Mechanisms of oxidant production in esophageal squamous cell and Barrett’s cell lines

Linda A. Feagins,¹ Hui Ying Zhang,¹ Xi Zhang,¹ Kathy Horni-Carver,¹ Tojo Thomas,¹ Lance S. Terada,¹ Stuart J. Spechler,¹ and Rhonda F. Souza¹²
¹Department of Medicine, Dallas Veterans Affairs Medical Center and the University of Texas Southwestern Medical School, and ²The Harold C. Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas

IN MOST INDIVIDUALS WITH GASTROESOPHAGEAL REFLUX DISEASE (GERD), reflux-damaged esophageal squamous epithelium heals through the process of regeneration in which new squamous cells replace the injured ones. In some individuals, however, the damaged squamous epithelium heals through a metaplastic process in which intestinal-type columnar cells replace injured squamous cells. This condition, called Barrett’s esophagus, predisposes to esophageal adenocarcinoma, a tumor whose incidence has increased more than sixfold in the past few decades (28, 36).

It is not clear why only a minority of individuals with GERD develop Barrett’s metaplasia. However, recent data suggest that differences in the molecular mechanisms triggered when esophageal squamous cells are exposed to refluxed gastric juice might determine whether reflux esophagitis heals by regeneration or by metaplasia. For example, acid perfusion of the esophagus has been shown to activate ERK1/2, a proliferative member of the mitogen-activated protein kinase (MAPK) pathway, in the esophageal squamous epithelium of patients who have GERD without Barrett’s esophagus, but not in patients with Barrett’s esophagus (35). Acid exposure also can induce esophageal squamous cells to express Dickkopf genes, which regulate proliferation and apoptosis, and significant differences in the expression levels of Dickkopf genes have been found in the esophageal squamous epithelium of GERD patients with and without Barrett’s esophagus (1). It is conceivable that such differences in reflux-induced expression of proliferation factors by esophageal squamous cells contribute to the pathogenesis of Barrett’s metaplasia.

Reactive oxygen-derived species (ROS) [e.g., superoxide anion (O2⁻), hydrogen peroxide (H2O2), hydroxyl radical (OH), peroxynitrite (ONOO⁻)] produced by inflammatory and epithelial cells have been implicated in the development of reflux esophagitis, Barrett’s esophagus, and adenocarcinoma (9, 16, 38). Evidence of oxidative injury of the esophageal epithelium, in the form of DNA adducts and reduced levels of antioxidant enzymes, may persist even after reflux esophagitis has been treated successfully with antireflux surgery (25, 30). Within epithelial cells, the oxidants are produced through the mitochondrial electron transport chain and through enzymatic generators including NADPH oxidase and nitric oxide synthase [NOS (13)]. Although ROS are highly reactive molecules that can damage cellular lipids, proteins, and DNA, ROS also can function as “second messengers” that activate redox-sensitive signal transduction pathways involved in regulating epithelial cell proliferation, differentiation, and apoptosis (3, 4, 13). The specific signal transduction pathways that are activated depend on which intracellular generator produces the ROS and on what types of ROS are produced (13, 22). Thus it is conceivable that differences in the mechanisms of reflux-induced oxidant production by esophageal squamous epithelial cells might affect their ability to regenerate and might contribute to the pathogenesis of Barrett’s esophagus.

In cultures of esophageal squamous epithelial cells, benign Barrett’s epithelial cells, and Barrett’s cancer cells, a number of studies have shown that acid and bile salts increase the

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production of ROS (8, 9, 16, 20, 33). However, none of those studies determined the intracellular mechanisms that produced the ROS. We hypothesized that there might be differences in the mechanisms of reflux-induced oxidant production by esophageal squamous epithelial cells in GERD patients with and without Barrett’s esophagus. To explore that hypothesis, we used inhibitors of the enzymatic generators of ROS to study the effects of various components of reflux (acid, bile acids, or both) on ROS production in telomerase-immortalized normal esophageal squamous cell lines derived from GERD patients with and without Barrett’s esophagus. We also studied those effects in a telomerase-immortalized nonneoplastic Barrett’s epithelial cell line (BAR-T) in Barrett’s-associated adenocarcinoma cell lines and in a number of nongastric cell lines.

