cAMP-response Element-binding Protein Mediates Acid-induced NADPH Oxidase NOX5-S Expression in Barrett Esophageal Adenocarcinoma Cells

Xiaoying Fu‡, David G. Beer§, Jose Behar‡, Jack Wands‡, David Lambeth¶, and Weibiao Cao1

From the 1Department of Medicine, Rhode Island Hospital and Brown Medical School, Providence, Rhode Island 02903, the 2Department of Surgery, Section of General Thoracic Surgery, University of Michigan Medical School, Ann Arbor, Michigan 48109, and the 3Department of Pathology, Emory University School of Medicine, Atlanta, Georgia 30322

Gastroesophageal reflux disease complicated by Barrett esophagus (BE) is a major risk factor for esophageal adenocarcinoma (EA). The mechanisms whereby acid reflux may accelerate the progression from BE to EA are not known. We found that NOX1 and NOX5-S were the major isoforms of NADPH oxidase in SEG1-EA cells. The expression of NOX5-S mRNA was significantly higher in these cells than in esophageal squamous epithelial cells. NOX5 mRNA was also significantly higher in Barrett tissues with high grade dysplasia than without dysplasia. Pulsed acid treatment significantly increased H2O2 production in both SEG1-EA cells and BE mucosa, which was blocked by the NADPH oxidase inhibitor apocynin. In SEG1 cells, acid treatment increased mRNA expression of NOX5-S, but not NOX1, and knockdown of NOX5 by NOX5 small interfering RNA abolished acid-induced H2O2 production. In addition, acid treatment increased intracellular Ca2+ and phosphorylation of cAMP-response element-binding protein (CREB). Acid-induced NOX5-S expression and H2O2 production were significantly inhibited by removal of extracellular Ca2+ and by knockdown of CREB using CREB small interfering RNA. Two novel CREB-binding elements TGACGAGA and TGACGCTG were identified in the NOX5-S gene promoter. Overexpression of CREB significantly increased NOX5-S promoter activity. Knockdown of NOX5 significantly decreased [3H]thymidine incorporation, which was restored by 10−13 M H2O2. Knockdown of NOX5 also significantly decreased retinoblastoma protein phosphorylation and increased cell apoptosis and caspase-9 expression. In conclusion, in SEG1 EA cells NOX5-S is overexpressed and mediates acid-induced H2O2 production. Acid-induced NOX5-S expression depends on an increase in intracellular Ca2+ and activation of CREB. NOX5-S contributes to increased cell proliferation and decreased apoptosis.

Esophageal adenocarcinoma has increased in incidence over the past 3 decades (1), at a rate exceeding that of any other cancer in the last 10 years (2, 3). Esophageal adenocarcinoma is characterized by a uniformly poor prognosis, with a median survival time following diagnosis of less than 18 months, and a 5-year survival rate of less than 20% in operable tumors (4). The major risk factor for esophageal adenocarcinoma is gastroesophageal reflux disease (GERD)2 complicated by Barrett esophagus (BE) (5). Approximately 10% of GERD patients develop BE, where esophageal squamous epithelium damaged by acid reflux is replaced by a metaplastic, intestinal type epithelium. The specialized intestinal metaplasia of BE is associated with a 30–125-fold increased risk for the development of esophageal adenocarcinoma, with the best estimates of cancer incidence of about 0.5–1.0% per year, i.e. one cancer per 100–200 patients for each year of observation (6–8). A middle-aged individual with BE for 20 years or more has an estimated 10–20% lifetime risk of developing esophageal adenocarcinoma, which is similar to the risk of lung cancer among heavy smokers or of liver cancer among chronic hepatitis B virus carriers (8). However, the mechanisms of the progression from metaplasia to adenocarcinoma are not fully understood.

Acid reflux may play an important role in the progression from metaplasia to dysplasia and to adenocarcinoma in patients with BE because of the following. 1) Cultured biopsy specimens of intestinal metaplastic cells demonstrate a significant increase in tritiated thymidine uptake when explants are briefly exposed to acid (9). 2) Long term inhibition of esophageal acid exposure by administration of proton pump inhibitors to patients with BE has been shown to decrease proliferation of metaplastic cells (10). 3) A prospective study has shown that proton pump inhibitor treatment significantly reduces the incidence of dysplasia in BE patients when compared with no therapy or treatment with H2 receptor antagonist (11).

