NADPH Oxidase NOX5-S Mediates Acid-induced Cyclooxygenase-2 Expression via Activation of NF-κB in Barrett’s Esophageal Adenocarcinoma Cells*

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We have shown that the NADPH oxidase NOX5-S may play an important role in the progression from Barrett’s esophagus to esophageal adenocarcinoma (EA) by increasing cell proliferation and decreasing apoptosis. However, the mechanism of the acid-induced NOX5-S-mediated increase in cell proliferation is not known. We found that, in SEG1 EA cells, the acid-induced increase in prostaglandin E2 (PGE2) production was mediated by activation of cyclooxygenase-2 (COX2) but not by COX1. Acid treatment increased intracellular Ca2+, and a blockade of intracellular Ca2+ increase inhibited the acid-induced increase in COX2 expression and PGE2 production. Knockdown of NOX5-S or NF-κB1 p50 by their small interfering RNA significantly inhibited acid-induced COX2 expression and PGE2 production in SEG1 cells. Acid treatment significantly decreased IκBα and increased luciferase activity when SEG1 cells were transfected with an NF-κB in vivo activation reporter plasmid, pNF-κB-Luc. In a novel Barrett’s cell line overexpressing NOX5-S, IκBα was significantly reduced, and luciferase activity increased when these Barrett’s cells were transfected with pNF-κB-Luc. Overexpression of NOX5-S in Barrett’s cells significantly increased H2O2 production, COX2 expression, PGE2 production, and thymidine incorporation. The increase in thymidine incorporation occurring in NOX5-S-overexpressing Barrett’s cells or induced by acid treatment in SEG1 EA cells was significantly decreased by COX2 inhibitors or small interfering RNA. We conclude that acid-induced COX2 expression and PGE2 production depend on an increase in cytosolic Ca2+ and sequential activation of NOX5-S and NF-κB in SEG1 cells. COX2-derived PGE2 production may contribute to NOX5-S-mediated cell proliferation in SEG1 cells.

Esophageal adenocarcinoma (EA)2 has increased in incidence over the past four decades (1, 2). The major risk factor for this lethal tumor is gastroesophageal reflux disease complicated by Barrett’s esophagus (BE) (3), where esophageal squamous epithelium damaged by acid reflux is replaced by a metaplastic, intestinal type epithelium. The prevalence of BE is about 1–2% in the general population (4). The specialized intestinal metaplasia of BE is associated with a 30–125-fold increased risk for the development of esophageal adenocarcinoma (5–7). However, the mechanisms of the progression from metaplasia to adenocarcinoma are not fully understood.

Reactive oxygen species (ROS) may be an important factor mediating acid reflux-induced damage. ROS may damage DNA, RNA, lipids, and proteins, leading to increased mutation and altered functions of enzymes and proteins (e.g. activation of oncogene products and/or inhibition of tumor suppressor proteins) (8, 9). Low levels of ROS, seen in nonphagocytic cells, were thought to be by-products of aerobic metabolism. More recently, however, superoxide-generating homologues of phagocytic NADPH oxidase catalytic subunit gp91phox (NOX1, NOX3–NOX5, DUOX1, and DUOX2) and homologues of other subunits (p41phox or NOXO1, p51phox, or NOXA1) have been found in several cell types (10–12), suggesting that ROS generated in these cells may have distinctive cellular functions related to immunity, signal transduction, and modification of the extracellular matrix. Two types of NOX5 have been described: NOX5-S and NOX5-L (13). NOX5-L has EF-hand motifs at its NH2 terminus (14), whereas NOX5-S having a much stronger signal than NOX1, with NOX5-S having a much stronger signal than NOX1.
whereas NOX5-L is not detected in these cells. The expression of NOX5-S mRNA is significantly higher in these cells than in esophageal squamous epithelial cells. NOX5-S mRNA is also significantly higher in Barrett’s tissues with high grade dysplasia than without dysplasia. We have also shown that acid-induced H2O2 production is mediated by the NADPH oxidase NOX5-S and that acid-induced NOX5-S expression depends on an increase in intracellular calcium and activation of cyclic AMP response element-binding protein (CREB) in SEG1 EA cells. Overproduction of ROS, derived from up-regulation of NOX5-S, increases cell proliferation and decreases apoptosis, possibly contributing to progression from intestinal metaplasia (Barrett’s esophagus) to dysplasia and to adenocarcinoma.

COX2 (cyclooxygenase-2) may also play a role in the progression from BE to EA, since 1) COX2 overexpression has been demonstrated in both Barrett’s metaplastic and adenocarcinoma cells (16); 2) COX2 expression increases significantly in ex vivo BE tissues pulsed with acid or bile salts, and this effect is attenuated by the selective COX2 inhibitor NS-398 (16); and 3) selective COX2 inhibitors significantly decrease the development of esophageal adenocarcinoma in a rat model of BE (17). The mechanisms mediating COX2-mediated tumorigenesis, however, are not fully understood. COX2 has been reported to play an important role in development of colorectal cancer (18), possibly by increasing cell migration and invasion (19) and decreasing apoptosis (20, 21). In EA cells, selective COX2 inhibitors significantly decrease proliferation and increase apoptosis (22, 23), suggesting that COX2-derived prostaglandin E2 may contribute to esophageal tumorigenesis, possibly by promoting cell proliferation and inhibiting apoptosis.

Whether acid-induced overexpression of NOX5-S increases cell proliferation through up-regulation of COX2 in Barrett’s esophageal adenocarcinoma cells, however, is not known. In the present study, we show that acid-induced COX2 expression depends on an increase in intracellular calcium and sequential activation of NADPH oxidase NOX5-S and NF-κB and that COX2 may contribute to a NOX5-S-mediated increase in cell proliferation in SEG1 cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Acid Treatment**—Human Barrett’s adenocarcinoma cell lines SEG1 were derived from human esophageal Barrett’s adenocarcinomas (24) and generously provided by Dr. David Beer. These cells are cultured in DMEM containing 10% fetal bovine serum and antibiotics.