MATERIALS AND METHODS

Cell culture. We used two telomerase-immortalized, normal esophageal squamous cell lines created from endoscopic biopsy specimens of normal esophageal squamous mucosa obtained from the distal esophagus of patients who had GERD with (NES-B3T) and without (NES-G2T) Barrett’s esophagus; one telomerase-immortalized, nonneoplastic, Barrett’s epithelial (BAR-T) cell line; and two Barrett’s-associated esophageal adenocarcinoma cell lines [SEG-1 and FLO-1 (generous gift from Dr. David Beer, University of Michigan, Ann Arbor, MI)] (19, 32). The cell lines were cultured in their respective growth media as previously described (19, 23, 32). All cells were maintained in monolayer culture at 37°C in humidified air with 5% CO_2. The telomerase-immortalized NES and Barrett’s cell lines were cocultured with a fibroblast feeder layer as previously described (29).

Acid and/or bile acid exposure. For individual experiments, the cells were cultured either in neutral full growth medium (pH 7.2) (neutral medium), acidic full growth medium brought to pH values ranging from 3.0–6.0 with 1 M HCl (acidic medium), neutral full growth medium containing the conjugated bile acid glycochenodeoxycholic acid (GCDA) (neutral bile salt medium) or acid full growth medium, containing GCDA and brought to pH values ranging from 3.0 to 6.0 with 1 M HCl (acidic bile salt medium). Neutral medium alone or one of the experimental media was added for 3 min to equally seeded wells of cells, then removed and replaced with neutral pH medium alone for the remainder of the experiment. The 3-min duration of the exposure was chosen to simulate a typical, physiological episode of gastroesophageal reflux (31). The desired pH of each of the media was monitored and found to be stable throughout each experiment. GCDA (one of the most prevalent bile salts found in esophageal aspirates from GERD patients, especially in those who do not take acid-suppressive medications) was used at a concentration of 200 μM, a physiologically relevant concentration, as previously described (18, 21, 24).

Detection of intracellular ROS and ROS generators. Equally seeded wells of cells were washed twice with Hanks’ buffered saline solution (HBSS; Invitrogen, Carlsbad, CA), incubated with 5 μM of a 2’,7’-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes, Carlsbad, CA) probe for 20 min at 37°C, and washed again with HBSS to remove any excess probe. Cells were exposed to neutral, acidic, neutral bile salt, or acidic bile salt medium as described above. After 20 min, cells were transferred to HBSS and fluorescent intensity was immediately detected with a fluorescent plate reader (Labsystems Fluoroskan, Ascent, FL). To determine the specific intracellular source of the ROS production, equally seeded wells of cells were incubated in the presence of inhibitors to NADPH oxidase [10 μM diphenyleneiodonium chloride (DPI) for 60 min], NOS [1 mM N^6^-methyl-L-arginine acetate salt (L-NMMA) for 30 min], and the mitochondrial electron transport chain (1 μM rotenone for 60 min) prior to incubation with H_2DCF-DA. To detect the production of nitric oxide (NO), cells were incubated with 10 μM of a 4-amino-5-methylamino-2’,7’-difluorofluorescein (DAF-FM, Molecular Probes) probe, a sensitive indicator of NO, for 30 min at 37°C, followed by washing with HBSS to remove excess probe prior to experimental exposures. As a positive control for ROS production, cells were treated with 200 μM hydrogen peroxide.

Statistical analyses. Quantitative data are expressed as means ± SE. Statistical analyses were performed by either the unpaired Student’s t-test or an ANOVA in combination with the Student-Newman-Keuls multiple-comparison test with the Instat for Windows statistical software package (GraphPad Software, San Diego, CA). Data from the experimental group were expressed as a percentage of the nontreated control cells. These results were then averaged prior to statistical analyses. P values ≤0.05 were considered significant for all analyses.

