Acid increases MAPK-mediated proliferation in Barrett’s esophageal adenocarcinoma cells via intracellular acidification through a Cl⁻/HCO₃⁻ exchanger

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Sarosi, George A., Jr., Kshama Jaiswal, Emily Herndon, Christie Lopez-Guzman, Stuart J. Spechler, and Rhonda F. Souza. Acid increases MAPK-mediated proliferation in Barrett’s esophageal adenocarcinoma cells via intracellular acidification through a Cl⁻/HCO₃⁻ exchanger. Am J Physiol Gastrointest Liver Physiol 289: G991–G997, 2005. First published August 4, 2005; doi:10.1152/ajpgi.00215.2005.—Abundant epidemiological evidence links acid reflux to adenocarcinoma in Barrett’s esophagus, but few studies have examined the cellular mechanisms by which acid promotes this neoplastic progression. We hypothesized that extracellular acid exposure causes intracellular acidification that triggers MAPK signaling and proliferation in Barrett’s epithelial cells. We tested that hypothesis in a Barrett’s-derived esophageal adenocarcinoma cell line (SEG-1). SEG-1 cells were exposed to varying concentrations of acid, and intracellular pH (pHi) was measured by 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein microfluorimetry. After acid exposure, ERK and p38 MAPK activation were measured by Western blot analysis and an immune complex kinase assay. Proliferation was measured by Coulter counter cell counts and a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide incorporation assay. Exposure of SEG-1 cells to solutions with a pH between 3 and 6.5 caused a rapid, reversible decrease in pHi, to a level approximately equal to extracellular pH. Acid exposure caused a rapid activation of both ERK and p38 MAPKs and also resulted in pH-dependent increases in cell number, with a maximum increase of 41% observed at pH 6.0. The MAPK activation and proliferation in SEG-1 cells induced by acid exposure could be blocked by pretreatment with disodium 4,4'-dithiobis(2-nitrophenyl)disulfonate (DIDS), which prevents intracellular acidification by inhibiting the Cl⁻/HCO₃⁻ exchanger. In conclusion, in SEG-1 cells, extracellular acid exposure causes intracellular acidification, which activates MAPK and causes proliferation. The magnitude of these effects is pH dependent, and the effects can be inhibited by preventing intracellular acidification with DIDS.

Barrett’s esophagus; mitogen-activated protein kinase; pH; proliferation

THE INCIDENCE OF ESOPHAGEAL ADENOCARCINOMA has increased by more than 600% over the last three decades in the United States. Adenocarcinoma is now the most common histological type of esophageal cancer, with a 5-yr survival rate of only 10% (8). Esophageal adenocarcinomas are believed to arise from Barrett’s esophagus, the condition wherein a metaplastic, intestinal-type epithelium replaces the squamous epithelium that normally lines the distal esophagus. Although there is strong epidemiological evidence linking gastroesophageal reflux disease to Barrett’s esophagus and esophageal adenocarcinoma, there is a paucity of data on the cellular mechanisms by which acid promotes neoplastic progression in the esophagus.

In a variety of cell types, acid exposure activates MAPK signaling, which can induce proliferation (21). Our group (15) has shown that acid activates p38 and ERK MAPKs in Barrett’s adenocarcinoma cells and that this acid-induced MAPK activation mediates cycle progression and suppression of apoptosis. Transient acid exposure also has been shown to cause proliferation in ex vivo cultures of Barrett’s metaplasia (3, 5). However, the molecular events linking acid exposure, MAPK activation, and cellular proliferation in Barrett’s esophagus remain unclear.

Cultured cells exposed to acid exhibit decreases in intracellular pH (pHi), and decreases in pHi have been shown to be associated with mitogenic signaling in both lymphocytes and epithelial cells (6). Changes in pHi have also been linked to apoptotic signaling (19). On the basis of these observations, we hypothesized that transient acid exposure decreases pHi in the epithelial cells of Barrett’s esophagus, thereby triggering MAPK signaling, which results in proliferation. We tested this hypothesis in a cell line derived from an adenocarcinoma in Barrett’s esophagus (SEG-1).

We found that 1) different doses of acid exposure (pH) result in different magnitude proliferative responses, 2) different magnitudes of acid exposure produce different degrees of pHi changes, and 3) inhibition of intracellular acidification via a Cl⁻/HCO₃⁻ exchanger (AE) inhibits acid-induced MAPK activation and proliferation in the Barrett’s-derived adenocarcinoma cell line SEG-1.

