Constitutive Cyclooxygenase-2 Expression in Healthy Human and Rabbit Gastric Mucosa

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ABSTRACT
Selective cyclooxygenase (COX)-2 inhibitors are expected to cause fewer gastric side effects because of sparing of COX-1-dependent prostaglandin (PG) synthesis in the gastric mucosa. However, the possible contribution of COX-2 to overall gastric PG biosynthesis is not known. This study demonstrates constitutive expression of COX-2 mRNA and protein in apparently healthy human and rabbit gastric mucosa. This basal expression of COX-2 protein in human gastric mucosa was increased by lipopolysaccharide and phorbol ester, indicating its up-regulation in response to appropriate stimuli. The functional significance of COX-2-dependent PG formation was studied in terms of PGE2 generation in the rabbit mucosa and its inhibition by the COX-2-selective inhibitor flosulide. There was concentration-dependent (IC50 = 107 ± 55 nm) and ultimately complete inhibition of PGE2 generation by flosulide. In addition, gastric mucosa generated 15-hydroxyeicosatetraenoic acid upon treatment with acetylsalicylic acid. The data suggest an important role for COX-2-dependent PG production in apparently healthy gastric mucosa and raise the issue of whether selective COX-2 inhibitors might also interfere with physiological PG formation and actions in the stomach.

The COX step is rate-limiting in PG biosynthesis. COX exists in two isoforms, referred to as COX-1 and COX-2 (reviewed by Otto and Smith, 1995). COX-1 is constitutively expressed in most cells and tissues and is thought to carry out ‘housekeeping’ functions, such as local regulation of blood flow in the stomach, heart, and kidney and control of hemostasis by modulation of platelet function. In contrast, COX-2 mRNA and protein are normally undetectable in most tissues but can be rapidly induced by proinflammatory or mitogenic stimuli, including cytokines, endotoxin, and growth factors (Habib et al., 1993; Lyons-Giordano et al., 1993; Hempel et al., 1994). Thus, COX-2 appears to play a major role in body defense mechanisms by producing PGs that modulate inflammatory and immune responses (Otto and Smith, 1995). Consequently, it has been proposed that selective inhibitors of COX-2 would suppress PG biosynthesis at sites of inflammation but would not impair COX-1-dependent biosynthesis of PGs that are involved in the control of body homeostasis, including the generation of vasodilatory and tissue-protective PGs in the gastrointestinal tract (Mitchell et al., 1994).

This concept was challenged recently by the detection of constitutive COX-2 activity in several tissues and organs, including the kidney (Harris et al., 1994), the brain (Breder et al., 1995), and tracheal epithelial cells (Walenga et al., 1996).

Interestingly, severe nephropathy was observed in COX-2-knockout mice (Morham et al., 1995), whereas these animals were still susceptible to inflammatory stimuli (Dinchuk et al., 1995). In contrast, COX-1-knockout mice did not develop gastric ulcers (Langenbach et al., 1995). These data suggest that the biological functions of COX-2 might be more complex than originally thought (Wu, 1998).

The present study investigated the possible constitutive expression of COX-2 mRNA and protein, as well as the cellular localization of protein, in apparently healthy human and rabbit gastric mucosa. We found the enzyme to be constitutively expressed in both species, mainly in smooth muscle cells of the muscularis mucosae and in fibroblast-like cells of the mucosa. In addition, PGE2 biosynthesis in the gastric mucosa was completely suppressed by the COX-2-selective inhibitor flosulide, suggesting that COX-2 activity accounts for a significant portion of endogenous PG formation. These data might have significant implications for the development and clinical use of COX-2-selective inhibitors.

Experimental Procedures

Materials. Reagents used for the experiments were as follows: acetylsalicylic acid (aspirin), Bayer (Leverkusen, Germany); flosulide, Schering AG (Berlin, Germany); anti-COX-1 antibodies, Cayman Chemicals (Ann Arbor, MI); anti-COX-2 antibodies, Dianova (Hamburg, Germany). Electrophoresis reagents were from Bio-Rad.

