Acid increases proliferation via ERK and p38 MAPK-mediated increases in cyclooxygenase-2 in Barrett’s adenocarcinoma cells

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Acid increases proliferation via ERK and p38 MAPK-mediated increases in cyclooxygenase-2 in Barrett’s adenocarcinoma cells. Am J Physiol Gastrointest Liver Physiol 287: G743–G748, 2004. First published July 1, 2004; 10.1152/ajpgi.00144.2004.—Cyclooxygenase-2 (COX-2) has been linked to neoplastic progression in Barrett’s esophagus. Acid exposure has been shown both to activate the MAPK pathways and to increase COX-2 protein expression in Barrett’s metaplasia, but it is not known whether these effects are interrelated. We hypothesized that acid-induced activation of the MAPK pathways mediates an increase in COX-2 expression in Barrett’s esophagus, and we tested this hypothesis in a Barrett’s-associated adenocarcinoma cell line (SEG-1). We exposed SEG-1 cells to acidic or neutral media in the presence and absence of two MAPK inhibitors: U-0126 (an ERK inhibitor) or SB-203580 (a p38 inhibitor). We quantitated COX-2 protein levels using an enzyme immunometric assay and COX-2 mRNA levels using real-time PCR. We also determined how acid affects the activity of the COX-2 promoter and mRNA stability. Compared with SEG-1 cells exposed to neutral media, acid-exposed cells exhibited a 2.8-fold increase in COX-2 mRNA levels within 30 min. Both U-0126 and SB-203580 attenuated the acid-induced increase in COX-2 mRNA. Acid significantly increased COX-2 protein expression and promoter activity, and both of these effects were abolished by treatment with U-0126 and SB-203580. Acid exposure also stabilized COX-2 mRNA levels, an effect that was abolished by U-0126 but not by SB-203580. We conclude that acid increases COX-2 expression through activation of the MAPK pathways. Acid-induced activation of both ERK and p38 causes a significant increase in COX-2 promoter activity, and acid-activated ERK stabilizes COX-2 mRNA. These findings suggest potential mechanisms whereby acid reflux might promote carcinogenesis in Barrett’s esophagus.

Barrett’s esophagus; messenger ribonucleic acid stability; cell viability

THE INCIDENCE OF ESOPHAGEAL ADENOCARCINOMA, one of the most deadly gastrointestinal cancers, has been rising rapidly for more than two decades. Symptomatic gastroesophageal reflux disease (GERD) and its sequela, Barrett’s esophagus, are the major recognized risk factors for this lethal tumor (10). However, it is not clear how these conditions contribute to esophageal carcinogenesis. One potential mechanism whereby GERD might promote carcinogenesis involves acid-induced expression of cyclooxygenase-2 (COX-2) in Barrett’s esophagus.

Data both from human and animal studies suggest an important role for COX-2 in gastrointestinal tumor formation (19, 25). Upregulation of COX-2 has been found in a number of human gastrointestinal tumors including colorectal, pancreatic, gastric, and esophageal adenocarcinomas (4, 13, 24, 26). Overexpression of COX-2 has been detected in the nonmalignant, metaplastic epithelium of Barrett’s esophagus (7, 26), and COX-2 has been shown to increase as the metaplastic cells progress through dysplasia to adenocarcinoma (15). In vitro studies (21–23) have demonstrated that overexpression of COX-2 reduces the rate of apoptosis, increases tumor cell invasiveness, and promotes angiogenesis. Selective inhibition of COX-2 by NS-398 decreases cell growth and increases the rate of apoptosis in human Barrett’s-associated esophageal adenocarcinoma cells (17), and COX-2 inhibitors have been shown to decrease the development of esophageal adenocarcinoma in an animal model of Barrett’s esophagus (2).

