Regulation and function of COX-2 gene expression in isolated gastric parietal cells

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The gastric parietal cells are highly specialized gastric epithelial cells that secrete gastric acid in response to stimulation with histamine, gastrin, and acetylcholine (19, 44, 45). Recent reports have indicated that, in addition to this well-established function, the parietal cells appear to exert important regulatory actions in the stomach through their paracrine and hormonal actions. In particular, transforming growth factor (TGF)-α, a polypeptide growth factor known to regulate important biological processes such as secretion, growth, and differentiation (20), is expressed and released by the parietal cells (3, 9, 25). These studies have suggested that production of TGF-α by the parietal cells might be an important mechanism for the paracrine regulation of gastric epithelial cell growth and differentiation (3, 9, 10, 25). Accordingly, the parietal cells appear to exert important regulatory actions in the stomach through their

Prostaglandins have been shown to inhibit gastric acid secretion and to regulate gastrointestinal mucosal blood flow (19, 45, 52). The importance of prostaglandins in gastrointestinal mucosal homeostasis has been underscored by the observation that inhibition of prostaglandin generation by the use of nonsteroidal anti-inflammatory drugs (NSAIDs) leads to the development of significant gastrointestinal mucosal damage (19, 52).

The genes that encode COX-1 and COX-2 exhibit considerable differences in regard to their regulation and biological functions (43). Whereas COX-1 appears to be constitutively expressed, COX-2 is rapidly induced in response to cytokines and growth factors through the coordinated activation of numerous intracellular signal transduction pathways (43). COX-2 expression has been documented in several pathological conditions of the gastrointestinal tract. In particular, COX-2 has been shown to regulate colon cancer cell growth and apoptosis (39) and to be expressed in a significant number of human gastric carcinomas (41). Furthermore, selective inhibition of COX-2 appears to delay the healing of gastric ulcers and to exacerbate inflammation-associated colonic injury in rats (32, 33, 40, 46).

The parietal cells are highly specialized gastric epithelial cells that secrete gastric acid in response to stimulation with histamine, gastrin, and acetylcholine (19, 44, 45). Recent reports have indicated that, in addition to this well-established function, the parietal cells appear to exert important regulatory actions in the stomach through their paracrine and hormonal actions. In particular, transforming growth factor (TGF)-α, a polypeptide growth factor known to regulate important biological processes such as secretion, growth, and differentiation (20), is expressed and released by the parietal cells (3, 9, 25). These studies have suggested that production of TGF-α by the parietal cells might be an important mechanism for the paracrine regulation of gastric epithelial cell growth and differentiation (3, 9, 10, 25). Accordingly, the parietal cells appear to exert important regulatory actions in the stomach through their

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ability to produce and secrete polypeptide growth factors. A recent study has demonstrated that Helicobacter pylori-induced acute and chronic antral inflammation in humans is associated with immunohistochemical detection of COX-2 in the parietal cells, suggesting that these cells might be an important source of prostaglandins during the onset and development of gastric inflammation (31).

We hypothesize that, in addition to their ability to express and secrete TGF-α, the parietal cells might also produce other agents, such as prostaglandins, that are known to exert multiple important cytoprotective actions in the gastric mucosa. Accordingly, we undertook studies to examine the expression and regulation of the COX-2 gene in isolated gastric parietal cells in primary culture. In particular, we investigated the intracellular mechanisms that regulate COX-2 gene expression in the parietal cells and the linkage of these events to the production of gastric prostaglandins.

Materials and Methods

Adenoviral vectors. The replication-defective adenoviral vector expressing a hemagglutinin (HA)-tagged mutated IκB (Ad.dom.neg.IκB) under the control of the cytomegalovirus (CMV) promoter, was a gift of D. Brenner (Chapel Hill, NC) (23, 24). The adenoviral vector expressing the β-galactosidase enzyme under the control of the CMV promoter (Ad.CMV-β-gal) was previously described (1).