ABBREVIATIONS: COX, cyclooxygenase; PBS, phosphate-buffered saline; PG, prostaglandin; 15-HETE, 15-hydroxyeicosatetraenoic acid; LPS, lipopolysaccharide; PMA, phorbol-12-myristate-13-acetate; PCR, polymerase chain reaction; RT, reverse transcription.
Laboratories (München, Germany). All other reagents were of analytical-grade purity and, unless otherwise indicated, were purchased from Sigma (Deisenhofen, Germany). Human stomach tissue was obtained during tumor gastrectomy surgery (three samples). These specimens were taken from stomach regions that were unaffected by the tumors. For immunohistochemical analysis, diagnostic biopsy material (10 samples) that had been found to be negative for signs of inflammation or tumor was used. All biopsy material was negative for Helicobacter pylori antigen. Gastric tissue was also obtained from New Zealand White rabbits.

**Preparation of gastric mucosa.** After removal, the tissues were rinsed with physiological saline solution and stored in ice-cold physiological saline solution. All additional procedures were performed on ice. The mucosa/submucosa layer was carefully cut away from the muscularis propria layer and was further processed as described below.

**RT-PCR.** Mucosal pieces (3 × 5 mm) were homogenized, and total RNA was prepared using the Trizol method (Gibco-BRL, Eggenstein, Germany). Poly(A)+ RNA was isolated using the POLYATtract mRNA Isolation System IV (Promega, Madison, WI). After RT, PCR was polymerase chain reaction performed using the following primers (at 0.15 μM): human COX-1 (481-base pair fragment), sense, 5′-CCGTCTCTCGTCGCCGAGCAGCAGGTAGCT-3′; antisense, 5′-AAAGCCGCGGCCCAGAGCTGGC-3′; human COX-2 (421-base pair fragment), sense, 5′-AATGAGTACCAGAAAATCT-3′; antisense, 5′-CATCTAGTCCGGCCAGGAATG-3′. The PCR was carried out using the GeneAmp RNA PCR kit (Perkin Elmer, Branchburg, NJ). The samples were prenatatured for 2 min at 94°C, and the cycle profiles (40 cycles) were as follows: human COX-1 and COX-2, 1 min at 95°C, 1 min at 60°C, and 1.5 min at 72°C. Fragments were identified by fragment size and restriction analysis, using CiaI, HaeIII, HinfI, NcoI, and PstI.

**Western blot analysis.** For Western blot analysis, gastric mucosa was homogenized in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-Cl, pH 8.0), supplemented with the protease inhibitors leuphctin (1 μg/ml), aprotinin (1 μg/ml), and phenylmethylsulfonyl fluoride (100 μM). Proteins (100 μg) were separated by sodium dodecyl sulfate (8%)-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore Inc., Bedford, MA). For immunodetection, the membranes were probed with anti-COX-2 antibodies (1/1000) or anti-COX-1 antibodies (1/1000), followed by incubation with peroxidase-conjugated secondary antibodies (anti-mouse IgG, 1/3000; Dianova, Hamburg, Germany). COX proteins were visualized by enhanced chemiluminescence (Amer sham Buchler, Braunschweig, Germany).

**Immunohistochemical analysis.** Gastric mucosal pieces (3 × 5 mm) were fixed in neutral buffered 4% formaldehyde and embedded in paraffin. Sections (5 μm) were mounted on poly-L-lysine-coated glass slides. Dried slides were baked at 56°C for 2 hr, dewaxed with xylol, and rehydrated. Slides were then heated in a microwave oven, in 10 min citrate buffer, pH 6.0, for 17 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Immunohistochemical analysis was performed using an avidin-biotin-peroxidase system (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Briefly, tissue sections were treated for 15 min with avidin, washed with PBS, treated for 15 min with biotin, and blocked for 20 min at room temperature with PBS/2% fat-free bovine serum albumin, to saturate nonspecific binding sites. Anti-COX-1 and anti-COX-2 antibodies were diluted in PBS (1/50) and incubated with the sections for 12 hr at 4°C, in a humidified chamber. The sections were rinsed with PBS and then incubated with the biotinylated secondary antibody (goat anti-mouse IgG, 1/200; Vector Laboratories) for 1 hr, in a humidified chamber. Diaminobenzidine was used as a substrate, and slides were counterstained with Meyer’s hemalum. Specificity was determined by substitution of the primary antibody with PBS.