MATERIALS AND METHODS

Cell culture. SEG-1, a Barrett’s-derived esophageal adenocarcinoma cell line, was obtained from Dr. David Beer (University of Michigan, Ann Arbor, MI) (14). SEG-1 cells were cultured in DMEM
(GIBCO-BRL; Gaithersburg, MD) supplemented with 10% fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 mg/ml), and amphotericin B (12.5 mg/ml) (from GIBCO-BRL). Cells were maintained in culture at 37°C in a humidified incubator with air and 5% carbon dioxide. We chose SEG-1 cells because they have been shown to increase proliferation and to activate ERK and p38 MAPKs in response to acid exposure (pH 4.0) (15). For individual experiments, cells were cultured in either serum-free media at neutral pH or serum-free media adjusted to pH levels between 3.0 and 6.5 using 1 M HCl.

**pH measurements.** SEG-1 cells were subcultured onto 22-mm round glass coverslips coated with rat tail collagen (Roche Applied Science; Indianapolis, IN). Cells were loaded with the fluorescent dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF)-AM (Molecular Probes; Eugene, OR) at a concentration of 2.5 µM for 20 min at room temperature. After a 10-min dye equilibration period, the coverslips were mounted in a custom Lucite superfusion chamber attached to an eight-well buffer reservoir. The superfusion chamber was then mounted on the stage of a Photon Technologies QL 103 cooled charge-coupled device imaging system, and cells were superfused with a modified high-K⁺ Ringer-HEPES (KRH) buffer containing (in mM) 118 NaCl, 4.7 KCl, 1.8 CaCl₂, 10 HEPES, 11 glucose, 0.9 NaH₂PO₄, and 0.8 MgSO₄ at a defined pH. Single cell pHᵢ was calculated from the ratios of BCECF fluorescence intensities at 440- and 490-nm wavelengths with an emission wavelength of 530 nm. pHᵢ was calibrated using an in situ calibration curve in SEG-1 cells using the high-K⁺ Ringer solution/nigerinic technique at standard pHs of 6.0, 6.5, 7.0, 7.5, and 8.0 (10).

**Inhibitors and treatments.** Disodium 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS), 5-(N-ethyl-N-isopropyl)amiloride (EIPA), and amiloride were obtained from Sigma (St. Louis, MO). DIDS was dissolved in 0.1 M KHCO₃ as a 100X stock solution, and cells were pretreated for 5 min before acid exposure for pHᵢ experiments and 15 min before acid exposure for cell counts and Western blot experiments. EIPA and amiloride were dissolved in DMSO to make 100X stock solutions, and cells were pretreated for 5 min before acid exposures.

**MAPK activity and phosphorylation assays.** Equally seeded SEG-1 cells were cultured in serum-free media for 24 h before acid exposure. After acid exposure at different pHs, cells were lysed in cold lysis buffer (Cell Signaling Technology; Beverly, MA), and protein was harvested at the appropriate time points. To assess the time course of acid-induced MAPK activation, cells were treated with acidic media for 3 min; the acidic media was then removed and replaced with neutral serum-free media for the remainder of the experiment. Cell number was determined 24 h after acid exposure by harvesting and counting the cells with a Coulter Z-1 particle counter. For proliferation, SEG-1 cells were equally seeded into a 96-well plate (10,000 cells/well) and cultured in serum-free media at pH 7.4 for 24 h. The media were removed, and cells were exposed to acidic media at differing pHs for 3 min. The acidic media were then removed and replaced with neutral serum-free media. Cell proliferation was assessed 24 h after the acid exposure using Cell Proliferation Kit I (Roche; Indianapolis, IN) per the manufacturer’s instructions. The assay is based on the ability of proliferating cells to cleave 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a formazan dye, which is then detected using a Multiscan EX (Lab Systems) at a wavelength of 560 nm.

**Statistical analysis.** All experiments were performed in at least triplicate. Statistical significance was assessed by ANOVA with the Tukey’s post hoc comparison using the Prism 3.0 software package (Graphpad Software; San Diego, CA).

