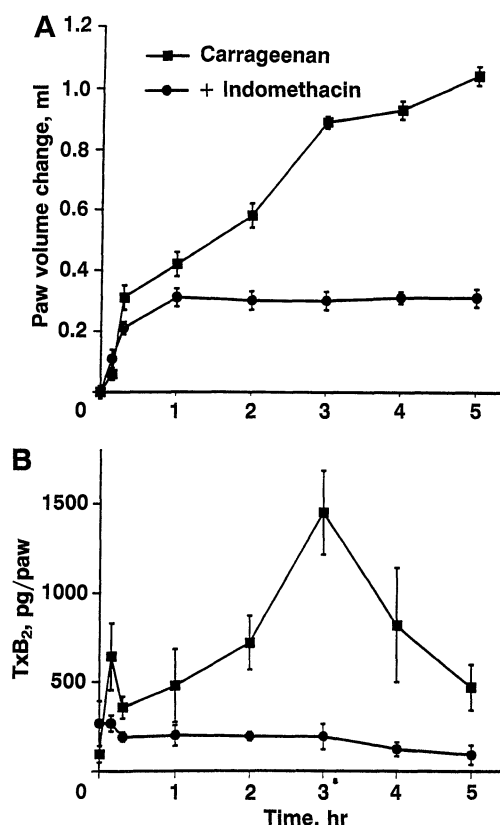


FIG. 1. Onset of edema correlates with TxB_2 production at the inflammatory site. Paw edema was induced by injecting 0.1 ml of 1% carrageenan in saline into the hindpaw of a Sprague-Dawley rat (Harlan Laboratories, Haslett, MI). Indomethacin (\bullet) or vehicle (0.5% methylcellulose in water) (\blacksquare) was administered orally 1 hr before the carrageenan injection. (A) Paw volume was measured at intervals thereafter. Each point represents the change in paw volume compared with control measurements taken prior to carrageenan injection ($n = 5$). (B) At selected times after challenge, rats were euthanized by CO_2 inhalation and their hind feet were removed. Paws were assayed for TxB_2 by ELISA. Data are expressed as mean \pm SEM.

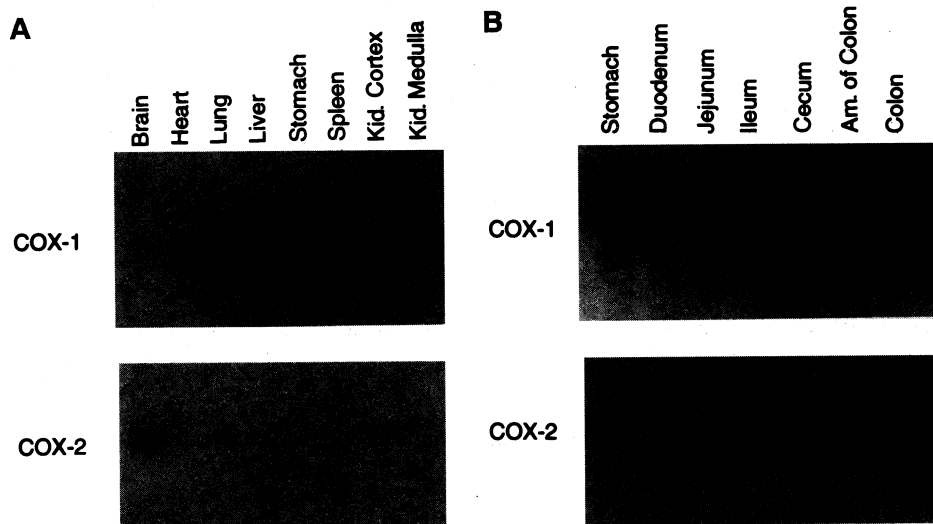


FIG. 2. Expression of COX-1 and COX-2 in normal tissues. Total RNA was extracted from normal rat tissues and hybridized with ^{32}P -labeled antisense RNA probe corresponding to rat COX-1 or rat COX-2. The protected band corresponding to COX-1 or COX-2 is shown. (A) Survey of mRNAs from brain, heart, lung, liver, stomach, spleen, kidney cortex, and kidney medulla. (B) Survey of mRNAs from the gastrointestinal tract, including stomach, duodenum, jejunum, ileum, cecum, ampulla of colon, and colon.