Human Barrett’s cell line was established and generously provided by Dr. S. J. Spechler and Dr. R. F. Souza. This cell line was derived from esophageal mucosal biopsies of patients with BE (intestinal metaplasia) and immortalized with telomerase as described previously (25). Cells were cultured in wells precoated with collagen IV (1 μg/cm2; BD Bioscience, Bedford, MA) and in Keratinocyte Medium-2 (Ca2+-free solution, Cambrex, Rockland, ME) supplemented with 1.8 mM CaCl2, 5% fetal bovine serum, 400 ng/ml hydrocortisone, 20 ng/ml epidermal growth factor, 0.1 μM cholera toxin, 20 μg/ml adenosine, 5 μg/ml insulin, 70 μg/ml bovine pituitary extract, and antibiotics. Fig. 1A shows Alcian blue staining-positive cells in cultured Barrett’s cell line, indicating that this cell line contains mucous-secreting cells (possibly Goblet cells). Mucin-2 and an intestine-specific transcription factor CDX2 were also detectable (Fig. 1B), confirming that these cells are intestinal metaplastic cells. Both cell lines were cultured at 37 °C in a 5% CO2 humidified atmosphere.

For acid treatment, SEG1 cells were exposed to acidic DMEM (pH 4.0), acidic DMEM (calcium-free and 1 mM EGTA) (pH 4.0), acidic medium plus NS-398 (10−6 M), acidic medium plus valeryl salicylate (10−5 M), or normal DMEM (control) for 1 h, washed, and cultured in fresh medium (pH 7.2, without phenol red) for an additional 24 h. For the NS-398 and valeryl salicylate group, NS-398 or valeryl salicylate was added to the culture medium in this additional 24-h culture. Finally, the culture medium and cells are collected for measurements. Acidic DMEM (pH 4.0, 250 μl) was added to each well in a 12-well plate, and the final pH was about 4.9 after a 1-h incubation.

**NOX5-S-overexpressing Stable Barrett’s Cell Line**—Barrett’s cells were transfected with NOX5-S plasmid or pCMV-Tag5A plasmid by using Lipofectamine 2000. From the second day after the transfection, NOX5-S or empty vector-transfected cells were selected with 200 μg/ml G418 for 4 weeks. These Barrett’s cells were cultured as described above.

**Mucosal Organ Culture**—Endoscopic mucosal biopsies were obtained from patients with documented BE undergoing endoscopy for cancer surveillance. As clinically recommended, one biopsy was taken from each quadrant every 2 cm in the entire length of the Barrett’s esophagus. All mucosal samples were divided in half using an aseptic technique. One half was used for histology and examined by a pathologist; the other was placed immediately in ice-cold culture medium and transported to the laboratory. BE mucosa confirmed to be intestinal metaplasia by pathological examination were used for the studies. The experimental protocols were approved by the Human Research Institutional Review Committee at Rhode Island Hospital.

Biopsies were cultured as described previously (16, 26, 27). Briefly, BE mucosal biopsy specimens were randomly assigned to acid, acid plus calcium-free, or control groups. The biopsy specimens were placed on a sterilized stainless wire mesh (Flynn & Enslow, Inc., San Francisco, CA) within a Falcon center-well organ culture dish (BD Biosciences) so that culture medium (0.9 ml) just covered the surface of the biopsy. Organ
culture dishes were then placed on racks in the Modular Incubator Chamber (Billups-Rothenberg, Inc., Del Mar, CA), perfused with 95% oxygen and 5% carbon dioxide, and then cultured at 37 °C. Organ culture was performed in RPMI 1640 supplemented with 10% fetal bovine serum, 5 μg/ml insulin, CaCl2 (1.377 mM), glutamine (2 mM), glucose (3.66 mg/ml), 500 units/ml streptomycin, and 250 units/ml penicillin. The final concentration of calcium in the medium was 1.8 mM. BE mucosal biopsy tissues were first equilibrated in culture for 2 h and then exposed to acidic medium (pH 4.0), acidic medium without calcium plus 1 mM EGTA, or control medium (pH 7.2) for 1 h. After washing twice, BE mucosa biopsies were cultured in fresh medium without phenol red (pH 7.2) for an additional 24 h. Finally the culture medium was collected for measurement of H2O2, and the levels of H2O2 were normalized for protein content.

Small Interfering RNA (siRNA) Transfection—24 h before transfection at 40–50% confluence, cells were trypsinized and diluted 1:5 with fresh medium without antibiotics (1–3 × 105 cells/ml) and transferred to 12-well plates (1 ml/well). Transfection of siRNAs was carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. Per well, 75 pmol of siRNA duplex of NOX5, COX2, NF-κB1 p50, or scrambled siRNA formulated into liposomes were applied; the final volume was 1.2 ml/well. After a 4-h transfection, the transfection medium was replaced with regular medium. 12 h (NOX5 siRNA) or 48 h (p50 siRNA) later, cells were exposed to acidic medium (pH 4, 1 h), washed, and cultured in fresh medium (pH 7.2, without phenol red) for an additional 24 h. For COX2 siRNA, cells were cultured without acid treatment for 48 h after transfection. Finally, the culture medium and cells were collected for measurements. Transfection efficiencies were determined by fluorescence microscopy after transfection of Block-it fluorescent oligonucleotide (Invitrogen) and were about 90% at 48 h.