2 The abbreviations used are: GERD, gastroesophageal reflux disease; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N′,N″,N‴-tetracetic acid; Fura 2AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxyl)-ethane-N,N′,N″,N‴-tetracetic acid pentaaacetoxymethyl; BE, Barrett esophagus; CREB, cyclic AMP-response element-binding protein; EA, esophageal adenocarcinoma; FBS, fetal bovine serum; GAPDH, glyceroldehyde-3-phosphate dehydrogenase; LCM, laser capture microdissection; NOX, NADPH oxidase; Rb, retinoblastoma protein; ROS, reactive oxygen species; RT, reverse transcription; siRNA, small interfering RNA; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco’s modified Eagle’s medium; RACE, rapid amplification of cDNA ends.
The mechanisms whereby acid reflux accelerates this progression are not known. 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) (12, 13). High levels of ROS are present in BE (14, 15) and in esophageal adenocarcinoma (13, 16). In addition, ROS levels are elevated, and antioxidant defenses are decreased in the metaplastic cells (14, 15), as evidenced by the reduced levels of glutathione and vitamin C and decreased activity of superoxide dismutase (15, 17). Besides metaplastic cells, other cells in BE mucosa may also produce ROS and affect metaplastic cells.

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 and p51phox or NOXA1) have been found in several cell types (18–20), 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 as follows: NOX5-S and NOX5-L (21). NOX5-L has EF-hand motifs at its N terminus (22), whereas NOX5-S does not (23). NOX5-L has the following four isoforms: α, β, δ, and γ (22). Whether these NADPH oxidases play a role in acid-induced H₂O₂ production has not been established, and the transcription factor responsible for NOX5 expression is not known.

We now show that NOX5-S is the major isoform of NADPH oxidase present in esophageal adenocarcinoma cells and that NOX5-S contributes to increased proliferation and decreased apoptosis of SEG1 esophageal adenocarcinoma cells. To our knowledge we are the first to report that cyclic AMP-response element-binding protein (CREB) is responsible for acid-induced expression of NOX5-S mediating acid-induced production of H₂O₂.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Acid Treatment**—Human esophageal squamous HET-1A cells (ATCC, Manassas, VA) were cultured in the bronchial epithelial cell medium (BEGM BulletKit, Cambrex, East Rutherford, NJ) containing a basal medium (BEBM) plus the additives (BEGM SingleQuots®) in wells precoated with a mixture of 0.01 mg/ml fibronectin, 0.03 mg/ml vitrogen 100, and fetal bovine serum (FBS).

Human Barrett adenocarcinoma cell lines SEG1 were derived from human esophageal Barrett adenocarcinomas (24) and generously provided by Dr. David Beer. These cells were cultured in DMEM containing 10% FBS and antibiotics.

Both cell lines were cultured at 37 °C in a 5% CO₂-humidified atmosphere. For acid treatment, cells were exposed to acidic DMEM, pH 4.0, DMEM (calcium-free and 1 mM EGTA), pH 4.0, or normal DMEM (control) for 1 h, washed, and cultured in fresh medium (pH 7.2, without phenol red) for an additional 24 h. The culture medium and cells were collected for measurements. Acidic DMEM (pH 4.0, 300 μl) was added to each well in a 12-well plate, and the final pH was about 4.9 after a 1-h incubation.

**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 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 half 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 (25, 26). Briefly, BE mucosal biopsy specimens were randomly assigned to acid, acid plus apocynin, acid plus vehicle, or control groups. The biopsy specimens were placed on a sterilized stainless wire mesh (Flynn & Enslow, Inc., San Francisco) 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) and perfused with 95% oxygen and 5% carbon dioxide and then cultured at 37 °C. Organ culture was performed in RPMI 1640 supplemented with 10% FBS, 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 with apocynin (10⁻⁴ M), 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. For the apocynin and vehicle group, apocynin (10⁻⁴ M) or vehicle was added to the culture medium in this additional 24-h culture. 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**—Twenty-four hours before transfection at 40–50% confluency, 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 per well). Transfection of siRNAs was carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. 75 pmol of siRNA duplex of NOX5 or CREB or scrambled siRNA formulated into liposomes were applied per well. 