Primary parietal cell preparation and culture. For preparation of primary parietal cells, we used a modification of the method of Soll and colleagues (8, 12, 34, 37, 44). The mucosal layer of freshly obtained canine gastric fundus was bluntly separated from the submucosa and rinsed in Hanks balanced salt solution containing 0.1% BSA. The cells were then dispersed by sequential exposure to collagenase (0.35 mg/ml) and 1 mM EDTA, and parietal cells were enriched by centrifugation of the cell suspension through a 40% Percoll (Pharmacia Biotech, Piscataway, NJ) at 24,000 g for 20 min. The cell fraction at ρ = 1.05 consisted of virtually all parietal cells, as determined by staining with a specific mouse monoclonal antibody against the hog H-1-K-ATPase α-subunit (a gift of A. Smolka, Charleston, SC) (47). The isolated parietal cells (2 × 10^6 cells/well) were cultured according to the method of Chen et al. (9) with some modifications (12, 34, 47). Briefly, the cells were cultured in Ham’s F-12 DMEM (1:1) containing 0.1 mg/ml gentamicin, 48 U/ml penicillin G, and 2% DMSO (Sigma, St. Louis, MO) on six-well culture dishes (Costar) coated with 150 μl of H2O-diluted (1:5) growth factor-reduced Matrigel (Becton Dickinson, Bedford, MA) for 30 min at room temperature and then incubated for 1 h in TBST and 5% dry milk, containing either a specific mouse monoclonal anti-HA antibody (1:1,000; Babco Berkeley Antibody, Richmond, CA) or a rabbit polyclonal anti-COX-2 antibody (1:1,000; Cayman Chemical, Ann Arbor, MI). At the end of the incubation period, the membranes were washed in TBST for 30 min at room temperature and then incubated for 1 h in TBST and 5% dry milk, containing either protein A, directly conjugated to horseradish peroxidase (1:2,500; Amersham Life Science, Arlington Heights, IL) for the COX-2 Western blots or a horseradish peroxidase-conjugated anti-mouse secondary antibody that was obtained from Calbiochem (Cambridge, MA). Labeling and hybridizations were carried out according to the manufacturer’s instructions as previously described (35).

Western blots. Parietal cell lysates (80 μg) were loaded on 10% SDS-polyacrylamide minigels and run at 200 V for 1 h. Protein concentrations were measured by the Bradford method (7). The gels were transferred onto Immobilon-P transfer membranes and blocked with 5% low-fat dry milk in TBST and 5% dry milk, containing 0.1% Tween 20. After transfer, the membranes were incubated in 10 ml of TBST (20 mM Tris, 150 mM NaCl, and 0.3% Tween) and 5% dry milk for 1 h and then incubated for 16–18 h at 4°C in 10 ml of TBST and 5% dry milk, containing either a specific mouse monoclonal anti-HA antibody (1:1,000; Babco Berkeley Antibody, Richmond, CA) or a rabbit polyclonal anti-COX-2 antibody (1:1,000; Cayman Chemical, Ann Arbor, MI). The Western blotting was visualized by horseradish peroxidase (Roche Diagnostics Inc., Indianapolis, IN) and chemiluminescence detection system according to the manufacturer’s instructions.

Immunohistochemistry. The parietal cells were transduced with Ad.dom.neg.IκB and cultured on slides for 16–18 h. At the end of the incubation period the cells were fixed in 4% formalin-PBS. The slides were blocked for 30 min with 20% donkey serum and incubated for 2 h with the anti-HA antibody. The slides were rinsed with PBS, and a 1:150 dilution of a FITC-conjugated donkey anti-mouse IgG secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) was added for 1 h. After a wash with PBS, the slides were mounted in Vectashield (Vector Laboratories, Burlington, CA) containing 5 μg/ml propidium iodide and were visualized by fluorescence microscopy.

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Amplification and purification of adenoviral vectors. Briefly, the recombinant adenoviruses were amplified as previously described using 293 cells (1). The viruses were subsequently concentrated and purified on a cesium chloride gradient (1). The concentration of the recombinant adenoviruses was assessed on the basis of the absorbance at 260 nm and on a limiting dilution plaque assay (1).

Detection of adenoviral-delivered β-galactosidase. For identification of parietal cells transduced with the adenoviral vector expressing β-galactosidase, the cells were cultured on slides for 16–18 h and stained with 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-gal) after 24 h of infection. The cells were washed with PBS and then fixed in 0.5% glutaraldehyde at room temperature for 10 min. After two washes with 1 mM MgCl₂ in PBS, the cells were incubated with PBS and observed with a light transmission microscope.