Reverse Transcription-PCR—Total RNA was extracted by TRIzol reagent (Invitrogen) for the cultured cells and extracted by the RNAqueous kit (Ambion Inc., Austin, TX) for the biopsy tissues according to the protocols of the manufacturers. 1.5 μg of total RNAs from cultured cells or 0.5 μg of total RNA from tissues was reversely transcribed by using a SUPERSCRIPT™ kit first strand synthesis system for reverse transcription-PCR (Invitrogen) or a Sensiscript RT kit (Qiagen, Valencia, CA), respectively.

Luciferase Assay—24 h before transfection, NOX5-S-overexpressing Barrett’s cells, control Barrett’s cells transfected with pCMV-Tag5A, or SEG1 cells were seeded in 24-well plates. Each well of cells was transfected with Lipofectamine 2000 (Invitrogen) and 0.2 μg of either pGL3-Basic (as no promoter control) or an NF-κB in vivo activation reporter plasmid pNF-κB-Luc, which contains five repeats of NF-κB binding element GGGGACCTTTCC in the enhancer element of the plasmid. For acid treatment, SEG1 cells were treated with acidic medium (pH 4, 1 h) 12 h after transfection and then cultured for an additional 24 h.

Luciferase activity was assayed 24 h (Barrett’s cells) or 36 h (SEG1 cells) after transfection. Cell extracts were prepared by lysing the cells with lysis buffer (Roche Applied Science). The lysate was centrifuged at 13,000 rpm for 10 min to pellet the cell debris. The protein concentration in the supernatants was determined. The luciferase activities in the cell lysates were measured using Luciferase assay substrate (Roche Applied Science) and normalized to protein content.

Quantitative Real Time PCR—Quantitative real time PCR was carried out on a Stratagene Mx4000® multiplex quantitative PCR system. The primers used were: COX2 sense (5’-CCT-GGCCCTTCTGGTAGAAA-3’), COX2 antisense (5’-GGACA-GGCCCTTACGGTATT-3’), NOX5 sense (5’-AAGACTCC-ATCACGGGGCTGCA-3’), NOX5 antisense (5’-CTCTCAG-CACCTTGGCCAGA-3’), GAPDH sense (5’-CATGACCAC-GTCCATG CCATCAC-3’), and GAPDH antisense (5’-AGGTCACCACCCCTGTGGCTGA-3’).

All reactions were performed in triplicate in a 25-μl total volume containing a 1× concentration of Brilliant® SYBR® Green QPCR Master Mix (Stratagene), a 100 nM concentration of each sense and antisense primer, 1 μl of cDNA, and 30 nM reference dye. Reactions were carried out in a Stratagene Mx4000® multiplex quantitative PCR system for one cycle at 94 °C for 5 min; 40 cycles at 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s; one cycle at 94 °C for 1 min; and one cycle at 55 °C for 30 s. Fluorescence values of SYBR Green I dye, representing the amount of product amplified at that point in the reaction, were recorded in real time at both the annealing step and the extension step of each cycle. The Ct, defined as the point at which the fluorescence signal was statistically significant above background, was calculated for each amplicon in each experimental sample using Stratagene Mx4000 software. This value was then used to determine the relative amount of amplification in each sample by interpolating from the standard curve. The transcript level of each specific gene was normalized to GAPDH amplification.

Western Blot Analysis—Cells was lysed in Triton X lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (v/v) Triton X-100, 40 mM β-glycerol phosphate, 40 mM p-nitrophehyphosphate, 200 μM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin. The suspension was centrifuged at 15,000 × g for 5 min, and the protein concentration in the supernatant was determined. Western blot was done as described previously (28). Briefly, after these supernatants were subjected to SDS-PAGE, the separated proteins were electrophoretically transferred to a nitrocellulose membrane at 30 V overnight. The nitrocellulose membranes were blocked in 5% nonfat dry milk and then incubated with appropriate primary antibodies followed by a 60-min incubation in horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). Detection was achieved with an enhanced chemiluminescence agent (Amer- sham Biosciences).

Primary antibodies used were mucin-2 antibody (1:1000), CDX2 antibody (1:1000), human COX1 antibody (1:1000), COX2 antibody (1:1000), actin antibody (1:1000), IkBα antibody (1:200), NF-κB1 p50 antibody, and GAPDH antibody (1:2000). NOX5 antibody prepared against a mixture of unique NOX5 peptides (NH2-YESFKASDPLGRGSKRC-COOH and
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NH₂-YRHQKRKHTCPS-COOH) was generously provided by Dr. David Lambeth (29) and used at a dilution of 1:1000.

Cytosolic Calcium Measurements—SEG1 cells were loaded with 1.25 μM Fura-2/AM for 40 min and placed in a 5-ml chamber mounted on the stage of an inverted microscope (Carl Zeiss). The cells were allowed to settle onto a coverslip at the bottom of the chamber. The bathing solution is the HEPES-buffered solution (pH 7.4) containing 112.5 mM NaCl, 3.1 mM KCl, 2.0 mM KH₂PO₄, 10.8 mM glucose, 24.0 mM HEPES (sodium salt), 1.9 mM CaCl₂, 0.6 mM MgCl₂, 0.3 mg/ml basal medium Eagle amino acid supplement, and 0.08 mg/ml soybean trypsin inhibitor. The Ca²⁺-free medium is the HEPES-buffered solution without CaCl₂ but with 200 μM 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), which completely blocked KCl-induced Ca²⁺ influx (30). When the Ca²⁺-free medium was used, the bathing solution was changed twice with Ca²⁺-free medium after the cells had settled to the bottom of the chamber. When thapsigargin or cyclopiazonic acid was used, cells were preincubated with thapsigargin (3 μM) or cyclopiazonic acid (1 μM) for 1 h and then utilized for acid treatment.