**RT-PCR**—Total RNA was extracted by TRIzol reagent (Invitrogen) for the cultured cells and extracted by RNAqueous...
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**TABLE 1**

| Primers       | Product size | Primer                        |
|---------------|--------------|-------------------------------|
| NOX1          | 98           | 5′-TGGTCTACATGGCATAAATCTTTT-3′ (sense) |
|               |              | 5′-AAAACTATTGGCTCAATGTTT-3′ (antisense) |
| NOX2          | 94           | 5′-GAGTGTCAGCCCTACATCGTT-3′ (sense) |
|               |              | 5′-AGTAGAAAATGACATTCTGTCA-3′ (antisense) |
| NOX3          | 91           | 5′-TTCCTTCTGCGTACCTGGCT-3′ (sense) |
|               |              | 5′-ATGCCAAAGCTCTGTCAAG-3′ (antisense) |
| NOX4          | 76           | 5′-ATCCGAAAGCTCTGTCAAG-3′ (sense) |
|               |              | 5′-GTCATACGCTGTCGAGC-3′ (antisense) |
| NOX5          | 144          | 5′-AAGAATCTACACCGGCTACCTG-3′ (sense, 1F) |
|               |              | 5′-AGCTTCACTGCTGTCAGCC-3′ (antisense, 1R) |
| DUOX1         | 108          | 5′-AGGTTGAAGCTGCTGTCAGCC-3′ (sense) |
|               |              | 5′-AGGTTGAAGCTGCTGTCAGCC-3′ (antisense) |

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 were reverse-transcribed by using a kit SUPERSCRIPT™ first-strand synthesis system for RT-PCR (Invitrogen), or a SensScript RT kit (Qiagen, Valencia, CA), respectively. Primers used are shown in Table 1.

**Cloning of NOX5 in SEG1 EA Cells**—The primers used for cloning of NOX5 in SEG1 EA cells were as follows: ATGGGCTACG-TGGTAGGTGGG (2F), ATGGGGAACCTGACCATCAGC (3F), TTGGGCCCATAGAAAGTGAGCA (2R), GTTGAGCC-CACAGTTGACG (3R), AGCCGCCACTACAGTACGCC (4R), AGTGCCAGGCCGTAGTGC (5R), and TCTCTTTGAAAAATCTCG (6R). Three primers (3R for RT and 4R and 5R for nested PCR) were used to amplify the 5′-RACE kit (Invitrogen). PCR products were gel-extracted and sequenced by W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT).

**Construction of NOX5-S Reporter Plasmid Construct**—The fragment (−1396 to −6 from ATG) of the NOX5-S promoter was amplified by PCR by using SEG1-EA cell genomic DNA as template with the primers containing an Xhol restriction site at the 5′ end and an HindIII restriction site at 3′ end. The primers used for the PCR were AACAATCATGCAAGCTGACCGGTCTCACCACG and AAAAGCTTCCGGGAAGCGCTGACCGTC. The PCR products were digested with Xhol and HindIII, and cloned into the luciferase reporter plasmid, pGL3-Basic (Promega).

**Luciferase Assay**—Twenty four hours before transfection, SEG1 EA 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 luciferase reporter plasmid containing the NOX5-S promoter fragment. In co-transfection experiments, 0.6 μg of CREB expression vector or pcDNA 3.1 vector (Invitrogen) was used. CREB expression vector was generously provided by Dr. Marc R. Montminy (The Salk Institute for Biological Studies, San Diego) (27).

Luciferase activity was assayed 24 h 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. Data reflect the fold change in luciferase activity in experimental cells over cells co-transfected with empty pGL3-Basic.

**Quantitative Real Time PCR**—Quantitative real time PCR was carried out on a Stratagene Mx4000® multiplex quantitative PCR system (Stratagene, La Jolla, CA). The primers used were specific for NOX1 as follows: sense 5′-TGGTCTACATGGCATTAAACTTTT-3′ and antisense 5′-AAAACTATTGGCTCAATGTTT-3′; NOX5, sense 5′-AAGAATCTACACCGGCTACCTG-3′ and antisense 5′-AGGTTGAAGCTGCTGTCAGCC-3′; and GAPDH, sense 5′-CATGACCACGTCCCATGGCCA-3′ and antisense 5′-AGGTTCAACCACCTCTGTCGATCA-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, La Jolla, CA), each sense and antisense primer at 100 nm, 1 μl of cDNA, and 30 nm reference dye. Reactions were carried out in a Stratagene Mx4000® multiplex quantitative PCR system (Stratagene, La Jolla, CA) 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 value, defined as the point at which the fluorescence signal was statistically significant above background, was calculated for each ampiclon 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. Transcript level of each specific gene was normalized to GAPDH amplification.