GABA receptor measurement. PGE₂ production was measured using an enzyme immunoassay kit (Cayman Chemical) according to the manufacturer’s instructions.

Data analysis. Data are expressed as means ± SE. Statistical analysis was performed using Student’s t-test. P values <0.05 were considered significant.

RESULTS

COX-2 plays an important role in gastric pathophysiology. Because the gastric parietal cells express receptors for carbachol, gastrin, and histamine, agents known to stimulate gastric acid secretion (19, 44, 45), we investigated the regulation of COX-2 gene expression in response to the gastric acid secretagogues. As depicted in Fig. 1, stimulation of the parietal cells with carbachol (100 μM) for 2 h induced COX-2 gene expression >10-fold (12.52 ± 1.95-fold induction over control, mean ± SE, n = 3). In contrast, stimulation of the parietal cells for 2 h with either gastrin (10 nM) or histamine (100 μM) induced COX-2 gene expression by only 2- and 1.7-fold, respectively (2.41 ± 0.65-fold over control for gastrin, n = 9, and 1.73 ± 0.13-fold over control for histamine, n = 3). Moreover, induction of COX-2 gene expression in response to carbachol in combination with either gastrin or histamine, or in the presence of both agents combined, was not statistically different from that observed in the presence of carbachol alone. Similarly, exposure of the parietal cells to a combination of gastrin and histamine for 2 h did not significantly affect COX-2 gene expression compared with the effects observed in the presence of either gastrin or histamine alone. Accordingly, exposure of the parietal cells to different combinations of gastric acid secretagogues failed to exhibit any significant synergistic effects.

Since carbachol was the most potent inducer of COX-2 gene expression in the parietal cells, we focused our efforts primarily on the understanding of the intracellular pathways that target the COX-2 gene in response to carbachol stimulation and to analyze the functional relevance of this phenomenon.

We investigated the kinetics of carbachol induction of COX-2 gene expression. In these studies, we observed that the action of carbachol (100 μM) was time

dependent, with a maximal stimulatory effect detected between 30 min and 6 h of incubation (Fig. 2, A and B). In addition, as shown in Fig. 2C, carbachol stimulated the expression of the COX-2 protein after 6 h of incubation. We confirmed the specificity of the stimulatory action of carbachol on COX-2 gene expression. As indicated in Fig. 2D, although carbachol (100 μM) potently induced the COX-2 gene, it failed to affect the expression of the COX-1 gene.

Carbachol is known to induce multiple signal transduction pathways in the gastric parietal cells (19, 37, 47, 48, 50). Accordingly, we examined the intracellular mechanisms that mediate carbachol induction of COX-2 gene expression. Carbachol-stimulated COX-2 gene expression was inhibited by the intracellular Ca²⁺ chelator BAPTA-AM (100 μM; 95%; Fig. 3A), the
PKC inhibitor GF-109203X (3.5 μM; 50%; Fig. 3B), and the p38 kinase inhibitor SB-203580 (10 μM; 50%; Fig. 3C). Addition of the specific mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) kinase 1 inhibitor PD-98059 (10 μM) led to a modest (30%) inhibitory effect (data not shown). The specificity of the effect of GF-109203X was confirmed by the observation that the inactive PKC inhibitor bisindolylmaleimide V had no effect on carbachol-stimulated COX-2 gene expression (data not shown). Since carbachol induces NF-κB activation (50), we examined the role of the NF-κB inhibitor PDTC (1 mM) on COX-2 gene expression. As shown in Fig. 3D, PDTC inhibited carbachol-stimulated COX-2 gene expression by 80%.