Ca²⁺ measurements were obtained using a modified dual excitation wavelength imaging system (IonOptix Corp. Milton, MA) as described previously (31). Ratio-metric images were masked in the region outside the borders of the cell, since low photon counts give unreliable ratios near the edges. We developed a method for generating an adaptive mask that follows the borders of the cell as Ca²⁺ changes. A pseudoisosbestic image (i.e., an image insensitive to Ca²⁺ changes) was formed in computer memory from a weighted sum of the images generated by 340-nm excitation and 380-nm excitation. This image was then thresholded (i.e., values below a selected level were considered to be outside the cell and assigned a value of 0). For each ratiometric image, the outline of the cell was determined, and the generated mask was applied to the ratiometric image. This method allows the imaging of the changes in Ca²⁺. Our algorithm has been incorporated into the IonOptix software.

This algorithm calculates the conversion of the ratios of fluorescence elicited by 340-nm excitation to 380-nm excitation to Ca²⁺ concentrations using techniques previously described in detail by Grynkiewicz et al. (32).

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FIGURE 3. NOX5-S expression and H$_2$O$_2$ production in a NOX5-S overexpressing stable cell line derived from telomerase-immortalized Barrett’s cells. A, typical example of Western blot analysis (A) and summarized data (B) showed that transfection of NOX5-S plasmid significantly increased NOX5-S protein expression, compared with control group, where cells were transfected with pcMV-Tag5A plasmid. C, transfection of NOX5-S plasmid significantly increased NOX5-S mRNA expression, measured by real time PCR. D, overexpression of NOX5-S significantly increased H$_2$O$_2$ production in Barrett’s cells, suggesting that NOX5-S may be constitutively active or activated by mediators present in culture medium.

RESULTS

Role of NOX5-S in Acid-induced COX2 Expression and PGE$_2$ Production—In SEG1 esophageal adenocarcinoma cells, acid treatment significantly increased COX2 (Fig. 2, A and B) but not COX1 expression (Fig. 2, C and D). In addition, acid treatment significantly increased PGE$_2$ production. This PGE$_2$ increase was inhibited by the COX2 inhibitor NS-398 but not by the COX1 inhibitor valeryl salicylate (Fig. 2F). The data suggest that acid-induced PGE$_2$ production is mediated by activation of COX2 but not by COX1.

We have previously shown that NOX5 siRNA effectively knock down NOX5 protein in SEG1 cells (27). Fig. 2 shows that knockdown of NOX5-S by transfection of SEG1 cells with NOX5 siRNA significantly decreased COX2 expression (Fig. 2, A and B) and PGE$_2$ production (Fig. 2E) at basal condition as well as in response to acid treatment. Knockdown of NOX5-S, however, had no effect on COX1 expression (Fig. 2, C and D).
The data suggest that NOX5-S may contribute to acid-induced COX2 expression and PGE2 production in EA cells.

To confirm the role of NOX5-S in COX2 expression, we established a NOX5-S-overexpressing stable cell line in telomerase-immortalized Barrett's cells. As expected, transfection of NOX5-S plasmid significantly increased NOX5-S mRNA (Fig. 3C) and protein expression (Fig. 3, A and B) in these cells. In addition, overexpression of NOX5-S significantly increased H2O2 production in Barrett's cells (Fig. 3D), suggesting that in these cells NOX5-S may be constitutively active or activated by mediators present in culture medium. In addition, in Barrett's cells, overexpression of NOX5-S significantly increased COX2 expression (Fig. 4, A and B) but did not affect COX1 expression (Fig. 4, C and D). Overexpression of NOX5-S significantly increased PGE2 production, an increase that was blocked by NS-398 but not by valeryl salicylate (Fig. 4E). The data suggest that overexpression of NOX5-S induces COX2 expression and PGE2 production.

When normal Barrett's cells were co-transfected with NOX5-S and pNF-κB-Luc plasmids, the luciferase activity increased significantly (Fig. 7C), suggesting that NOX5-S activates NF-κB.

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Role of NF-κB in Acid-induced COX2 Expression—Since NF-κB-dependent COX2 expression has been indicated in other preparations (34–36), we examined whether NF-κB mediates acid-induced COX2 expression. Transfection of SEG1 EA cells with NF-κB p50 siRNA significantly reduced the p50 protein expression (Fig. 5, A and B) and significantly decreased COX2 expression and prostaglandin E2 production at the basal condition as well as in response to acid treatment (Fig. 5, C–E), suggesting that acid-induced COX2 expression may depend upon activation of NF-κB.

Pulsed acid treatment significantly decreased the expression of 1κβα (Fig. 6, A and B), supporting acid-induced activation of NF-κB. To confirm this conclusion, we transfected SEG1 cells with NF-κB cis-reporter plasmid pNF-κB-Luc, which contains five repeats of NF-κB binding element GGG-GACTTTCC in the enhancer element of the plasmid. Acid treatment significantly increased the luciferase activity (Fig. 6C), indicating that acid treatment induces activation of NF-κB.

Since acid up-regulates the expression of NOX5-S, we examined whether NOX5-S activates NF-κB. In the NOX5-S-overexpressing Barrett's cell line, the expression of 1κβα was significantly decreased (Fig. 7, A and B).

Role of Ca2+ in Acid-induced COX2 Expression and PGE2 Production—We have shown that acid-induced NOX5-S expression depends on increased intracellular calcium (27). Since NOX5-S contributes to acid-induced COX2 expression, we examined whether an intracellular calcium increase mediates acid-induced COX2 expression.

As previously shown, acid treatment significantly increased intracellular Ca2+ concentration in Fura-2/AM-loaded SEG1 cells, an increase that was blocked by removal of extracellular Ca2+ (Fig. 8, A and B). Acid treatment increased intracellular Ca2+ in a time-dependent manner (Fig. 8C), and depletion of intracellular Ca2+ stores by the Ca2+-ATPase inhibitors thapsigargin (37) and cyclopiazonic acid (38) did not affect the acid-induced Ca2+ increase (Fig. 8, C and D), suggesting that the
acid-induced Ca\textsuperscript{2+} increase depends on Ca\textsuperscript{2+} influx. Fig. 8, A and B, have been published (27) and are included here for readers’ convenience.