Acid reflux is widely assumed to promote esophageal carcinogenesis, and acid exposure has been shown to increase the level of COX-2 protein expression in ex vivo cultures of biopsy specimens from Barrett’s esophagus (15). One recent study (9) has shown that biopsy specimens of Barrett’s metaplasia from patients treated with selective COX-2 inhibitors in combination with acid suppressive therapy exhibit a significant decrease in cell proliferation compared with those from patients on acid suppressive therapy alone. Thus acid reflux-induced expression of COX-2 is a potential mechanism for tumor formation in Barrett’s esophagus. Although the molecular mechanisms underlying this acid-induced increase in COX-2 expression are not known, an understanding of those mechanisms conceivably could lead to the development of novel chemopreventive measures for patients with Barrett’s esophagus. There are data to suggest that these mechanisms involve the MAPK pathways that transmit signals from the cell surface to the nucleus. (8)

The MAPK signaling cascade comprises three principal signal-transduction pathways: ERK, JNK, and p38 (8). These three MAPKs respond to different stimuli. ERKs are stimulated principally by mitogens, growth factors, and phorbol esters, whereas JNKs and p38 are activated by cell stressors.
Such as heat shock, osmotic stress, and cytokines (6, 8). Activation of the ERK and p38 pathways by nonacidic stimuli has been shown to increase the expression of COX-2 (11, 14, 27). We have previously demonstrated (18) that acid exposure induces ERK and p38 activation in metaplastic Barrett’s cells in vivo and in a Barrett’s-associated adenocarcinoma cell line (SEG-1) in vitro. Therefore, we hypothesized that acid exposure increases COX-2 expression in Barrett’s esophagus by activation of these MAPK pathways. To test this hypothesis, we have used SEG-1 cells to study the effects of acid exposure on MAPK-mediated induction of COX-2 protein and mRNA expression, on the COX-2 promoter, and on posttranscriptional COX-2 mRNA stability.

MATERIALS AND METHODS

Cell culture. An esophageal adenocarcinoma cell line (SEG-1) derived from a Barrett’s-associated adenocarcinoma of the esophagus was used for the experiments (generous gift from Dr. David Beer, University of Michigan) (16). SEG-1 cells have a population doubling time of 21 h (R. Souza, unpublished observation) and a low baseline rate of apoptosis (<5%) (1). SEG-1 cells have been shown to express COX-2 at baseline and to activate the ERK and p38 MAPK pathways in response to acid exposure (17, 18). Our choice of SEG-1 cells for the present study was based on these observations. The cells were maintained in culture as previously described (18). For individual experiments, cells were cultured either in serum-free media at neutral pH or in serum-free media brought to a pH of 6.0 using 1 M HCl acid (acidic media).

Inhibitors of MAPK. The ERK pathway was blocked using the specific MEK1 and -2 inhibitor U-0126 (Calbiochem, San Diego, CA). U-0126 was used at a concentration of 10 μM, which is above the IC₅₀ for both MEK1 and -2 inhibition. The p38 pathway was blocked using SB-203580 (Calbiochem) as previously described (18). Cells were pretreated with U0196 for 15 min or SB-203580 for 1 h, then exposed to acidic or neutral media containing the MAPK inhibitors.

Transient transfection assays. A plasmid containing the COX-2 5’-untranslated region promoter construct (−1,432/+59) attached to a luciferase reporter was used for the transfection studies (6).

![Figure 1](http://ajpgi.physiology.org/content/287/6/G744/F1)

**Fig. 1.** A: effect of acid exposure alone or in the presence of the selective cyclooxygenase-2 (COX-2) inhibitor NS-398 on cell viability determined using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based assay. B: effect of acid exposure alone or in the presence of NS-398 on cell number (*P < 0.05 compared with control cells in neutral media). NS, not significant.

![Figure 2](http://ajpgi.physiology.org/content/287/6/G744/F2)

**Fig. 2.** A: effect of acid exposure alone or in the presence of NS-398 on proliferation using a BrdU assay. B: effect of acid exposure alone or in the presence of NS-398 on apoptosis using a cell death ELISA (*P < 0.05 compared with control cells in neutral media).
pSV-β-gal (Promega, Madison, WI) plasmid was used to equalize for transfection efficiency. Cells were cultured in serum-free media for 48 h followed by the addition of 3.5 mM thymidine overnight to arrest cells at the G1/S boundary. Six hours following the removal of thymidine, cells were cotransfected with 40 ng of the pSV-β-gal plasmid and 10 μg of pSV-β-gal (Promega) using a Gene Pulser II electroporator (Bio-Rad, Hercules, CA) set at 300 mV and delivering 1,000 μF. The activities of luciferase and β-galactosidase were measured in cellular extracts as described previously (12).