**RESULTS**

**Extracellular acid exposure produces pH-dependent intracellular acidification.** The resting pHᵢ of BCECF-loaded SEG-1 cells in modified Krebs-Ringer buffer at pH 7.4 was 7.32 ± 0.1. Exposure to KRH buffer acidified with 1 M HCl to a pH of 4.0 for 3 min resulted in a rapid drop in pHᵢ, followed by a rapid return to baseline pH after removal of the acidified buffer (Fig. 1A). Similar results were observed at all pH levels tested. The cells demonstrated a rapid drop in pHᵢ with acid exposure to a level roughly equal to extracellular pH (pHₑ; Fig. 1, B and C). With removal of the acid, the pHᵢ returned to the preexposure baseline level.

**Acid exposure produces pH-dependent increases in cell number.** To examine the effects of pH on cell number, SEG-1 cells were exposed to serum-free media at a pH of 4, 5, or 6 for 3 min and then returned to neutral pH serum-free media for 24 h. At this time, cells were harvested and counted. Compared with control, acid exposure at all three pH levels significantly increased cell numbers at 24 h (Fig. 2). Interestingly, acid exposure at pH 6.0 caused a significantly greater increase than acid exposure at pH 4.0 (Fig. 2). To further characterize the effects of pH on proliferation, we used the MTT assay to serve as a loading control. In addition to the activity assays, MAPK activity was also assessed by Western blot analysis for phosphorylated p38 and ERK with total p38 and ERK immunoblotting used as loading controls. Equal volumes of whole cell lysate were separated by SDS-PAGE and transferred overnight to nitrocellulose membranes.
examine a wide range of acid doses. We found pH-dependent increases in cell proliferation over the examined pH range compared with control cells (Fig. 3). The largest increase in cell proliferation occurred with acidic media at pH 6.0; this result was significantly greater than the increase observed at pH 4.0.

Acid exposure produces increases in MAPK activation. Prior work has suggested that brief acid exposure can activate both the ERK and p38 MAPK signaling pathways in several cell types (21). However, a careful examination of the effects of acid dose on MAPK activation in Barrett’s adenocarcinoma had not been done. SEG-1 cells were exposed to pH-adjusted media at either pH 4 or 6 for 3 min, and cell lysates collected immediately (time 0) and at various time points after the completion of the acid exposure. ERK and p38 MAPK activation was assayed by both MAPK immune complex kinase assay and phospho-MAPK and total MAPK Western blot analysis. After exposure to both pH 4 and 6, ERK and p38 activities increased immediately and remained elevated at 3 min after acid exposure. Both ERK and p38 activity returned to baseline by 15 min after the acid exposure with both doses of acid exposure (Fig. 4, A and B).

DIDS, an inhibitor of Cl⁻/HCO₃⁻ exchange, blocks intracellular acidification. A variety of molecular mechanisms exist to transport protons into and out of cells to allow cells to carefully regulate their pH. The three best-studied mechanisms include the Na⁺/H⁺ exchangers (NHE), AE, and Na⁺-dependent HCO₃⁻ cotransporters (NBC). The diuretic amiloride and its analogs strongly inhibit NHE family members, and the compound DIDS is a well-characterized inhibitor of both AE and NBC. We sought to determine which ion transport mechanism was responsible for intracellular acidification in the SEG-1 cell line. We first investigated the role of NHE in intracellular acidification by examining the effects of amiloride and the more potent analog EIPA on acidification in SEG-1. High doses of both amiloride and EIPA had no effect on intracellular acidification across the pH range of 4–6 (data not shown). In contrast, DIDS, at doses ranging from 100 to 500 μM, strongly inhibited intracellular acidification (Fig. 5, A and B). Further-
more, we found that removal of Na\(^+\) from the buffer did not affect intracellular acidification in the presence of extracellular acid, indicating that AE rather than NBC was mediating the intracellular acidification (data not shown).

**DIDS inhibits acid-induced MAPK activation.** On the basis of the prior observation that acid exposure causes intracellular acidification and MAPK activation, and that DIDS inhibits intracellular acidification, we next examined the ability of DIDS to inhibit acid-induced MAPK activation. Because of the observation that 500 μM DIDS blocked most of the pH\(_i\) drop induced by pH 6.0 acid exposure, SEG-1 cells were exposed to pH 6 for 3 min in the presence and absence of 500 μM DIDS, and MAPK activation was assessed by MAPK activity assays. Rapid activation of p38 and ERK was observed immediately after acid exposure (time 0) and persisted for at least 3 min after acid exposure. DIDS pretreatment significantly (P < 0.05) inhibited p38 (Fig. 6) and ERK activation after acid exposure (Fig. 7). Similar results were observed when MAPK activities were measured by Western blot analysis for phosphorylated and total MAPKs (data not shown).