To confirm the involvement of NF-κB in carbachol induction of COX-2 gene expression, we transduced the gastric parietal cells with 100 moi of the adenoviral vector Ad.dom.neg.IkB, which overexpresses a HA-tagged IkBα in which serines 32 and 36 were substituted with alanines. Because of this mutation, IkBoS32A/S36A acts as a repressor of NF-κB (24). Ad.dom.neg.IkB expression was monitored by Western blots with an anti-HA antibody (data not shown). Immunohistochemical staining of Ad.dom.neg.IkB-transduced parietal cells with an anti-HA antibody demonstrated expression of Ad.dom.neg.IkB in the parietal cells (Fig. 4C). The specificity of the immunohistochemistry was demonstrated by incubating the transduced parietal cell with the secondary antibody without the primary antibody (Fig. 4D). Additional experiments were performed by transducing the parietal cells with 100 moi of the control adenoviral vector Ad.CMV-β-gal, which expresses β-galactosidase. As shown in Fig. 4B, histochemical staining for β-galactosidase demonstrated that >80% of the parietal cells were transduced with Ad.CMV-β-gal. Control experiments were conducted in noninfected cells, in which no cells stained with X-gal (Fig. 4A). To demonstrate that transduction of the parietal cells with adenoviral vectors does not affect their function, we conducted studies in which we compared the effect of carbachol on [14C]aminopyrine uptake between transduced and nontransduced parietal cells. In these studies, we observed that Ad.CMV-β-gal did not alter the function of the parietal cells because carbachol-stimulated uptake of [14C]aminopyrine was similar in both infected and noninfected cells (data not shown; see Ref. 49).

Transduction of the parietal cells with Ad.dom.neg.IkB produced a partial (70%) inhibition of carbachol stimulation of COX-2 gene expression (Fig. 5, A and B). In contrast, this inhibition was almost complete (90%) in the presence of Ad.dom.neg.IkB in combination with SB-203580, suggesting that p38 kinase and NF-κB act in concert to regulate COX-2 gene expression in the gastric parietal cells (Fig. 5, A and B). To confirm the specificity of the inhibitory action of Ad.dom.neg.IkB on COX-2 gene expression, we examined the effect of this construct on the expression of the highly inducible early response gene c-jun in response to carbachol stimulation. As shown in Fig. 5C, transduction of the parietal cells with Ad.dom.neg.IkB significantly inhibited carbachol-stimulated COX-2 gene expression but failed to affect the expression of c-jun. Thus Ad.dom.neg.IkB exerts specific inhibitory effects on COX-2 gene expression in the gastric parietal cells.

Because activation of COX-2 leads to prostaglandin production (43), we examined the effect of both Ad.dom.neg.IkB and SB-203580 on PGE2 release in response to carbachol stimulation. Carbachol induced a statistically significant increase in PGE2 release in Ad.CMV-β-gal-transduced parietal cells (620 ± 190
Fig. 3. Signal transduction pathways regulating carbachol stimulation of COX-2 gene expression in isolated gastric parietal cells. Aliquots of total RNA extracted following exposure of the parietal cells to carbachol (100 μM), either alone or in combination with the intracellular Ca\(^{2+}\) chelator 1,2-bis(2-aminophenoxy)ethane-N,N',N,N'-tetraacetic acid (BAPTA)-AM (100 μM; A), the protein kinase C (PKC) inhibitor GF-109203X (3.5 μM; B), the p38 kinase inhibitor SB-203580 (10 μM; C) and the nuclear factor (NF)-κB inhibitor 1-pyrrolidinecarbodithioic acid (PDTC; 1 mM; D), were examined by Northern blot analysis using a \(^{32}\)P-labeled human cDNA probe for COX-2. Bar graphs represent results obtained from densitometric analysis of blots from several parietal cell preparations. Data are expressed as percentage of carbachol-stimulated stimulated COX-2 gene expression in the absence of the inhibitors (means ± SE).
pg/ml in untreated cells vs. 2,050 ± 581 pg/ml in cells incubated for 2 h in the presence of 100 μM carbachol, n = 6). As shown in Fig. 6, either transduction of the parietal cells with Ad.dom.neg.IκB or addition of SB-203580 led to 70 and 60% inhibition, respectively, of carbachol-stimulated PGE2 release. In addition, treatment of the parietal cells with SB-203580 in combination with Ad.dom.neg.IκB induced a >80% inhibition of
carbachol-stimulated PGE₂ release. Accordingly, carbachol activates both NF-κB and p38 kinase to regulate prostaglandin release.