RESULTS

Acidic medium and acidic bile salt medium increase ROS production by BAR-T cells. BAR-T cells were incubated with H_2DCF-DA followed by treatment with a single, 3-min exposure to neutral medium, acidic medium, neutral bile salt medium, or acidic bile salt medium. Acidic medium induced a pH dose-dependent increase in ROS production, with significant increases in ROS observed following exposure to acidic medium at pH 3.0 and 4.0 (Fig. 1A). To ensure that the
H₂DCF-DA probe was not being activated by the low pH rather than by the production of ROS, we incubated the probe alone (in the absence of any cells) with the media at the various pHs and found no increase in probe fluorescence (data not shown). Acidic bile salt medium at pH 5.0, 4.0, and 3.0 also significantly increased ROS production; acidic bile salt medium at a pH of 4.0 produced the highest levels of ROS (Fig. 1B). After reviewing these data, we chose to use acidic medium alone or acidic bile salt medium at a pH of 4.0 for all further experiments.

To determine the relative potencies of acid and acidic bile acids for inducing ROS production in Barrett’s cells, BAR-T cells were incubated with H₂DCF-DA and then treated with a single, 3-min exposure to neutral medium, acidic medium, acidic bile salt medium (pH 4), or neutral bile salt medium. In agreement with our previous observations, we found that both acidic medium and acidic bile salt medium, but not neutral bile salt medium, significantly increased ROS production (Fig. 2). Moreover, acidic bile salt medium induced a significantly greater increase in ROS than acidic medium alone (Fig. 2).

**Mechanisms of ROS generation in BAR-T cells differ between acidic medium and acidic bile salt medium.** Having found that acidic medium and acidic bile salt medium increase ROS in BAR-T cells, we sought to determine the underlying mechanisms by pretreating the cells with DPI (NADPH oxidase inhibitor), rotenone (mitochondrial electron transport chain inhibitor), and L-NMMA (NOS inhibitor). We found that pretreatment with L-NMMA, but not with DPI or rotenone, blocked the acid-induced increase in ROS production (Fig. 2A). To demonstrate that NO was being generated by acid exposure, we incubated the BAR-T cells with DAF-FM, an NO-sensitive probe, and found a significant increase in probe fluorescence following acid exposure (Fig. 3B). These observations show that acid exposure induces ROS production through the NOS generator in BAR-T cells. In contrast, we found that the increase in ROS production induced by acidic bile salt medium was blocked by pretreatment with DPI, but not by rotenone or L-NMMA (Fig. 4). These observations show that exposure to acidic bile salt medium induces ROS production through the NADPH oxidase generator in BAR-T cells.

In response to acidic bile salt medium, ROS are produced through different pathways in esophageal squamous cells from GERD patients with and without Barrett’s esophagus. We next determined the effects of the various media on ROS production in telomerase-immortalized normal esophageal squamous epithelial cell lines that were derived from patients who had GERD with Barrett’s esophagus (NES-B3T) and GERD without Barrett’s esophagus (NES-G2T). In both squamous cell lines, we found that ROS production increased significantly after exposure to acidic bile salt medium, but not after exposure to acidic medium or neutral bile salt medium (Fig. 5). We found the same effects in another human, telomerase-immortalized, normal esophageal squamous cell line derived from the proximal esophagus of a patient with Barrett’s metaplasia [EPC-2 hTERT (generous gift of Dr. Anil Rustgi, University of Pennsylvania, Philadelphia, PA)] [data not shown (15)].

We found that pretreatment with DPI and L-NMMA, but not rotenone, prevented the increase in ROS production induced by acidic bile salt medium in NES-G2T cells (Fig. 6A). Using the NO sensitive probe DAF-FM, we confirmed that acidic bile
salt medium causes a significant increase in NO generation by NES-G2T cells (Fig. 6B). In NES-B3T cells, in contrast, pretreatment with DPI, but not with L-NMMA or rotenone, blocked the increase in ROS production after the combination of acid and bile salts. Graphs show means ± SE for at least 3 individual determinations in each group. ***P < 0.001 compared with control.

Neither acidic medium nor acidic bile salt medium induce ROS production in Barrett’s-associated adenocarcinoma cells. In two Barrett’s-associated adenocarcinoma cell lines (SEG-1 and FLO-1), we detected no increase in ROS production after exposure to acidic medium and acidic bile salt medium (data not shown). However, both cancer cell lines demonstrated a significant increase in ROS production following treatment with H2O2 (data not shown). These data show that malignant Barrett’s cells respond differently to acid and bile salts than benign Barrett’s epithelial cells.