**Determination of cell viability, cell number, proliferation, and apoptosis.** Equally seeded wells of SEG-1 cells were cultured in serum-free media for 24 h using 96-well plates for the cell viability and proliferation assays and 24-well plates for cell number and apoptosis assays. Cells were then pretreated with 10 μM NS-398 (Biomol, Plymouth Meeting, PA) for 30 min followed by exposure to acidic media alone or acidic media containing 10 μM NS-398 (3). The acidic media were removed and replaced with neutral serum-free media alone or neutral serum-free media containing 10 μM NS-398 for 24 h. Cell viability was assessed using the Cell Proliferation Assay Kit I (Roche, Indianapolis, IN) per the manufacturer’s instructions. The assay is based on the ability of metabolically active cells to cleave 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a formazan dye, which is then detected using a Multiscan EX (Lab Systems, Vantaa, Finland) at a wavelength of 560 nm. Cell proliferation was assessed using the cell proliferation ELISA (Roche) per the manufacturer’s instructions. The assay is based on the measurement of BrdU incorporation during DNA synthesis, which is detected using a Multiscan EX at a wavelength of 450 nm. Cell number and apoptosis were determined as previously described (18).

**COX-2 enzyme immunometric assay for protein expression.** Equally seeded wells of SEG-1 cells were cultured in serum-free media for 48 h in six-well plates. Cells were then exposed to acidic media alone or acidic media containing MAPK inhibitors. Cells were then lysed in buffer containing 10 mM Tris, pH 8.0, 0.15 M NaCl, 1% Nonidet P-40, and 1 mM EDTA and assayed for COX-2 protein using the TiterZyme enzyme immunometric assay (EIA) for human Cyclooxygenase-II kit (Assay Designs, Ann Arbor, MI) per the manufacturer’s instructions.

**SYBRgreen real-time PCR for COX-2 mRNA expression.** Total cellular RNA was isolated from SEG-1 cells using TRIzol reagent (Life Technologies, Gaithersburg, MD) treated with RNase free DNase (Ambion, Houston, TX), and cDNAs were synthesized using the SuperScript II kit (Life Technologies) per the manufacturer’s instructions. Primers for COX-2 and cyclophilin, an internal control, were synthesized using the Primer Design software package (BioRad; primer sequences: 1) COX-2 sense 5′-GAA GCC TTC TCT AAC CTC TCC TAT-3′; 2) COX-2 antisense 5′-CTC ATT TGA ATC AGG AAG CTG CT-3′; 3) cyclophilin sense 5′-TGC CAT CGC CAA GGA GTA G-3′; and 4) cyclophilin antisense 5′-TGC ACA GAC GGT CAC TCA AA-3′). With the use of SYBRgreen Supermix (Bio-Rad), real-time PCR was performed using the iCycler (Bio-Rad) at the default conditions and analyzed per the manufacturer’s instructions.

Each assay included a relative standard curve, a no-template control, a no-reverse-transcribed control, and cDNA samples in triplicate. The relative standard curve was generated by serial fivefold dilutions of cDNAs obtained from SEG-1 cells treated with 50 ng/ml PMA (Sigma, St. Louis, MO) for 4.5 h to induce high levels of COX-2 expression as previously described (17, 20). The relative expression level of COX-2 was expressed as the ratio of COX-2 to cyclophilin in arbitrary units (5). All quantitative real-time PCR experiments were performed in duplicate, and the results were averaged.

**Statistical analyses.** Statistical analyses were performed using ANOVA and the Student-Newman-Keuls multiple-comparisons test.

**Fig. 3:** Effect of acid alone, with the ERK inhibitor U-0126, or with the p38 inhibitor SB-203580 on COX-2 protein levels determined using a COX-2 EIA assay (*P < 0.05 compared with control cells in neutral media).