**Measurement of PG production.** To measure PG production, rabbit mucosal tissue was cut into approximately equal pieces (3 × 5 mm), weighed, and preincubated for 15 min in Dulbecco’s modified Eagle medium containing penicillin (100 units/ml)/streptomycin (0.1 mg/ml) (Gibco BRL, Eggenstein, Germany), to remove PGs possibly generated by mechanical irritation of the tissue during the preparation procedures. Mucosal tissues were then placed in Dulbecco’s modified Eagle medium for 5 hr at 37°C, in the absence or presence of flusulide (0.01–3 μM) or aspirin (0.01–5 mM). PG formation was measured, in terms of PG-like formed, by radioimmunoassay of aliquots of the incubation mixtures (Bothwell et al., 1982).

**Measurement of 15-HETE production.** 15-HETE production was analyzed by two-dimensional thin-layer chromatography according to the method of Miller et al. (1994), with some modifications. Briefly, rabbit gastric mucosa was homogenized in Tris buffer (50 mM, pH 8.0). An aliquot (500 μl) of this preparation was added to 500 μl of reaction buffer (10 mM Tris, pH 8.0, 1 mM tryptophan, 1 mM epinephrine, 0.3 mM glutathione), in the absence or presence of acetyllysaliacylic acid (5 mM). The reaction was started by addition of 0.5 μCi of [14C]arachidonic acid (New England Nuclear, Boston, MA). After 20 min at 37°C, 500 μl of 10% formic acid was added. PGs were extracted with 8 ml of ethyl acetate and finally redissolved in 100 μl of ethyl acetate. Two-dimensional thin-layer chromatography (LK6D plates; Whatman, Clifton, NJ) was performed using ethyl acetate/isooctane/acetic acid/water (11.5:2:10) for the first dimension and chloroform/methanol/acetic acid (90:8:6) for the second dimension. Radioactive spots were visualized by autoradiography using En3Hance spray (Dupont de Nemours, Bruxelles, Belgium). Products were identified by comigration with PG standards (Biomol, Springhouse, PA).

**Results**

**Constitutive expression of COX-1 and COX-2 in human gastric mucosa.** The expression of COX-1 and COX-2 mRNA in human gastric mucosa was demonstrated by RT-PCR. The mRNA for both COX isoforms was found in all gastric mucosal specimens examined (data not shown). Consistent with these findings, COX-1 and COX-2 protein was detected in mucosal tissue by Western blotting (Fig. 1). All biopsy specimens (10 samples) were positive for both COX isoforms.

**Cellular localization of COX-1 and COX-2 proteins in human gastric mucosa.** The cellular localization of COX-1 and COX-2 in human gastric mucosa was studied by immu-
Fig. 2. Immunohistochemical localization of COX-2 in human gastric mucosa. COX-2-positive cells were detected in the mucosal layer (a and b), in the muscularis mucosae (c), and in endothelial cells of mucosal blood vessels (d, arrows). Scale bars, 20 μm.
nohistochemical analysis. All diagnostic biopsy samples used for these studies were negative for signs of inflammation or malignancy and were negative for *H. pylori* antigen. COX-1 immunoreactivity was most prominent in the muscularis mucosae layer. Furthermore, both endothelial and smooth muscle cells of arterial and venous vessels showed strong COX-1 signals (data not shown). The immunohistochemical localization of COX-2 protein was similar. The enzyme protein was found primarily in the muscularis mucosae layer. Immunopositive cells were identified as smooth muscle cells, whereas cells of the mucosal glands were negative. COX-2 immunoreactivity was also detected in fibroblast-like cells of the mucosa and endothelial cells of both arteries and veins (Fig. 2).

**Induction of COX-2 in human gastric mucosa by LPS and PMA.** To establish that COX-2 protein expression could be further stimulated by activation of transcription factors, gastric mucosal samples (3 x 5 mm) were placed in culture medium and stimulated with LPS (10 μg/ml) or PMA (1 μM) for 9–24 hr (Inoue et al., 1995). In these experiments, a smaller amount of protein (25 μg) was loaded onto the gel, for easier detection of any stimulation. Both LPS and PMA markedly enhanced the expression of COX-2 protein (Fig. 3).

**Expression of COX-1 and COX-2 in rabbit gastric mucosa.** Because only very limited amounts of human stomach tissue were available, studies on the functional significance of COX-2-dependent PG formation were conducted in rabbits. First, constitutive COX-1 and COX-2 expression in rabbit gastric mucosa was verified by RT-PCR and Western blotting, respectively (data not shown). The immunohistochemical localization of COX isoforms was similar to that observed in human tissue (data not shown).