**DIDS inhibits acid-induced cell number increases.** SEG-1 cells were exposed to acid at pH 6.0 in the presence or absence of 500 μM DIDS, and proliferation was determined by MTT incorporation. Acid exposure at pH 6.0 produced a significant increase in cell proliferation that was completely blocked by pretreatment of the cells with 500 μM DIDS (Fig. 8). In contrast, DIDS treatment had no effect on serum-induced cell proliferation, demonstrating that this effect did not result from a nonspecific inhibition of proliferation.

**DISCUSSION**

In the Barrett’s-derived adenocarcinoma cell line SEG-1, we have demonstrated that extracellular acid exposure produces significant increases in cell number and that the magnitude of this increase depends on the strength of the acid exposure. We have also shown that acid exposure produces intracellular acidification, and the degree of pH\(_i\) change also depends on the strength of acid exposure. We found that acid entry occurs by a DIDS-inhibitable pathway in a Na\(^+\)-free buffer suggesting...
Fig. 6. Effect of DIDS pretreatment on acid-induced p38 activation in SEG-1 cells. Top: p38 activity is measured by immune complex kinase assay with the exogenous substrate ATF. Western blot analysis for total p38 on the same lysates confirms equivalent amounts of p38 in each sample. Time 0 is defined as the time point immediately after a 3-min acid exposure, whereas the 3- and 15-min lysates were collected 3 or 15 min after the acid exposure. Bottom: histogram showing the average of 3 repetitions of the experiment expressed as p38 activity fold change over media control (Fold Cont). Acid treatment of SEG-1 produces a marked increase in p38 activity. Pretreatment of SEG-1 with 500 μM DIDS causes a significant inhibition of acid-induced p38 activation as measured by p38 activity. *P < 0.05 vs. time 0 without DIDS.

Fig. 7. Effect of DIDS pretreatment on acid-induced ERK activation in SEG-1 cells. Top: ERK activity is measured by immune complex kinase assay with the exogenous substrate ELK. Western blot analysis for total ERK on the same lysates confirms equivalent amounts of ERK in each sample. Time 0 is defined as the time point immediately after a 3-min acid exposure, whereas the 3- and 15-min lysates were collected 3 or 15 min after the acid exposure. Bottom: histogram showing means ± SE of 3 repetitions of the experiment expressed as ERK activity fold change over media control. Again, DIDS pretreatment causes a significant reduction in acid-induced ERK activation. *P < 0.05 vs. the 3-min time point without DIDS; #P < 0.05 vs. the 15-min time point without DIDS.
that an AE is responsible for intracellular acidification. The drop in pH_i with acid exposure is accompanied by activation of ERK and p38 MAPKs, and inhibition of intracellular acidification with DIDS blocks MAPK activation. Finally, inhibition of intracellular acidification with DIDS also inhibits acid-induced cell number increases.

Prior investigators have shown that transient acid exposure results in proliferation in both ex vivo cultures of Barrett’s biopsies (3) and Barrett’s-derived adenocarcinoma cell lines (15). Similar results have been observed in other epithelial cell-derived cell lines, including colon cancer cell lines (4), and rabbit esophageal epithelial cells (9). Most of the prior investigators have picked a single strong acid exposure, usually pH 3.5–4, to study the effects of acid on cell proliferation on the basis of the clinical parameters used to define acid reflux in clinical 24-h pH monitoring. However, a systematic evaluation of the effects of pH on cell number increases in Barrett’s adenocarcinoma has not been performed. The effects of lower doses of acid exposure on Barrett’s epithelium is particularly relevant in an era when most patients with Barrett’s esophagus and esophageal adenocarcinoma are treated with proton pump inhibitors. Although these drugs effectively suppress acid secretion, direct measurement of the pHi of reflux is rarely performed on therapy, and it has been shown that symptom control successfully predicts the elimination of acid reflux in patients with Barrett’s esophagus only 50–60% of the time (7, 11). Our findings of an inverted U-shaped curve of acid dose on proliferation centered around a pH of 6 suggests that two competing effects may be at work. The proliferative effects induced by exposure to strong acid may be countered by the acid’s direct toxic effects. At intermediate pH levels, the proliferative effect predominates. Our observation of an optimal pH for acid-evoked proliferation centered around pH 6.5–6.8 is in agreement with the observations of Jimenez et al. (9) in rabbit esophageal squamous cells.