Some nonsquamous cells can produce ROS after exposure to acidic medium. To determine whether ROS production in response to acid and bile salts is a unique property of esophageal cells, we performed similar experiments in nonsquamous cell lines including a human umbilical vein endothelial cell line (HUVEC), a normal rat intestinal epithelial cell line (IEC-6), and two human colorectal cancer cell lines (Caco2 and SW48). We found that treatment with acidic and acidic bile salt media caused no significant increase in ROS production in HUVEC, IEC-6, or Caco2 cells (data not shown). However, acidic medium, but not acidic bile salt medium, significantly increased ROS production in SW48 cells (data not shown).

DISCUSSION

A number of animal models for GERD have linked the generation of ROS with the development of reflux esophagitis, Barrett’s esophagus, and esophageal adenocarcinoma (5, 6, 10, 11, 14, 27). In normal human bronchial epithelial cells, oxidative stress has been shown to induce the expression of the MUC5AC and acetylated tubulin, proteins that characterize the intestinal metaplasia of Barrett’s esophagus (2). In Barrett’s metaplasia, high levels of ROS are associated with signs of oxidative injuries such as lipid peroxidation and oxidative DNA damage (8, 26, 38). These observations suggest that ROS produced in the esophagus in response to gastroesophageal reflux play a role in the pathogenesis of esophagitis and Barrett’s metaplasia and in the progression from metaplasia to carcinoma.

It is not known why only a minority of individuals with GERD develop Barrett’s metaplasia, but differences in the molecular mechanisms triggered when esophageal squamous cells are exposed to refluxed gastric juice might determine whether reflux esophagitis heals by regeneration or by metaplasia. A number of investigators have documented differences between GERD patients with and without Barrett’s esophagus in the response of their esophageal squamous epithelium to acid exposure. For example, significant differences have been found between such patients in the acid-induced expression of MAPK and Dickkopf genes, which are known to mediate cellular proliferation (1, 35). We hypothesized that there might
be differences in the mechanisms of reflux-induced oxidant production by esophageal squamous epithelial cells in GERD patients with and without Barrett’s esophagus, and we explored that hypothesis using inhibitors of the enzymatic generators of ROS to study the effects of various components of reflux on ROS production in benign and malignant esophageal cell lines.

We found that the combination of acid with a conjugated bile salt induced ROS production in all of our nonneoplastic, telomerase-immortalized esophageal squamous cell lines. As hypothesized, however, we found differences in the mechanisms of oxidant production between the squamous cells derived from patients with and without Barrett’s esophagus. In the NES-B3T cell line (from a GERD patient with Barrett’s esophagus), ROS was produced by NADPH oxidase but not by NOS, whereas both NADPH oxidase and NOS contributed to ROS production by the NES-G2T cell line (from a GERD patient without Barrett’s esophagus).

ROS are known to activate signal transduction pathways involved in both cellular proliferation and differentiation. In human bronchial epithelial cells, for example, ROS activate the epidermal growth factor receptor signaling cascade, and ROS have been shown to increase proliferation in ras-transformed fibroblasts and in NIH3T3 cells (2, 17, 37). Moreover, which signal transduction pathways are activated by the ROS depends in part on the intracellular generators involved in their production (13, 22). In neonatal myocardial cells, for example, ROS generated by the NADPH oxidase system decrease cell viability, whereas oxidants released from the mitochondrial system have no apparent viability effects (22). Therefore, ROS produced by one intracellular generator can trigger different pathways than ROS produced by another generator, and those differences can result in disparate effects on proliferation, differentiation, and apoptosis (13). Our finding that acidic bile acids cause ROS production by different generators in esophageal squamous cells from GERD patients with and without Barrett’s esophagus suggests the possibility that reflux-induced ROS production may have different effects on esophageal proliferation and differentiation pathways in different individuals. We speculate that, perhaps, some of those differences might impair the ability of the squamous cells to regenerate in response to reflux injury, and such impairment might contribute to epithelial repair through metaplasia (i.e., Barrett’s esophagus). However, further studies are needed to elucidate the biological implications of our findings.