To examine in more detail the role of COX-2 in carbachol-stimulated PGE₂ production, we examined the effects of both NS-398 (10 μM), a selective COX-2 inhibitor (43), and indomethacin (10 μM), an agent known to inhibit both COX-1 and COX-2 (43), on PGE₂ release from the isolated parietal cells. Carbachol induced a statistically significant increase in PGE₂ release in nontransduced parietal cells (688 ± 203 pg/ml in untreated cells vs. 2,601 ± 874 pg/ml in cells incubated for 4 h in the presence of 100 μM carbachol, n = 6). In contrast, no significant increase in PGE₂ release was observed in response to either gastrin or histamine (data not shown). As indicated in Fig. 7, treatment of the parietal cells with NS-398 completely blocked carbachol stimulation of PGE₂ production without inhibiting basal PGE₂ release. In contrast, indomethacin led to a complete inhibition of carbachol-stimulated PGE₂ release and to a 50% reduction in basal PGE₂ production. Thus, in the gastric parietal cells, COX-2 regulates PGE₂ production in response to carbachol stimulation.

DISCUSSION

The gastric parietal cells are complex biological structures whose behavior is regulated by a broad array of hormones, neurotransmitters, and growth factors (19, 35, 44, 45). In particular, the parietal cells express receptors for gastrin, acetylcholine, and histamine agents known to stimulate gastric acid secretion (19, 44, 45).

The cholinergic agonist carbachol is one of the most potent activators of the canine gastric parietal cells (19). Carbachol is known to interact with specific M₃ muscarinic receptors present on the parietal cell surface to stimulate gastric acid secretion and PGE₂ production (19, 38, 44, 45). In this study, we dissected the mechanisms and the intracellular signal transduction pathways that mediate carbachol stimulation of PGE₂ production. In particular, we have demonstrated that carbachol-stimulated PGE₂ generation is mediated by the ability of this agent to rapidly induce COX-2 gene expression. In contrast, we have reported that gastrin and histamine have extremely weak stimulatory actions on COX-2 gene expression and that both agents fail to regulate PGE₂ production. Accordingly, induction of COX-2 is a signaling event that is specifically activated by carbachol but not by the other gastric acid secretagogues.

Previous work from our laboratory has indicated that activation of the parietal cell M₃ muscarinic receptor leads to the induction of numerous protein kinases. In particular, we and others have shown that carbachol induces the ERKs (36, 47, 48), the c-Jun NH₂-terminal kinases (JNKs) (35), and p38 kinase (37), molecules known to regulate multiple important physiological functions in the parietal cells. Whereas the ERKs and p38 kinase appear to be involved in the process of gastric acid production (37, 48), activation of the JNKs seems to be an important step in the response of the parietal cells to stress and inflammation (35). Here, we demonstrate that p38 kinase, and to a
COX-2 gene in the parietal cells in response to carbachol activation of NF-κB. Recent studies have demonstrated that NF-κB plays a crucial role in the regulation of numerous genes that are involved in the inflammatory response because it modulates the expression of proinflammatory cytokines, chemokines, immune receptors, adhesion molecules, and enzymes that generate mediators of inflammation (2). In unstimulated cells, NF-κB is present in the cytoplasm complexed with a repressor protein known as IκB, which prevents it from moving into the nucleus to regulate gene transcription (2). On cell stimulation, IκB is rapidly phosphorylated by the IκB kinases, which are responsible for the specific phosphorylation of IκB on serine residues (27). Phosphorylated IκB is then rapidly degraded by the proteasome, leading to the movement of NF-κB to the nucleus, where it binds to specific DNA sequences present in the promoters of numerous target genes (2, 27).