**Fig. 4:** A: effects of acid alone on COX-2 mRNA levels determined using SYBRgreen real-time PCR. B: effects of treatment with SB-203580. C: effects of treatment with U-0126. Data are the means ± SE of duplicate wells assayed in triplicate and are representative of at least 2 separate experiments. The dotted line denotes the baseline.
with the Instat for Windows statistical software package (GraphPad Software, San Diego, CA). *P values < 0.05 were considered significant for all analyses.

RESULTS

Acid-induced COX-2 expression increased cell viability, number, and proliferation but not apoptosis. Acid exposure resulted in a significant increase in cell viability and cell number (Fig. 1). Acid exposure significantly increased the rate of proliferation but did not affect the rate of apoptosis (Fig. 2). Treatment with the COX-2 inhibitor NS-398 in neutral media significantly increased the basal rates of both proliferation and apoptosis (Fig. 2) but had no apparent effects on cell viability and cell number (Fig. 1). These results might be explained by the fact that the concomitant increase in both proliferation and apoptosis cancelled out any apparent effects of NS-398 on cell viability and number. In contrast to these effects in neutral media, NS-398 inhibited the acid-induced increases in cell viability, number, and proliferation with no significant affect on apoptosis. These findings suggest that COX-2 contributes to acid-mediated increases in SEG-1 cell survival by increasing the rate of proliferation and not by decreasing the rate of apoptosis.

Acid activation of ERK and p38 increased COX-2 protein expression. Acid treatment of SEG-1 cells caused a modest but significant increase in COX-2 protein expression compared with non-acid-treated controls (Fig. 3). To determine whether this effect is mediated by the MAPK pathways, cells were pretreated with either the ERK inhibitor U-0126 or the p38 inhibitor SB-203580. Pretreatment with U-0126 but not SB-203580 lowered the basal rate of COX-2 protein expression (Fig. 3). Pretreatment with either U-0126 or SB-203580 prevented the increase in COX-2 protein expression with acid exposure (Fig. 3). These results suggest that acid-induced activation of both ERK and p38 contribute to the increased expression of COX-2 protein in SEG-1 cells exposed to acid.

Acid activation of ERK and p38 increased COX-2 mRNA. SEG-1 cells were exposed to acidic media and expression levels of COX-2 mRNA were assessed using SYBRgreen real-time PCR at time 0 (baseline), 30, 60, 90, and 180 min. Compared with non-acid-treated cells, COX-2 mRNA expression increased 2.8-fold at 30 min, 2.9-fold at 60 min, and 1.9-fold at 90 min; by 180 min, levels had returned to baseline values (Fig. 4A). Pretreatment with both U-0126 and SB-203580 blunted the acid-induced rise in COX-2 mRNA, suggesting that this effect is mediated by the MAPK pathways (Fig. 4, B and C).

Acid activation of ERK and p38 increased COX-2 promoter activity. Transient transfections were performed using a COX-2 promoter-luciferase construct to investigate a possible mechanism whereby acid-induced MAPK activity might modulate COX-2 expression. Exposure of transfected SEG-1 cells to acidic media caused a modest but significant increase in COX-2 promoter activity (P < 0.05) that was abolished by treatment with U-0126 and SB-203580 (Fig. 5). To determine whether these effects were specific for Barrett’s cancer cells, we performed similar experiments in additional cell lines.

![Fig. 5](image_url)
including a human umbilical vein endothelial cell line (HUVEC), a squamous carcinoma cell line (Hela), and another human Barrett’s-associated esophageal adenocarcinoma cell line (FLO). We found that treatment with acidic media led to a significant increase in COX-2 promoter activity in the FLO cells (P < 0.05) but not in the HUVEC or the Hela cells (data not shown).