terization of NSAIDs was employed—carrageenan-induced rat paw edema (14, 15). Following the injection of carrageenan into the paw, changes in paw volume that correspond to edema occurred rapidly and in a biphasic manner (Fig. 1A). Paw volume increased substantially during the 5 hr following treatment, with an initial phase of edema formation within 30 min followed by a second phase that was sustained up to 5 hr. The initial increase in paw volume was not inhibited by indomethacin and may have been the result of local bradykinin production insensitive to the action of antiinflammatory NSAIDs (16), whereas the marked edema produced in the second phase (0.5–5 hr) was blocked quantitatively by indomethacin. Further, the increase in paw volume coincided with an increase in tissue TxB_2 . (Fig. 1B), and as was observed for paw volume, pretreatment of animals with indomethacin prevented this carrageenan-induced tissue TxB_2 production. After 3 hr the TxB_2 levels observed in the paws began to decline although the edema was sustained. Perhaps the pathophysiological consequences of the marked release of proinflammatory TxB_2 in the paw over time, along with the probable production of other inflammatory mediators at the site, contributed to the sustained edematous effect observed at 5 hr.

The hypothesis that COX-2 is expressed *in vivo* at the site of inflammation whereas COX-1 is expressed constitutively in normal tissues was examined directly by using molecular reagents. Total RNA was obtained from both normal rat tissues and inflamed paws previously injected with carrageenan, and COX-1 and COX-2 mRNAs were quantified by RNase protection. COX-1 mRNA was readily detectable in all normal tissues examined—especially in lung, liver, spleen, kidney, and stomach—whereas levels of COX-2 mRNA were substantially lower, with the exception of brain (Fig. 2A). Since the therapeutic usefulness of commercial NSAIDs is limited primarily by the toxicity that results from inhibition of PGs in the kidney, stomach, and intestinal tract, the distribution of COX isozymes in these tissues was of particular interest. Significantly more COX-1 mRNA was detected in the renal medulla than in the cortex, consistent with the abundant production of physiologically important renal medullary PGE_2 in the normal kidney (23). Likewise, further evaluation of COX expression in the gastrointestinal tract revealed measureable amounts of COX-1 mRNA from stom-

ach to colon, whereas COX-2 expression was much lower (Fig. 2B).

COX-1 mRNA was detectable in normal paws before injection of carrageenan (Fig. 3), with no apparent change in the expression of COX-1 for up to 3 hr following the administration of the proinflammatory stimulus. In contrast, COX-2 mRNA was virtually undetectable in the uninjected paws, but after injection with carrageenan, COX-2 mRNA was slightly detectable within 60 min, with a substantial induction observed at 3 hr (Fig. 3). The induction of COX-2

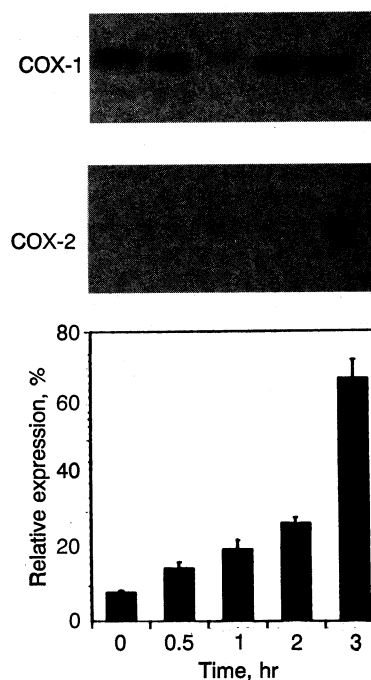


FIG. 3. COX-2 mRNA is induced *in vivo* at the inflammatory site. Total RNA was extracted from carrageenan-injected paws at 0, 0.5, 1, 2, and 3 hr and hybridized with ^{32}P -labeled antisense RNA probe as described in Fig. 2. Relative expression of COX-2 was determined as the expression of COX-2 normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase in the same RNA samples; results were determined by densitometry in three experiments, and the data are shown as mean \pm SEM ($n = 3$).