**Suppression of PGE2 production in rabbit gastric mucosa by flosulide and aspirin.** The functional relevance of the COX-2 isoform in PG production was studied by measuring the inhibition of PGE2 generation by the COX-2-selective inhibitor flosulide, compared with aspirin. Flosulide caused concentration-dependent and ultimately complete inhibition of gastric PGE2 production (IC50 = 107 ± 55 nM), indicating that COX-2 was the major COX isoform contributing to endogenous PG generation. In contrast, aspirin, a more COX-1-selective compound (Mitchell et al., 1994), inhibited PGE2 formation completely only at 1 mM. These data are summarized in Fig. 4.

**15-HETE formation by aspirin-treated rabbit gastric mucosa.** It is known that high concentrations of aspirin inhibit COX-2-dependent PG production. However, in contrast to COX-1, COX-2 is still able to generate 15-HETE after aspirin treatment (Lecomte et al., 1994). We therefore measured possible 15-HETE production by aspirin (5 mM)-treated gastric mucosa. Untreated gastric tissue released mainly PGE2 and PGD2, as well as PGF2α and 6-keto-PGF1α, to a lesser extent, into the culture medium (Fig. 5). When the tissue was treated with aspirin, PG formation was completely inhibited. However, aspirin-treated gastric mucosa released 15-HETE (Fig. 5), demonstrating the presence of functionally active COX-2.

**Discussion**

COX-2 mRNA was previously found in normal gastric tissue (O'Neill and Ford-Hutchinson, 1993; Ristimäki et al., 1997). However, no study has yet detected COX-2 protein in human gastric mucosa.
human gastric mucosa (O'Neill and Ford-Hutchinson, 1993; Kargman et al., 1996; Ristimäki et al., 1997), and there was only a low level of COX-2 protein expression in rat stomach tissue (Iseki, 1995). Accordingly, it was concluded that PGs produced in normal gastrointestinal tissue are mainly, if not entirely, derived from the COX-1 isozyme (Kargman et al., 1996).

This study is the first to demonstrate the constitutive expression of both COX-2 mRNA and protein in apparently healthy human and rabbit gastric mucosa. Gastric PG E2 production was potently inhibited by flosulide at nanomolar concentrations (IC50 = 107 ± 5 nM). At these concentrations, the compound is assumed to act as a COX-2-selective inhibitor. In contrast, the IC50 values for COX-1 inhibition by flosulide range between 10 and 100 μM (Klein et al., 1994, 1996; Cromlish and Kennedy, 1996; Greig et al., 1997; Wong et al., 1997). We have studied the effects of flosulide on platelet aggregation and thromboxane formation (Muck et al., 1998). In those studies, flosulide inhibited both aggregation and thromboxane formation, with an IC50 of 1–3 μM (Weber A-A, Zimmermann KC, and Schrör K, unpublished observations). At concentrations that inhibited gastric PG formation, no effects on platelet COX were seen. The presence of COX-2 was also demonstrated by 15-HETE production (Lecomte et al., 1994) in aspirin-treated homogenates of rabbit gastric mucosa. These findings, taken together, demonstrate the presence and an important functional role of COX-2 in apparently healthy gastric mucosa.

Gastrointestinal ulcers are often accompanied by mucosal inflammation. Wallace and Cirino (1994) hypothesized that these inflammatory reactions might cause COX-2 expression in the stomach. Similar considerations might apply to local inflammation. Wallace and Cirino (1994) hypothesized that COX-2 expression is induced by known activators of the COX-2 promoter, up-regulated by known activators of the COX-2 promoter, and induced by known activators of the COX-2 promoter in human gastric mucosa (O'Neill and Ford-Hutchinson, 1993; Ristimäki et al., 1997). We have demonstrated that, in addition to its constitutive expression, COX-2 protein can be up-regulated by known activators of the COX-2 promoter, such as LPS or PMA (Inoue et al., 1995). Therefore, COX-2-derived PG formation might be involved in physiological functions of the mucosa (i.e., inhibition of secretion or cytoprotection), and might become up-regulated in ulcerations or inflammation of the gastric mucosa, to facilitate healing processes (Kobayashi and Arakawa, 1995; Schmässmann et al., 1998).

Taken together, these findings indicate that COX-2 is an important enzyme generating vasodilatory and possibly cytoprotective PGs in the gastric mucosa. This raises the issue of whether selective COX-2 inhibitors might disturb physiological PG actions in the human stomach.

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