**Western Blot Analysis**—Cells were lysed in Triton X lysis buffer containing 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 40 mM β-glycerol phosphate, 40 mM p-nitrophenyl phosphate, 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 the appropriate primary antibodies followed by 60 min of incubation in horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). Detection was achieved with an enhanced chemiluminescence agent (Amersham Biosciences). The primary antibodies used were as follows: phospho-CREB antibody (Ser-133) (1:1000), CREB antibody (1:1000), normal rabbit IgG (1:1000), human caspase-9 monoclonal antibody (1:1000), phospho-Rb (Ser-780) antibody (1:1000), Rb antibody (1:1000), and GAPDH antibody (1:2000). NOX5 antibody prepared against a mixture of unique NOX5 peptides (NH2-YES-FKAASPLGRGSKRC-COOH; and NH2-YRHQKRKHTCPS-COOH) was generously provided by Dr. David Lambeth (29) and was used at a dilution of 1:1,000.
Cytosolic Calcium Measurements—SENG1 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, Germany). 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 122.5 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 BME 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, bathing solution was changed twice with Ca2+-free medium after the cells had settled to the bottom of the chamber.

Ca2+ measurements were obtained using a modified dual excitation wavelength imaging system (IonOptix Corp. Milton, MA) as described previously (31). Ratiometric images were masked in the region outside the borders of the cell because 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 pseudosiosbestic 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 described in detail previously by Grynkiewicz et al. (32).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed using the ChIP assay kit (Upstate, Charlottesville, VA) following manufacturer’s protocol. Briefly, SEG1 cells grown in plastic dishes for 2 days (~1 × 106 cells) were treated with acidic medium, pH 4.0, for 1 h and then treated with 1% formaldehyde for 10 min to cross-link CREB to DNA. After removal of the formaldehyde, the cells were washed with ice-cold phosphate-buffered saline containing 0.1% EDTA and protease inhibitors (1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor mixture) and gently scraped into a conical tube, centrifuged for 5 min at 700 × g at 4°C. Pelleted cells were resuspended in 400 μl of lysis buffer (10 mM HEPES, pH 7.9, 60 mM KCl, 0.5% (v/v) Nonidet P-40) with protease inhibitors and incubated on ice for 10 min. Nuclei were recovered by centrifugation at 1000 × g for 10 min and resuspended in 400 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing protease inhibitors. The mixture was incubated on ice for 10 min, and the lysate was sonicated eight times for 10 s each time on ice to shear the genomic DNA to lengths of 0.2–1 kb. The debris was removed by centrifugation, and the supernatant was then diluted 10-fold with ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 16.7 mM NaCl, and protease inhibitors, pH 8.0). Five hundred microliters of the diluted lysate was kept for input control. The chromatin solution was precleared with salmon sperm DNA/protein A-agarose for 1 h at 4°C. Anti-CREB (Upstate) antibody was added, respectively, to the supernatant fraction and incubated overnight at 4°C with rotation. The mixture was then incubated with 60 μl of salmon sperm DNA/protein A-agarose slurry for 1 h at 4°C with rotation. Normal rabbit IgG (Upstate) or c-Myc antibody (SeroTec, Raleigh, NC) was used instead of the specific antibody for the negative control. The protein A-agarose-antibody-histone complex was pelleted by gentle centrifugation (1000 × g at 4°C for 1 min). The pellet was washed sequentially (3–5 min per wash) on a rotating platform with 1 ml each of low salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl, pH 8.0), high salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl, pH 8.0), LiCl washing buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After the final wash, the pellet was eluted by two 15-min incubations with 250 μl of freshly made elution buffer (1% SDS and 50 mM NaHCO3). Two fractions of elutes were combined, and 20 μl of 5 M NaCl was added to the supernatant. Cross-linking was reversed by heating at 65°C for 4 h, followed by the addition of 10 μl of 0.5 mM EDTA, 20 μl of 1 M Tris-HCl, pH 6.5, and 2 μl of 10 mg/ml proteinase K. The sample was incubated at 45°C for 2 h, and DNA was then extracted by phenol chloroform extraction followed by ethanol precipitation. The DNA pellet was resuspended in 50 μl of H2O, and 5 μl was used for PCR analysis. PCR was carried with the primer pairs that targeted the −192 to +103 region of the human NOX5 promoter (sense 5′-GGCAGTGGCTCCATGACC-3′ and antisense 5′-ATCCGGGTGATTCCTGCG-3′) at 94°C for 5 min, 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s for 35 cycles followed by a 7-min extension at 72°C. Results were visualized in 2% agarose gels stained with ethidium bromide.