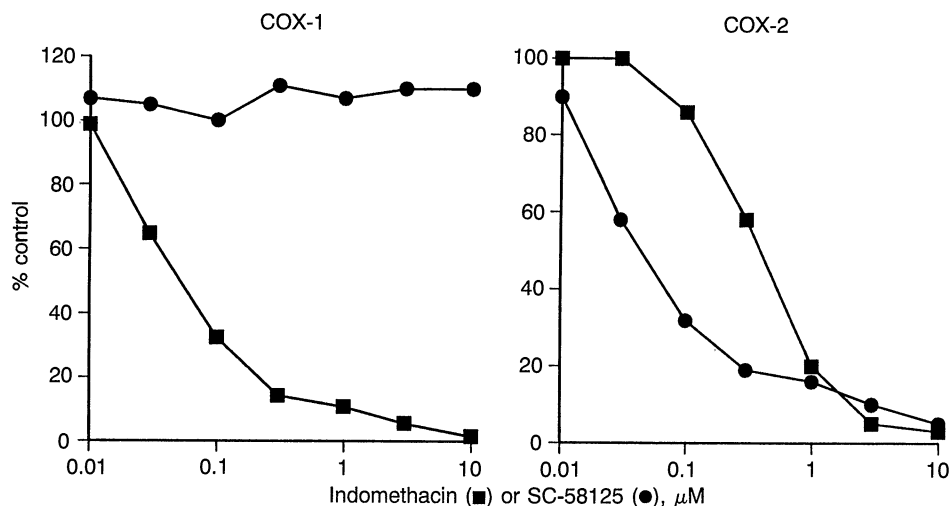


Fig. 4. SC-58125 selectively inhibits COX-2 versus COX-1. Mouse COX-1 and COX-2 were engineered into the baculovirus expression vector pVL1393; expression of COX protein was determined by assessing PG synthetic capability in homogenates from cells incubated 3 days with COX-1 or COX-2 baculovirus-infected Sf9 cells. Indomethacin (■) or SC-58125 (●) (0.001–10 μ M) was preincubated with homogenates (2–10 μ g of protein) for 10 min prior to addition of arachidonic acid (10 μ M). PGE₂ formed during a 10-min incubation was detected by ELISA (Cayman Chemicals, Ann Arbor, MI).

mRNA coincided with the appearance of TxB₂ and edema in the tissue. These results indicated that an inflammatory stimulus specifically increased COX-2 mRNA *in vivo*, which correlated with the time-dependent increase in proinflammatory PG formation and the formation of edema.

Indomethacin is a potent antiinflammatory drug, but the therapeutic usefulness of this and other efficacious NSAIDs is often limited by gastrointestinal ulcerogenicity (24). Our hypothesis to explain this pharmacological profile is that the current NSAIDs inhibit both the constitutive COX-1 found in uninvolved tissues and the proinflammatory COX-2 at the site of inflammation. Therefore, a selective inhibitor of COX-2 would potentially spare gastric PG production while providing antiinflammatory and analgesic properties. To examine this hypothesis directly, murine and human COX-1 and COX-2 were cloned and expressed in insect cells utilizing a baculovirus expression system, and the relative ability to inhibit the

recombinant enzyme *in vitro* was examined. Indomethacin was a potent inhibitor of both murine and human recombinant COX-1 (murine IC₅₀ = 10 nM) and COX-2 (murine IC₅₀ = 300 nM) (Fig. 4), indicating that this drug is a nonselective inhibitor, as previously reported (17, 25). In contrast, SC-58125 potently and selectively inhibited COX-2 (IC₅₀ = 0.05 μ M) without inhibition of COX-1 (IC₅₀ > 10 μ M). A similar profile of potency and selectivity for indomethacin and the selective COX-2 inhibitor SC-58125 was observed for the human recombinant enzyme (26).

In vivo the antiinflammatory and analgesic properties of the nonselective NSAID indomethacin were evaluated against the COX-2-selective agent SC-58125. As reported above, indomethacin reduced the edema observed 3 hr after injection of carrageenan into the paw (ED₅₀ = 2.0 mg/kg, orally). Likewise, SC-58125 suppressed the edematous response observed 3 hr after carrageenan injection, when COX-2 expression was maximal (Fig. 5). An important aspect of the clinical efficacy of the NSAIDs is their ability to relieve pain, especially the pain associated with inflammation. In addition to the edema observed following administration of carrageenan to the paw, there was also hyperalgesia produced (16) that was blocked by indomethacin (ED₅₀ = 2.0 mg/kg) (Table 1). The selective COX-2 inhibitor SC-58125 was as efficacious as indomethacin, suggesting that COX-2 is involved in the inflammatory hyperalgesia and that selective inhibition of COX-2 is sufficient for analgesic activity *in vivo* (Fig. 5).