Activation of NF-κB appears to be an important event in numerous inflammatory processes of the gastrointestinal tract. Induction of this transcription factor has been reported in H. pylori gastritis (28), inflammatory bowel disease (51), and pancreatitis (22). Accordingly, we have investigated the role of NF-κB in carbachol induction of COX-2 gene expression. For these experiments, we have taken advantage of both pharmacological and biochemical tools. In particular, we have transduced the parietal cells with an adenoviral vector overexpressing an IκB molecule that acts as an inhibitor of NF-κB. Through these studies, we have demonstrated that PKC, intracellular Ca^{2+}, and activation of NF-κB are crucial for the induction of the COX-2 gene in the parietal cells in response to carbachol stimulation. In fact, the PKC inhibitor GF-109203X, the intracellular Ca^{2+} chelator BAPTA-AM, the NF-κB inhibitor PDTC, and dominant-negative IκB all blocked carbachol stimulation of COX-2 gene expression. Our observations confirm those of other studies, which have indicated that activation of NF-κB is an important mechanism for the regulation of COX-2 gene expression. In particular, induction of NF-κB appears to modulate COX-2 gene expression in intestinal epithelial cells and in rheumatoid synovocytes treated with tumor necrosis factor (TNF)-α (16, 23) and in human myometrial cells stimulated with interleukin-1β (4).

Another important observation of our study is that, in the gastric parietal cells, blockade of either p38 kinase or NF-κB leads to only partial inhibition of carbachol induction of COX-2 gene expression. In contrast, exposure of the parietal cells to both SB-203580 and dominant-negative IκB leads to an almost complete inhibitory effect. These data suggest that both the p38 kinase signal transduction pathway and an IκB-dependent mechanism of NF-κB activation act in concert to regulate COX-2 gene expression in response to carbachol stimulation. These findings confirm earlier reports from our laboratory in which we observed that, in the gastric parietal cells, inhibition of p38 kinase by SB-203580 does not affect carbachol stimulation of the IκB kinases (data not shown). Similarly, we demonstrated that BAPTA-AM, which potently blocks carbachol induction of the IκB kinases, fails to inhibit the stimulatory effect of carbachol on p38 kinase activation (37).

The functional relevance of these findings was underscored by the observation that inhibition of carbachol-stimulated COX-2 gene expression by SB-203580 and dominant-negative IκB correlates with inhibition of PGE_{2} production. Thus NF-κB and p38 kinase are important mechanisms employed by carbachol to regulate prostaglandin production in the stomach. Although one report has suggested that macrophages and capillary endothelial cells might be the major producers of PGE_{2} (11), our study clearly indicates that the canine parietal cells can express COX-2 and release prostaglandins in response to carbachol stimulation.

Since cholinergic neurotransmitters are released in the inflamed gastrointestinal mucosa (14, 15), carbachol induction of COX-2 gene expression in the parietal cells might be one of the events that are activated in the gastric epithelium during the onset and development of inflammation. Indeed, several reports have demonstrated that activation of cholinergic efferent pathways might play an important role in the regulation of the inflammatory response. Cholinergic agonists have been shown to inhibit lipopolysaccharide-induced TNF-α release from human macrophages and to block TNF-α synthesis in the liver during lethal endotoxemia in rats (5). Moreover, the COX-2 gene appears to be potently induced in the gastric mucosa of rats during the recovery from cold-restraint-induced gastritis (30), a pathophysiological condition mediated by the activation of efferent cholinergic mechanisms (29).

Prostaglandins regulate important functions such as secretion, growth, and differentiation through the activation of both autocrine and paracrine mechanisms (19, 45, 52). Accordingly, we speculate that COX-2-mediated PGE_{2} production by the parietal cells might represent an important step for the activation of cytoprotective pathways in the stomach.

Previous studies conducted in isolated canine parietal cells (45) have shown that PGE_{2} has no effect on
carbachol-stimulated aminopyrine uptake. In contrast, PGE2 is known to inhibit histamine-stimulated gastric acid secretion. Since the parietal cells are regulated by the coordinated action of carbachol, histamine, and gastrin (19, 44, 45), it is possible that carbachol induces the release of PGE2 to modulate the secretory function of the gastric parietal cells. Thus activation of the parietal cell M3 muscarinic receptor appears to lead to the simultaneous induction of gastric acid secretion and of cytoprotective mechanisms such as stimulation of COX-2 gene expression and of PGE2 production.

In conclusion, we have reported that carbachol induces COX-2 gene expression in the parietal cells through signaling pathways that involve intracellular Ca2+ concentration, PKC, p38 kinase, and activation of NF-kB. The functional significance of this effect appears to be the stimulation of PGE2 release.

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COX-2 GENE EXPRESSION IN GASTRIC PARIENTL CELLS