In benign Barrett’s epithelial (BAR-T) cells, we have found that acid alone and acid in combination with a conjugated bile salt both can induce the production of ROS, but through different mechanisms. Pretreatment with the NOS inhibitor L-NMMA prevented ROS production after acid exposure alone, suggesting that NO is the reactive species induced by acid. We confirmed this event using an NO-sensitive probe. In contrast, exposure of BAR-T cells to a combination of acid and a conjugated bile salt caused ROS production that could be blocked by pretreatment with DPI, indicating the involvement of the NADPH oxidase system. In Barrett’s-associated esophageal adenocarcinoma cells, however, neither acid alone nor acid in combination with a conjugated bile salt induced ROS production. These findings in Barrett’s cancer cell lines have been confirmed by other investigators, although unconjugated bile salts and acid exposures in excess of 10 min have been

Fig. 6. Acidic bile acid medium induces ROS production via NADPH oxidase and NOS in esophageal squamous cells derived from a GERD patient without Barrett’s esophagus. A: NES-G2T cells were pretreated with 10 μM DPI, 1 μM rotenone, or 1 mM L-NMMA prior to exposure to acidified bile salts. DPI and L-NMMA, but not rotenone, blocked the increase in ROS production. B: acidic bile acid medium significantly increased NO production as detected by DAF-FM, an NO-sensitive probe. Graphs show means ± SE for at least 4 individual determinations in each group. ***P < 0.001 compared with control.

Fig. 7. Acidic bile acid medium induces the production of ROS via NADPH oxidase in esophageal squamous cells derived from a GERD patient with Barrett’s esophagus. NES-B3T cells were pretreated with 10 μM DPI, 1 μM rotenone, or 1 mM L-NMMA prior to exposure to acidic bile acid medium. DPI, but not rotenone or L-NMMA, blocked the increase in ROS production. Graphs show means ± SE for at least 4 individual determinations in each group. ***P < 0.001 compared with control.
shown to increase ROS production in those transformed cells (7, 8, 20). Using benign Barrett’s epithelial cells (QhTERT), Clemons et al. (7) recently detected acid-induced DNA damage, presumably mediated by the generation of intracellular ROS, suggesting a mechanism whereby oxidant production may contribute to the progression from Barrett’s metaplasia to adenocarcinoma.

Traditionally, in vitro studies on Barrett’s esophagus have used neoplastic, transformed cell lines, which have extensive DNA abnormalities that might affect the cells’ responses to acid and bile salts. Such cells are not ideal for assessing the effects of reflux components on benign esophageal tissues. Indeed, our observation that acid and bile salts exert disparate effects on benign and malignant Barrett’s cells highlights this issue. A strength of our investigation is the use of telomerase-immortalized cells for assessing the effects of reflux components on the esophagus. Unfortunately, relatively few such lines are available for study. This factor may limit the generalizability of our findings and, therefore, caution should be used when interpreting our data.

In conclusion, we have found differences in the mechanisms of oxidant production induced by components of gastric juice between esophageal squamous cell lines derived from GERD patients with and without Barrett’s esophagus. In addition, we have found that different components of gastric juice induce ROS production through different mechanisms in a benign Barrett’s epithelial cell line. Whereas different ROS produced by different intracellular generators are known to activate different signal transduction pathways involved in cellular proliferation, we speculate that the differences in oxidant production that we have found may exert disparate effects on proliferation and differentiation pathways, effects that might contribute to the pathogenesis of Barrett’s esophagus. Further studies are needed to confirm this speculation.

GRANTS

This work was supported by the American College of Gastroenterology Clinical Research Award (L. A. Feagins); the Office of Medical Research, Department of Veteran’s Affairs, Dallas, TX (R. F. Souza, L. S. Terada); the Harris Methodist Health Foundation, Dr. Clark R. Gregg Fund (R. F. Souza, K. Hormi-Carver); and the National Institutes of Health (DK63621 to R. F. Souza, 5T32DK-07745 to L. A. Feagins, and HL61897 and HL67256 to L. S. Terada).

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