In SEG1 cells, acid-induced COX2 expression and PGE\textsubscript{2} production were significantly decreased by removal of extracellular calcium (Fig. 8, E and F), suggesting that acid-induced COX2 expression and PGE\textsubscript{2} production may depend on a cytosolic Ca\textsuperscript{2+} increase.

To test whether acid up-regulates COX2 expression in Barrett’s esophageal mucosa, we cultured human BE mucosal biopsies in an oxygen-enriched environment and exposed these biopsy tissues to acid (pH 4). Similarly, acid treatment significantly increased COX2 expression in BE mucosal biopsies. Acid-induced COX2 expression was significantly decreased by the removal of extracellular calcium (Fig. 9A), suggesting that calcium mediates acid-induced COX2 expression in BE mucosa. Similarly, PGE\textsubscript{2} levels in the culture medium significantly increased after acid exposure, when compared with control (Fig. 9B). This PGE\textsubscript{2} increase was blocked by the removal of extracellular calcium (Fig. 9B), suggesting that calcium is involved in acid-induced PGE\textsubscript{2} production in BE mucosa.

COX2 Mediates the Acid-induced Increase in Cell Proliferation—In SEG1 cells, pulsed acid treatment significantly increased thymidine incorporation. This increase in thymidine incorporation was significantly reduced by NS-398 but not by valeryl salicylate (Fig. 10A). In these experiments, NS-398 effectively blocked the acid-induced increase in PGE\textsubscript{2} production (Fig. 2F). The data suggest that COX2 may mediate the acid-induced increase in cell proliferation.

We have shown that pulsed acid treatment increases cell proliferation in SEG1 EA cells via activation of NADPH oxidase NOX5-S (27). Therefore, we examined whether COX2 contributes to NOX5-S-mediated increase in cell proliferation.

In Barrett’s cells, overexpression of NOX5-S significantly increased the thymidine incorporation. This increase in thymidine incorporation was significantly decreased by NS-398 but not by valeryl salicylate (Fig. 10B). In the culture medium collected in these experiments, NS-398 has been demonstrated to abolish the
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***DISCUSSION***

We have previously shown that NOX1 and NOX5-S are the major isoforms of NADPH oxidase in SEG1-esophageal adenocarcinoma cells. The expression of NOX5-S mRNA is significantly higher in these cells than in esophageal squamous epithelial cells. NOX5 mRNA is also significantly higher in Barrett’s tissues with high grade dysplasia than without dysplasia, suggesting that NOX5-S may play an important role in the progression from BE to esophageal adenocarcinoma. Pulsed acid treatment significantly increases H$_2$O$_2$ production in both SEG1-EA cells and BE mucosa. In SEG1 cells, acid treatment increases mRNA expression of NOX5-S but not NOX1, and knockdown of NOX5 by NOX5 siRNA abolishes acid-induced H$_2$O$_2$ production, suggesting that NOX5-S mediates acid-induced H$_2$O$_2$ production. In addition, acid treatment increases intracellular calcium and phosphorylation of CREB. Acid-induced NOX5-S expression and H$_2$O$_2$ production are significantly inhibited by removal of extracellular calcium and by knockdown of CREB using CREB siRNA, suggesting that acid-induced NOX5-S expression depends on intracellular Ca$^{2+}$ increase and activation of CREB. Overproduction of ROS derived from up-regulation of NOX5-S increases cell proliferation and decreases apoptosis. In this study, we examined whether NADPH oxidase NOX5-S up-regulates COX2 expression, thereby causing increase in cell proliferation in SEG1 EA cells.

**Role of NADPH Oxidase NOX5-S in Acid-induced COX2 Expression and PGE$_2$ Production**—We have shown that acid treatment increases NOX5-S expression and H$_2$O$_2$ production in SEG1 cells (27). H$_2$O$_2$ is known to induce COX2 expression in other preparations (41, 42). Therefore, we examined whether NOX5-S contributes to acid-induced COX2 expression.

In SEG1 EA cells, acid-induced PGE$_2$ production may depend on activation of COX2 but not of COX1, since 1) acid-induced PGE$_2$ production was inhibited by the COX2 inhibitor NS-398 (39, 43) but not by the COX1 inhibitor valeryl salicylate (44) (Fig. 2F), and 2) acid treatment significantly increased COX2 expression (Fig. 2, A and B) but not COX1 expression (Fig. 2, C and D). Our data are consistent with a previous report (45).

In SEG1 EA cells, knockdown of NOX5-S by NOX5 siRNA significantly decreased acid-induced COX2 expression and PGE$_2$ production (Fig. 2, A, B, and E). However, knockdown of NOX5-S did not affect COX1 expression (Fig. 2, C and D). The data suggest that NOX5-S may mediate acid-induced COX2 expression and PGE$_2$ production in SEG1 EA cells. This result was further supported by our findings that overexpression of NOX5-S by transfection with NOX5-S plasmid significantly increased COX2 expression as well as PGE$_2$ production in Barrett’s cells (Fig. 4, A, B, and E) but did not affect COX1 expression (Fig. 4, C and D) and that PGE$_2$ production induced by overexpression of NOX5-S was blocked by COX2 inhibitors NS-398 (Fig. 4E), CAY10404 (Fig. 11E), and COX2 siRNA (Fig. 11C) but not by COX1 inhibitor valeryl salicylate (Fig. 4E). It is known that low doses of H$_2$O$_2$ are mitogenic and promote cell proliferation, whereas intermediate doses result in growth arrest, such as replicative senescence, and high doses cause cell

increase of PGE$_2$ production in response to overexpression of NOX5-S (Fig. 11C). In these cells used in Fig. 11C, COX2 siRNAs significantly lessened the thymidine incorporation at the basal condition as well as in response to the overexpression of NOX5-S (Fig. 11D). These data suggest that the NOX5-S-induced increase in cell proliferation at least in part depends on activation of COX2.