Although the hypothesis that extracellular acid exposure causes a drop in pH_i may seem obvious, prior work has suggested that the pH_i response to extracellular acid varies considerably by cell type (1, 2, 17, 20). For example, the magnitude of pH_i changes per unit change in pH_o change ranges from 0.25 units in rat esophageal squamous cells to 0.75 units in rat vascular smooth muscle cells. Our findings of a rapid change in pH_i to approximately pH_o show that Barrett’s-derived adenocarcinoma SEG-1 cells resemble vascular smooth muscle cells more than other gastrointestinal epithelial cells in their response to acid exposure. There are at least two possible explanations for our finding that pH_i approaches pH_o in SEG-1 cells. One explanation is that Barrett’s-associated cancer epithelial cells are intrinsically more sensitive to pH changes than native squamous or columnar epithelia. Alternatively, our observation may be a consequence of studying pH_i in dispersed cell culture rather than in an intact epithelium. However, no prior studies appear to have been performed in cells derived from Barrett’s metaplastic epithelium, and it is technically very difficult to collect intact sheets of Barrett’s metaplasia for study. For these reasons, it may be difficult to determine which explanation is correct.

Multiple pH_i regulatory mechanisms exist in eukaryotic cells, but the three most commonly studied include NHE, AE, and NBC. Our observation that intracellular acidification is inhibited by DIDS in a Na^+-independent fashion suggests that the transporter responsible for intracellular acidification is a member of the AE family (12). Although not previously described in cells derived from Barrett’s metaplasia, this acid entry mechanism has been shown to mediate intracellular acidification in rabbit esophageal squamous epithelial cells (17).

Prior work has suggested that extracellular acid exposure can activate MAPK signaling in several cell types including an epidermoid carcinoma, murine fibroblasts, and SEG-1 cells (15, 21). However, those investigations did not examine the precise mechanism by which cellular acid exposure induces MAPK activation. Conceivably, acid could activate signaling either by exerting an effect on the cell membrane or by entering the cell cytosol and activating intracellular signaling cascades. Our findings that inhibition of intracellular acidification by DIDS blocks both proliferation and activation of ERK and p38 MAPKs strongly suggests that acid-induced reductions in pH_i mediate MAPK activation and proliferation. This suggests that acid exposure may trigger proliferation in Barrett’s esophagus by causing a decrease in pH_i. Changes in pH_i have been linked to MAPK activation by other investigators, although the majority of reports show MAPK activation as a consequence of intracellular alkalization (13). However, a few reports (16, 22) suggest that both ERK and p38 activation can occur as a result of intracellular acidification in plant and tumor cells.

In summary, we report that acid exposure causes intracellular acidification, activation of ERK and p38 MAPKs, and proliferation in a Barrett’s-derived esophageal adenocarcinoma cell line. We also demonstrated that blocking intracellular acidification with DIDS inhibits acid-induced MAPK activation and proliferation, strongly suggesting that the proliferative effects of acid are mediated by intracellular effects via an AE rather than by extracellular effects on the cell membrane. Our findings suggest a mechanism by which acid reflux in Barrett’s esophagus can promote tumor formation by stimulating proliferation. Our finding that maximal stimulation of proliferation occurs with mild rather than strong acid exposure suggests that incomplete acid suppression may be worse that no acid suppression. Conceivably, this could explain why the widespread use of acid-suppressive medications has not resulted in a decrease in the incidence of esophageal adenocarcinoma.

**Fig. 8.** Cell proliferation as determined by MTT incorporation 24 h after a 3-min exposure to acidic media at pH 6 in the absence and presence of DIDS. Acid exposure produces a statistically significant increase in proliferation that is completely inhibited by preincubation of the cells with 0.5 mM DIDS. Of note, DIDS does not produce a baseline inhibition of cell growth and has no effect on serum-induced cell proliferation.
REFERENCES