Gel Mobility Shift Assay—Gel shift assay was performed using the gel shift assay kit (Promega, Madison WI) following the manufacturer’s protocol. Radiolabeled probe oligonucleotides derived from human NOX5 promoter (−103 to −82 and −123 to −102) were generated using end-labeling by phosphorylation with [γ-32P]ATP and T4 polynucleotide kinase. Probes were purified by centrifugation through Sephadex G-25 columns (Roche Applied Science). Gel shift assay was performed by incubating 2 μg of HeLa nuclear extract with 1 fmol of radio-labeled probe in a 10-μl reaction buffer containing 10 mM Tris, pH 7.5, 0.05 mg/ml poly(dI-dC)poly(dI-dC), 4% glycerol, 0.5 mM EDTA, 0.1 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1 mM magnesium chloride, and 1 mM sodium chloride for 20 min at room temperature. For competition experiments and supershift assay with normal rabbit IgG (control) or rabbit CREB antibody, the competing unlabeled probes or antibodies were preincubated for 20 min at room temperature with the nuclear extracts before the addition of the radiolabeled probes. The wild type competitor 1 (−123 to −102, NOX5-pWT1) (5′-TCGCTGCGTACGAGAGCTG-3′ and 3′-AGCGACGGACTGCTCTTGAC-5′) or mutant...
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FIGURE 1. ROS production in response to acid exposure. A and B, in organ-cultured Barrett mucosal biopsies (n = 3) (A) and in the human esophageal adenocarcinoma cell line SEG1 (n = 12–18) (B), pulsed acid treatment significantly increased H$_2$O$_2$ content in culture medium. The acid-induced H$_2$O$_2$ productions were significantly decreased by an NADPH oxidase inhibitor apocynin, suggesting that NADPH oxidase may participate in production of H$_2$O$_2$. C, in the human SEG1 cells (n = 5), pulsed acid treatment significantly increased superoxide content in culture medium. The acid-induced superoxide production was significantly decreased by an NADPH oxidase inhibitor apocynin, suggesting that NADPH oxidase may contribute to acid-induced production of superoxide.

A cryostat at 7 µm, mounted on precleaned nonadhesive glass slides, fixed for 1–2 min in ice-cold acetone, and stained for 15–30 s with HistoGene™ LCM frozen section staining kit (Arcturus Engineering, Mountain View, CA) to minimize the RNA degradation. As a preliminary step, the integrity of each tissue sample was checked by scraping a stained section from a slide and extracting the RNA with PicoPure™ RNA isolation kit (Arcturus). The quality was evaluated by using the Agilent Lab-on-a-Chip system with the BioAnalyzer 2100 (Agilent). When the ribosomal RNA was judged to be intact, fresh sections were cut and stained. Stained sections were dehydrated. Air-dried sections were subjected to LCM using an AutoPix automated LCM instrument (Arcturus). Total RNAs were extracted using PicoPure™ RNA isolation kit from the microdissected cells. The quality of the ribosomal RNA was examined as described above to ensure there was no RNA degradation.

Amplex® Red Hydrogen Peroxide Fluorescent Assay—Levels of H$_2$O$_2$ in culture medium were measured by using Amplex® Red H$_2$O$_2$ assay kit (Molecular Probes, Inc., Eugene, OR). This assay uses the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to detect H$_2$O$_2$. In the presence of peroxidase, the Amplex Red reagent reacts with H$_2$O$_2$ in a 1:1 stoichiometry to produce the red fluorescent oxidation product resorufin. Fluorescence is then measured with a fluorescence microplate reader using excitation at 540 nm and emission detection at 590 nm.