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NOX5-S and COX2 in Esophageal Adenocarcinoma

FIGURE 8. Role of Ca²⁺ in COX2 expression and PGE₂ production in SEG1 cells. A, typical cells; B, summarized data show that in Fura-2/AM-loaded SEG1 cells, exposure of cells to acidic physiologic salt solution (pH 4) for 30 min significantly increased intracellular calcium concentration. This increase was significantly decreased by removal of extracellular Ca²⁺ with Ca²⁺-free medium plus 200 μM BAPTA. A and B have been published (27) and are included here for the reader’s convenience. C, acid treatment increased intracellular Ca²⁺ in a time-dependent manner, and that depletion of intracellular Ca²⁺ stores by Ca²⁺-ATPase inhibitor thapsigargin (37) had no effect on acid-induced Ca²⁺ increase (control n = 20, thapsigargin n = 17). D, depletion of intracellular Ca²⁺ stores by Ca²⁺-ATPase inhibitor cyclopiazonic acid (CPA) (38) had no effect on acid-induced Ca²⁺ increase (control n = 20, cyclopiazonic acid n = 10). The data suggest that acid-induced COX2 expression may depend on intracellular Ca²⁺ increase. E, in SEG1 cells, acid-induced COX2 mRNA expression was significantly decreased by removal of extracellular calcium (n = 5). F, acid-induced PGE₂ production was significantly reduced by removal of extracellular calcium (n = 5), suggesting that acid-induced COX2 expression and PGE₂ production may depend on intracellular Ca²⁺ increase. Cultured cells were exposed to acidic medium (pH 4.0) for 1 h and then cultured at pH 7.2 for an additional 24 h. Finally, the culture medium and cells were collected for measurement. COX2 mRNA was measured by real-time PCR. The differences between different groups were tested using ANOVA. *, p < 0.01, compared with the pH 7.4 or 7.2 group. **, p < 0.01; #, p < 0.02; ##, p < 0.05, compared with the pH 4 group.

Death via either apoptotic or necrotic mechanisms (46). We have shown that NOX5-S is overexpressed in SEG1 cells (27). To avoid the possibility that transfection with NOX5-S plasmid would produce too much H₂O₂, which might cause cell apoptosis in SEG1 cells, we transfected Barrett’s cells with NOX5-S plasmid.

Overexpression of NOX5-S significantly increased H₂O₂ production in Barrett’s cells (Fig. 3D), suggesting that NOX5-S may be constitutively active or activated by mediators present in culture medium. However, functional regulation of NOX5-S by its subunits is not known and needs to be further explored.

siRNA significantly decreased COX2 expression and PGE₂ production at basal condition as well as in response to acid treatment (Fig. 5, C–E), suggesting that NF-κB1 may be responsible for COX2 expression in SEG1 cells. This result is further supported by our findings indicating that NF-κB is activated by acid treatment, since in SEG1 esophageal adenocarcinoma cells, pulsed acid treatment significantly decreased the expression of iκBα (Fig. 6, A and B) and increased the luciferase activity (Fig. 6C) in SEG1 cells transfected with NF-κB cis-reporter plasmid pNF-κB-Luc, which contains five repeats of NF-κB binding element GGGGACTTTCC in the enhancer element of the plasmid.

Role of NF-κB in Acid-induced COX2 Expression—In SEG1 EA cells, transcription factors responsible for acid-induced COX2 expression are not known. It has been reported that NF-κB mediates COX2 expression induced by interleukin-1β and tumor necrosis factor (34–36). In addition, NF-κB binding sites have been identified in the promoter region of the COX2 gene (47). Therefore, we examined the role of NF-κB in acid-induced COX2 expression.

NF-κB is thought to be a family of Rel domain-containing proteins, including Rel A (also called p65), Rel B, c-Rel, and NF-κB1 (p105/p50), and NF-κB2 (p100/p52). p105 and p100 are larger precursor proteins containing IκB (an inhibitor of κB)-like ankyrin repeat sequences in their carboxyl termini. Because of their IκB-like ankyrin repeat sequences, these precursors are retained in the cytoplasm and require proteolytic processing to generate their mature DNA-binding proteins, p50 and p52, respectively (48). In the cytoplasm NF-κB is in an inactive state, and its activity is regulated by at least two pathways. In the first pathway, a heterotrimer of p50, p65, and IκB is degraded in a ubiquitin-dependent reaction, leading to the translocation of the p65-p50 dimers to the nucleus (48). In the second pathway, the dimers consisting of p100 and Rel B undergo proteolytic removal of the IκB-like COOH-terminal domain of p100, allowing Rel B-p52 dimers to translocate to nucleus, where NF-κB activates gene transcription (48).

We found that in SEG1 cells, knockdown of NF-κB1 p50 by p50
removal of extracellular Ca$^{2+}$ (Fig. 8, A and B), suggesting that intracellular Ca$^{2+}$ increase may be due to Ca$^{2+}$ influx. This was further consolidated by the findings that depletion of intracellular Ca$^{2+}$ stores by Ca$^{2+}$-ATPase inhibitors thapsigargin (37) and cyclopiazonic acid (38) had no effect on acid-induced Ca$^{2+}$ increase (Fig. 8, C and D). The mechanisms of acid-induced Ca$^{2+}$ influx are not known and need to be further explored. Since acid increases intracellular Ca$^{2+}$, causing up-regulation of NADPH oxidase NOX5-S, we examined the role of calcium in acid-induced COX2 expression.