Acid activation of ERK increased COX-2 mRNA stability. The acid-induced elevation of COX-2 mRNA levels that we observed in SEG-1 cells (Fig. 4A) could have been caused by increased mRNA transcription, enhanced mRNA stability, or both. To explore the effects on mRNA stability, SEG-1 cells were treated with 50 μM 5′,6-dichloro-1-b-d-ribofuranosylbenzimidazole, an inhibitor of mRNA synthesis, and mRNA levels were assessed over 90 min. COX-2 message half-life was between 30 and 60 min in cells exposed to neutral pH media (Fig. 6A). In acid-exposed cells, in contrast, COX-2 message half-life was not achieved even by the 90-min time point, suggesting that acid enhanced mRNA stability. This acid-induced stabilization of COX-2 mRNA was significantly altered by treatment with U-0126 but not by SB-203580 (Fig. 6B). These data suggest that stabilization of COX-2 mRNA following acid exposure is mediated by activation of ERK but not p38.

DISCUSSION

In SEG-1 cells from a human Barrett’s-associated adenocarcinoma, we found that acid exposure caused a significant increase in cell viability and number. Acid significantly increased cell proliferation but had no significant effect on the rate of apoptosis. The proproliferative effect of acid was abolished by treatment with the selective COX-2 inhibitor NS-398, suggesting that the acid-induced increase in cell survival was mediated, at least in part, by effects of COX-2 on cell proliferation.

We next sought to determine whether acid exposure increased the expression of COX-2 and, if so, whether the increased COX-2 expression was mediated by the MAPK pathways. Indeed, we found that acid exposure caused a significant increase in COX-2 protein and that we could block that increase by treating the cells with inhibitors of the ERK and p38 MAPKs. We then used SYBRgreen real-time PCR to determine whether the increase in COX-2 protein expression was associated with an increase in COX-2 mRNA levels. We found that acid increased COX-2 mRNA levels threefold, an effect that could be blunted by the inhibition of both the ERK and p38 MAPKs. Together, these experiments suggest that acid exposure activates the ERK and p38 MAPK pathways, which results in an increase in COX-2 mRNA and protein levels, which in turn causes an increase in proliferation of Barrett’s adenocarcinoma cells.

The acid-induced, MAPK-mediated increase in COX-2 mRNA levels that we observed in our SEG-1 cells could have resulted from increased COX-2 transcription, enhanced COX-2 mRNA stability, or both. Both of these mechanisms have been reported to underlie MAPK-mediated increases in COX-2 expression stimulated by nonacidic agents. For example, human epidermal keratinocytes treated with transforming growth factor-α exhibit increased COX-2 mRNA stability that is mediated by p38 activation and increased mRNA transcription that is mediated by ERK (11). In rat intestinal epithelial cells treated with either chenodeoxycholate or ceramide, both p38 and ERK signaling increased COX-2 mRNA stability (27). We found that acid both stabilized COX-2 mRNA and increased the activity of the COX-2 promoter in SEG-1 cells. The enhanced COX-2 mRNA stabilization was evident between 30 and 60 min following acid exposure and was abolished by inhibition of ERK but not p38. In contrast, both ERK and p38 inhibition significantly decreased acid-induced activity of the COX-2 promoter. We found that COX-2 mRNA levels peaked within the first 30 min of acid exposure, whereas enhanced stability of mRNA was not apparent until after 30 min. Therefore, the early acid-induced rise in COX-2 mRNA levels is most likely due to enhanced promoter activity and gene transcription, whereas decreased mRNA degradation may contribute to the elevated mRNA levels observed from 30 to 90 min after acid exposure.

In conclusion, we have shown that acid exposure of SEG-1 cells increases the expression of COX-2 through activation of the ERK and p38 MAPK pathways. Moreover, we found that the acid-activation of ERK and p38 increases the expression of COX-2 through different mechanisms. Whereas both ERK and p38 signaling increase activity of the COX-2 promoter, only ERK stabilizes COX-2 mRNA. These findings suggest molecular pathways whereby acid exposure may contribute to carcinogenesis in Barrett’s esophagus. We speculate that the episodic acid-exposure characteristic of GERD facilitates the neoplastic progression of Barrett’s esophagus by increasing COX-2 expression through activation of the MAPK pathways. Furthermore, our data support a potential chemopreventive role for aggressive antisecretory therapy in patients with Barrett’s esophagus to minimize esophageal acid exposure and the resultant increase in COX-2.

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REFERENCES