Measurement of Superoxide Production—The measurement of superoxide released by cells was performed by detection of ferricytochrome c reduction as described previously (33). Briefly, SEG1 EA cells were pretreated without or with 100 µM apocynin for 30 min. Cells were then treated with acidic medium, pH 4.0, or normal medium, pH 7.2, for 1 h, washed, and cultured at pH 7.2 for additional 4 h. Then 20 µmol/liter cytochrome c with or without 500 units/ml superoxide dismutase was added to the cells and incubated at 37 °C in a 5% CO$_2$ incubator for 1 h. The reaction medium was then removed, and absorbance was read in an ELISA plate reader at 550 nm. Superoxide production was calculated using an

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absorption coefficient of 21.1 mm^{-1}cm^{-1} and normalized to protein content.

**Protein Measurement**—The amount of protein was determined by colorimetric analysis (Bio-Rad Protein Assay) according to the method of Bradford (34).

**Materials**—Fura 2AM and BAPTA were purchased from Molecular Probes (Eugene, OR); [γ-32P]ATP and [3H]thymidine were from PerkinElmer Life Sciences; human NOX5 siRNA was from Ambion Inc. (Austin, TX); and CREB siRNA was from Upstate (Charlottesville, VA). Phospho-CREB antibody (Ser-133), CREB antibody, and normal rabbit IgG were bought from Upstate, and phospho-Rb (Ser-780) antibody was from Cell Signaling (Beverly, MA), and GAPDH antibody was from Ambion. Hydrocortisone, epidermal growth factor, cholera toxin, adenine, insulin, bovine pituitary extract, Triton X-100, Nonidet P-40, phenylmethylsulfonyl-fluoride, dl-dithiothreitol, basal medium Eagle (BME) amino acid supplement, HEPES sodium, and other reagents were purchased from Sigma.

**Statistical Analysis**—Data are expressed as means ± S.E. Statistical differences between two groups were determined by Student’s t test. Differences between multiple groups were tested using analysis of variance (ANOVA) and checked for significance using Fisher’s protected least significant difference test.

**RESULTS**

**Acid-induced Production of ROS**—Acid reflux has been suggested as a risk factor in the progression from Barrett metaplasia to adenocarcinoma (9, 35). Therefore, we examined whether acid treatment induces production of ROS. Acid treatment remarkably increased H2O2 production (Fig. 1A) in human Barrett biopsies as well as H2O2 and superoxide production in the esophageal adenocarcinoma cell line SEG1 (Fig. 1, B and C). The data suggest that pulsed acid treatment causes production of ROS in esophageal adenocarcinoma cells as well as in BE mucosal biopsies.

To determine whether ROS production induced by acid treatment is mediated by NADPH oxidases, we used the
NADPH oxidase inhibitor apocynin (36–38). Apocynin is thought to inhibit the translocation of NADPH oxidase subunits p47\textsuperscript{phox} and p67\textsuperscript{phox} from the cytosol to the membrane, thereby inhibiting the activity of NADPH oxidase (39). Apocynin significantly decreased the H\textsubscript{2}O\textsubscript{2} production in response to acid treatment both in Barrett mucosal biopsies (Fig. 1A) and in SEG1 cells (Fig. 1B). Apocynin also significantly decreased acid-induced superoxide production in SEG1 cells (Fig. 1C). These data suggest that NADPH oxidase may be involved in acid-induced ROS production. Vehicle had no effect on acid-induced H\textsubscript{2}O\textsubscript{2} production (data not shown).

**NADPH Oxidase as a Source of ROS in Esophageal Adenocarcinoma Cells**—RT-PCR demonstrated that NOX1 and NOX5 were the two NADPH oxidases expressed in SEG1 cells, with NOX5 having a much stronger signal than NOX1 (Fig. 2A). NOX1 and NOX5 were also detectable in nontransformed human esophageal squamous cell line HET-1A and in Barrett metaplastic cells obtained from esophageal mucosal biopsies using laser capture microdissection (data not shown).