In SEG1 cells, acid-induced COX2 expression and PGE$_2$ production were significantly decreased by removal of extracellular calcium (Fig. 8, E and F), suggesting that acid-induced COX2 expression and PGE$_2$ production may depend on intracellular Ca$^{2+}$ increase. Similarly, acid treatment significantly increased COX2 mRNA expression and PGE$_2$ production in cultured human BE mucosal biopsies, an increase that was blocked by removal of extracellular calcium (Fig. 9, A and B), indicating that SEG1 cells might be a suitable in vitro model to study acid-induced changes. These data further support the possibility that NADPH oxidase NOX5-S mediates acid-induced COX2 expression.

**Role of Ca$^{2+}$ in Acid-induced COX2 Expression and PGE$_2$ Production**—We have shown that acid treatment significantly increased intracellular Ca$^{2+}$ concentration in Fura-2/AM-loaded SEG1 cells, an increase that was blocked by removal of extracellular Ca$^{2+}$ (Fig. 8, A and B), suggesting that intracellular Ca$^{2+}$ increase may be due to Ca$^{2+}$ influx. This was further consolidated by the findings that depletion of intracellular Ca$^{2+}$ stores by Ca$^{2+}$-ATPase inhibitors thapsigargin (37) and cyclopiazonic acid (38) had no effect on acid-induced Ca$^{2+}$ increase (Fig. 8, C and D). The mechanisms of acid-induced Ca$^{2+}$ influx are not known and need to be further explored. Since acid increases intracellular Ca$^{2+}$, causing up-regulation of NADPH oxidase NOX5-S, we examined the role of calcium in acid-induced COX2 expression.

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whether COX2 contributes to a NOX5-S-mediated increase in cell proliferation.

Overexpression of NOX5-S in Barrett’s cells significantly increased the thymidine incorporation (Fig. 10B), suggesting that overexpression of NOX5-S may increase cell proliferation. This result is consistent with our previous finding that knockdown of NOX5-S significantly decreases the acid-induced increase in cell proliferation and retinoblastoma protein phosphorylation (27). This is also consistent with other reports showing that blockade of NOX5 inhibits cell proliferation (51). We also found that an increase in thymidine incorporation induced by overexpression of NOX5-S was significantly decreased by COX2 inhibitors NS-398 (Fig. 10B) and CAY10404 (Fig. 11F) and by knockdown of COX2 with COX2 siRNA (Fig. 11D) but not by COX1 inhibitor valeryl salicylate (Fig. 10B). Meanwhile, NS-398 (Fig. 4E), CAY10404 (Fig. 11E), and COX2 siRNA almost abolished the increase of PGE2 production induced by overexpression of NOX5-S. These data suggest that the NOX5-S-mediated increase in cell proliferation may partially depend on COX2-derived PGE2 production.

We conclude that acid-induced COX2 expression depends on intracellular calcium increase and sequential activation of NADPH oxidase NOX5-S and NF-κB. COX2 may contribute to NOX5-S-mediated increase in cell proliferation. It is possible that acid reflux present in patients with Barrett’s esophagus may cause an increase of intracellular Ca2+ in metaplastic cells and activation of NADPH oxidase NOX5-S and NF-κB, causing overexpression of COX2 and overproduction of PGE2. Overproduction of PGE2 together with other possible mechanisms may increase cell proliferation, contributing to progression from intestinal metaplasia (Barrett’s esophagus) to dysplasia and to adenocarcinoma. Since COX2 inhibitors have severe side effects (e.g. acute myocardial infarction) (52), NOX5-S might be a better potential target to treat and/or prevent esophageal adenocarcinoma.

REFERENCES

1. Blot, W. J., Devesa, S. S., Kneller, R. W., and Fraumeni, J. F., Jr. (1991) J. Am. Med. Assoc. 265, 1287–1289
2. Shaheen, N. J. (2005) Gastroenterology 128, 1554–1566
3. Lagergren, J., Bergstrom, R., Lindgren, A., and Nyren, O. (1999) N. Engl. J. Med. 340, 825–831
4. Ronkainen, J., Aro, P., Storskrubb, T., Johansson, S. E., Lind, T., Bolling-Sternevald, E., Vieth, M., Stolte, M., Talley, N. J., and Agreus, L. (2005) J. Med. Assoc. 340, 825–831
Gastroenterology 129, 1825–1831

5. Kim, R., Weissfeld, J. L., Reynolds, J. C., and Kuller, L. H. (1997) Cancer Epidemiol. Biomarkers Prev. 6, 369–377

6. Haggitt, R. C. (1994) Hum. Pathol. 25, 982–993

7. Wild, C. P., and Hardie, L. J. (2003) Nat. Rev. Cancer 3, 676–684

Ohshima, H., Tatemichi, M., and Sawa, T. (2003) Arch. Biochem. Biophys. 417, 3–11

9. Farhadi, A., Fields, J., Banan, A., and Keshavarzian, A. (2002) Am. J. Gastroenterol. 97, 22–26

10. Lambeth, J. D. (2004) Nat. Rev. Immunol. 4, 181–189

11. Banti, B., Maturana, A., Jaconi, S., Arnaudeau, S., Laforgue, T., Sinha, B., Ligeti, E., Demaurex, N., and Krause, K. H. (2000) Science 287, 138–142

12. Suh, Y. A., Arnold, R. S., Lassegue, B., Sinha, X., Sorescu, D., Chung, A. B., Griendling, K. K., and Lambeth, J. D. (1999) Nature 401, 79–82

13. Vignais, P. V. (2002) Cell. Mol. Life Sci. 59, 1428–1459

14. Banti, B., Molnar, G., Maturana, A., Steger, K., Hegedus, B., Demaurex, N., and Krause, K. H. (2001) J. Biol. Chem. 276, 37594–37601