We recently reported that carrageenan administration to the subcutaneous rat air pouch induces a rapid inflammatory response characterized by a cellular influx and production of PGs in the pouch exudate attributed to COX-2 activity (17). Therefore, this model provides a direct *in vivo* biochemical measurement for COX-2 activity at an inflammatory site. Additionally, the *in vivo* effect of COX-1 inhibition was assessed by monitoring stomach PG production following the

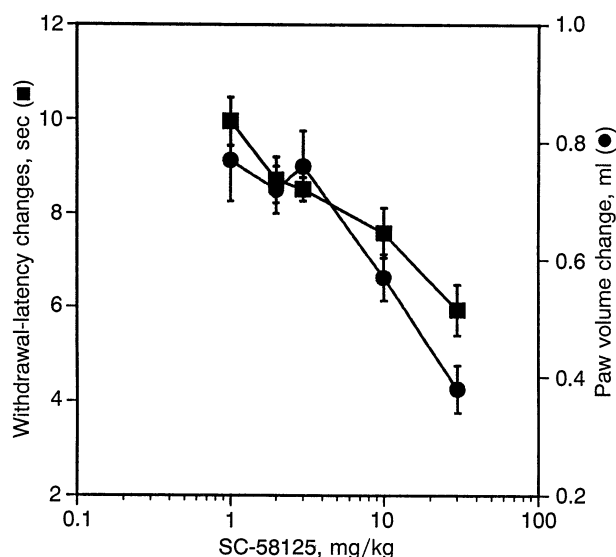


Fig. 5. Concentration-dependent inhibition of edema (●) and hyperalgesia (■) by a selective COX-2 inhibitor. SC-58125 was administered by gavage to rats (0.1–40 mg/kg) 1 hr prior to carrageenan injection. Paw volume and withdrawal latency were determined 3 hr later.

Table 1. *In vivo* pharmacology of COX inhibitors

Drug	ED ₅₀ , mg/kg			
	Pouch exudate PG	Stomach PG	Analgesia/edema	GI toxicity
Indomethacin	0.4	0.4	2.0	8.0
SC-58125	0.1	>10	10	>600

Drugs were administered by gavage. GI, gastrointestinal.

administration of inhibitors. Indomethacin inhibited the production of both pouch exudate PGs and stomach PGs at similar doses. In this study, the selective COX-2 inhibitor SC-58125 completely suppressed pouch PG synthesis ($ED_{50} = 0.1$ mg/kg) without reducing gastric PG production ($ED_{50} > 10$ mg/kg) (Table 1).

To evaluate the relationship between gastric PG production, lesions, and COX-2 inhibition, SC-58125 and indomethacin were administered to rats at doses that significantly exceeded those used therapeutically. Five hours after gavage of either indomethacin or SC-58125, the presence of gastric glandular mucosal damage was determined with a stereomicroscope. As expected, oral administration of indomethacin caused acute gastric glandular mucosal damage in the rats ($ED_{50} \approx 7$ mg/kg, $n = 6$), whereas oral administration of the COX-2-selective inhibitor SC-58125 at 60 times the ED_{50} for edema (400 mg/kg, $n = 6$) resulted in no visible gastric glandular mucosal lesions. These results are in accord with the recent observation (17) that indomethacin (administered by gavage at 0.03–10 mg/kg) inhibited PG production (measured as 6-keto-PGF_{1 α}) in the stomach with an $ED_{50} \approx 0.4$ mg/kg, whereas there was no detectable inhibition of stomach PGs by SC-58125 at doses up to 10 mg/kg (Table 1). In addition to gastric toxicity, we have also examined the ability of indomethacin and SC-58125 to cause intestinal ulcers (27). Administration of indomethacin at 16 mg/kg caused intestinal lesions in all rats tested ($ED_{50} = 10$ mg/kg, intragastrically, $n = 7$). Intestinal lesions were not observed following administration of SC-58125 at doses up to 200 mg/kg ($n = 7$).

Although the commercially available NSAIDs are antiinflammatory, gastrointestinal and renal toxicity limits their use. Apparently these actions are due to inhibition of proinflammatory PGs that are produced by COX-2 as well as to physiologically important PGs produced by COX-1. Results described here demonstrate that, while COX-1 was constitutively expressed in many tissues, COX-2 was expressed locally in response to a proinflammatory stimulus and that the induction of COX-2 mRNA coincided with the synthesis of proinflammatory PGs, the development of edema, and the hyperalgesic response. A selective inhibitor of COX-2 blocked PG production and resultant edema *in vivo* that followed an inflammatory insult; this occurred in the absence of detectable gastric side effects. Furthermore, inhibition of COX-2 was sufficient to produce a maximal analgesic effect *in vivo*. Current nonselective NSAID therapy represents the major therapeutic approach to the treatment of pain and discomfort in chronic and acute inflammation. Development of selective COX-2 inhibitors that achieve maximal antiinflammatory and analgesic activity with reduced gastric toxicity may provide a major advance in the treatment of these patients.

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