NOX5 may be NOX5-L or NOX5-S. The sequence of NOX5-S is the same as the sequence at the 3’ end of NOX5-L. The primers (4F, 5’-GGAGGATGCCAGGTGGCTCCGGT-3’ and 4R) covering a region of NOX5-L, but not NOX5-S, were used to determine which NOX5 is present in esophageal adenocarcinoma cells. The 4F and 4R primers are also common to NOX5 isoforms \textsuperscript{2,3,4,5}. These primers detected longhand NOX5\textsuperscript{1} in human spleen (22). However, the two primers failed to detect the PCR product even after 35 PCR cycles in SEG1 cells (Fig. 2B), suggesting that NOX5 in adenocarcinoma cells is NOX5-S. To further confirm this result, we did 5’-RACE and Western blot analysis. Fig. 2C shows

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**FIGURE 3.** A, in SEG1 EA cells, NOX5 siRNA significantly decreased NOX5 mRNA expression ($n = 3–6$). B, typical example of Western blot analysis. C, summarized data showed that transfection with NOX5 siRNA significantly decreased NOX5-S protein expression in SEG1 cells ($n = 3$). D, NOX5 siRNA did not affect NOX1 mRNA expression ($n = 3$). These data confirmed that NOX5 siRNA knocked down NOX5 effectively and selectively. E, knockdown of NOX5 also significantly decreased production of H\textsubscript{2}O\textsubscript{2} ($n = 3$), suggesting that NOX5 may contribute to basal production of H\textsubscript{2}O\textsubscript{2}. Transfection of siRNAs was carried out with Lipofectamine 2000. Per well, 75 pmol of siRNA duplex of NOX5 or scrambled siRNA formulated into liposomes were applied. After a 4-h transfection, the transfection medium was replaced with regular medium. 36 h later, culture medium and cells were collected for measurements. *, $p < 0.03$, ANOVA, compared with scrambled group; **, $p < 0.001$, t test.
that only one band (around 150bp) was detected by 5' RACE using the primers abridged universal amplification primer and 5R (+11 to +31 from ATG). The size of this PCR product was consistent with NOX5-S but not with NOX5-L. NOX5-S in SEG1 cells was sequenced, and this sequence was 9 bp longer at the 5' end than previously reported (GenBank accession number AF317889) (data not shown). Western blot analysis detected a band between 50 and 75 kDa, which is consistent with the size of NOX5-S (64 kDa), further confirming that NOX5-S may be the major isoform of NOX5 present in SEG1 EA cells (Fig. 2D).

The levels of mRNA expression of NADPH oxidase were examined in different cell types by real time RT-PCR. NOX5 mRNA expression was significantly greater in the esophageal adenocarcinoma cell line SEG1, when compared with the esophageal squamous cell line HET-1A (Fig. 2F). In contrast, NOX1 mRNA levels were not statistically different between SEG1 and HET-1A (data not shown), suggesting that NOX5-S is selectively overexpressed in esophageal adenocarcinoma cells.

In addition, NOX5 was detectable in esophageal mucosal biopsies from Barrett patients with dysplasia by RT-PCR using primers common to NOX5-L and NOX5-S (1F and 1R) or specific to NOX5-L (4F and 4R) (data not shown). Real time PCR using primers 1F and 1R showed that levels of NOX5 mRNA were significantly higher in Barrett esophageal mucosa with high grade dysplasia than in normal mucosa and Barrett mucosa without dysplasia (Fig. 2F). However, the levels of NOX5-L were not different in these tissue biopsies (data not shown). These data suggest that both NOX5-L and NOX5-S may be present in Barrett mucosa with high grade dysplasia and that NOX5-S might be overexpressed in mucosal biopsies from Barrett patients with high grade dysplasia.

To examine whether NOX5-S contributes to basal production of $H_2O_2$, we used NOX5 siRNA to knock down NOX5. NOX5 siRNA significantly decreased NOX5-S expression at the mRNA level (Fig. 3A) as well as at the protein level (Fig. 3, B and C) 24 h after transfection, but did not affect NOX1 expression (Fig. 3D), confirming that NOX5 siRNA knocked down NOX5-S effectively and selectively. Knockdown of NOX5-S significantly decreased basal production of $H_2O_2$ (Fig. 3E), suggesting that NOX5-S contributes to basal production of $H_2O_2$.