15. Cheng, G., Cao, Z., Xu, X., van Meir, E. G., and Lambeth, J. D. (2001) Gene (Amst.) 269, 131–140

16. Shirvani, V. N., Ouatu-Lascar, R., Kaur, B. S., Omary, M. B., and Triadafilopoulos, G. (2000) Gastroenterology 118, 487–496

17. Buttar, N. S., Wang, K. K., Leontovich, O., Westcott, J. Y., Pacifico, R. J., Anderson, M. A., Krishnadath, K. K., Lutzke, L. S., and Burgart, L. J. (2002) Am. J. Physiol. 283, C1212–C1224

18. Gupta, R. A., and Dubois, R. N. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5110–5115

19. Tsujii, M., Kawano, S., and Dubois, R. N. (1997) J. Biol. Chem. 272, 5767–5772

20. Tsujii, M., and Lu, D. (1995) Arch. Biochem. Biophys. 224, 266–273

21. Cao, W., Chen, Q., Xu, X., van Meir, E. G., and Lambeth, J. D. (2001) Cancer Res. 51, 273–286

22. Barbieri, S. S., Cavalca, V., Eligini, S., Brambilla, M., Caiani, A., Tremoli, E., and Colli, S. (2004) Free Radic. Biol. Med. 37, 156–165

23. Kim, H., Rhee, S. H., Kokkotou, E., Na, X., Savidge, T., Moyer, M. P., Pothoulakis, C., and LaMont, J. T. (2005) J. Biol. Chem. 280, 21237–21245

24. Johnson, J. L., Wimsatt, J., Buckel, S. D., Dyer, R. D., and Maddipati, K. R. (1995) Arch. Biochem. Biophys. 324, 26–34

25. Bhattacharyya, D. K., Lecomte, M., Dunn, J., Morgans, D. J., and Smith, W. L. (1995) Arch. Biochem. Biophys. 317, 19–24

26. Souza, R. F., Sooriakumaran, P., and Krause, K. H. (2002) J. Cell. Physiol. 192, 1–15

27. Martindale, J. L., and Holschbach, R. (2000) Am. J. Physiol. 278, G734–G748

28. Tazawa, R., Xu, X. M., Wu, K. K., and Wang, L. H. (1994) Biochem. Biophys. Res. Commun. 203, 190–199

29. Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. (2002) Nat. Rev. Cancer 2, 301–310

30. Cao, W., Yreea, M. D., Kirber, M. T., Fiocchi, C., and Priloc, V. E. (2004) Am. J. Physiol. 286, G833–G843

31. Cao, W., Chen, Q., Sohn, U. D., Kim, N., Kirber, M. T., Harnett, K. M., Behar, J., and Biancani, P. (2001) Am. J. Physiol. Cell Physiol. 280, C980–992

32. Grymkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450

33. Bradford, M. M. (1976) Ann. Biochem. 72, 248–254

34. Yan, X., Wu Xiao, C., Sun, M., Tsang, B. K., and Gibb, W. (2002) Biol. Reprod. 66, 1667–1671

35. Tsai, S. H., Liang, Y. C., Chen, L., Ho, F. M., Hsieh, M. S., and Lin, J. K. (2002) J. Cell. Biochem. 84, 750–758

36. Paik, J. H., Ju, J. H., Lee, J. Y., Boudreau, M. D., and Hwang, D. H. (2000) J. Biol. Chem. 275, 28173–28179

37. Ghosh, T. K., Bian, J. H., Short, A. D., Rybak, S. L., and Gill, D. L. (1991) J. Biol. Chem. 266, 24690–24697

38. Demaurex, N., Lew, D. P., and Krause, K. H. (1992) J. Biol. Chem. 267, 2318–2324

39. Barnett, J., Chow, J., Ives, D., Chion, M., Mackenzie, R., Osen, E., Nguyen, B., Tsing, S., Bach, C., Freire, J., Chan, H., Sigal, E., and Ramesha, C. (1994) Biochim. Biophys. Acta. 1209, 130–139

40. Habebe, A. G., Praveen Rao, P. N., and Knaus, E. E. (2000) Drug Dev. Res. 51, 273–286

41. Cao, W., Xu, X., van Meir, E. G., Spechler, S. J. (2001) Nat. Rev. Cancer 1, 401–410

42. Martindale, J. L., and Holbrook, N. J. (1995) Methods Cell Biol. 42, 165–187

43. Brown, J. J., Jr., and Lambeth, J. D. (2004) Gastroenterology 126, 2036–2046

44. Bhattacharyya, D. K., Lecomte, M., Dunn, J., Morgans, D. J., and Smith, W. L. (1995) Arch. Biochem. Biophys. 317, 19–24

45. Souza, R. F., Sooriakumaran, P., and Krause, K. H. (2002) J. Cell. Physiol. 192, 1–15

46. Martinide, J. L., and Holschbach, R. (2000) Am. J. Physiol. 287, G743–G748

47. Martindale, J. L., and Holschbach, R. (2000) J. Cell. Physiol. 192, 1–15

48. Tazawa, R., Xu, X. M., Wu, K. K., and Wang, L. H. (1994) Biochem. Biophys. Res. Commun. 203, 190–199

49. Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. (2002) Nat. Rev. Cancer 2, 301–310

50. Flohe, L., Brigelius-Flohe, R., Saliou, C., Traber, M. G., and Packer, L. (1997) Free Radic. Biol. Med. 22, 1115–1126

51. Gu, Y., Xu, Y. C., Wu, R. F., Nwaruak, E. F., Souza, R. F., Flores, S. C., and Terada, L. S. (2003) J. Biol. Chem. 278, 12710–12717

52. Brar, S. S., Kennedy, T. P., Sturrock, A. B., Huecksteadt, T. P., Quinn, M. T., Whorton, A. R., and Hoidal, J. R. (2002) Am. J. Physiol. 282, C1212–C1224

53. Sooriakumaran, P. (2006) Postgrad. Med. J. 82, 242–245