Role of NOX5-S in Acid-induced Production of ROS—To identify which isoform(s) of NADPH oxidases is (are) responsible for ROS production induced by acid treatment, we examined mRNA expression of NADPH oxidases with or without acid treatment. Because only NOX1 and NOX5-S were found in esophageal adenocarcinoma cells, as shown in Fig. 2A, we focused on NOX1 and NOX5-S. Acid treatment significantly increased NOX5-S expression in a time-dependent manner (Fig. 4A), without changing that of NOX1 (Fig. 4B), suggesting that NOX5-S may be responsible for acid treatment-induced $H_2O_2$ production in esophageal adenocarcinoma cells. To confirm this result, we transfected the SEG1 cells with NOX5 siRNA to knock down expression of NOX5. Transfection with NOX5 siRNA inhibited both acid-induced up-regulation of NOX5-S (Fig. 4C) and production of $H_2O_2$ (Fig. 4D), when compared with the control cells transfected with scrambled NOX5 siRNA. These data suggest that acid-induced NOX5-S up-regulation may mediate increased $H_2O_2$ production in esophageal adenocarcinoma cells.

$Ca^{2+}$ and CREB-mediated Up-regulation of NOX5-S—We found that exposure of Fura 2AM-loaded SEG1 cells to acidic physiologic salt solution, pH 4, for 30 min significantly increased intracellular $Ca^{2+}$ concentration (Fig. 5A and B). This $Ca^{2+}$ increase was significantly reduced by removal of extracellular calcium with a $Ca^{2+}$-free medium plus 200 $\mu M$ BAPTA. These data suggest that acid treatment increases intracellular calcium. In addition, removal of extracellular $Ca^{2+}$ significantly reduced acid-induced NOX5-S expression (Fig. 5C) as well as production of $H_2O_2$ in SEG1 esophageal adenocarcinoma cells (Fig. 5D), suggesting that increased intracellular $Ca^{2+}$ mediates acid-induced NOX5-S expression and $H_2O_2$ production.

The transcription factors regulating expression of NOX5-S are not known. Because exposure to acid increases the levels of intracellular calcium, it is likely that calcium-dependent transcription factors may be involved in NOX5-S expression. After analyzing the genomic DNA sequence of NOX5, we found two possible cAMP-response elements in the NOX5-S promoter, TGACGAGA and TGACGCTG.
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Therefore, we examined the role of CREB in acid-induced NOX5-S expression by knocking down CREB using CREB siRNA.

We found that acid treatment significantly increased CREB expression (Fig. 6A). In addition, co-transfection of SEG1 cells with CREB siRNA significantly decreased the basal levels of CREB mRNA (Fig. 6A) and CREB protein (Fig. 6, B and C). In addition, CREB siRNA significantly reduced acid-induced CREB mRNA expression in SEG1 cells (Fig. 6A). These data suggest that CREB siRNA effectively knocked down CREB. More importantly, knockdown of CREB with CREB siRNA blocked acid-induced NOX5-S expression (Fig. 6D) and H₂O₂ production (Fig. 6E). Furthermore, acid treatment significantly increased CREB phosphorylation (Fig. 7, A and B) in a time-dependent manner, and this increased phosphorylation was reduced by removal of extracellular calcium (Fig. 7, C and D). These data suggest that CREB may be involved in acid-induced NOX5-S expression.

We examined CREB binding to two possible CREs in the NOX5-S promoter by ChIP assay. NOX5 DNA was detectable in the immunoprecipitated chromatin sample of SEG1 cell lysate by PCR using the primers targeting the −192 to +103 (position from ATG) region of the NOX5-S promoter (Fig. 8). This pair of primers covers two possible CREB-binding sites as described above. The PCR product was sequenced and is specific for the NOX5-S promoter. The data suggest that CREB binds to the NOX5-S promoter. This result was confirmed by gel mobility supershift assay. In the gel shift assay, one prominent complex was detectable with either the oligonucleotide NOX5-pWT1 (containing TGACGAGA; see Fig. 9A) or NOX5-pWT2 (containing TGACGCTG; see Fig. 9B). Competition experiments with increasing concentrations of either unlabeled (cold) NOX5-pWT1 oligonucleotide or unlabeled NOX5-pWT2 significantly reduced binding. The addition of the mutant oligonucleotides NOX5-pMUT1 or NOX5-pMUT2 had less effect on binding (Fig. 9, A and B). In the supershift assay, the supershifted band was detected with a CREB antibody but not with immunoglobulin G (Fig. 9C). These data suggest that CREB binds to the sites TGACGAGA and TGACGCTG on the NOX5-S promoter region. To further confirm the role of CREB in the NOX5-S expression, a reporter plasmid of NOX5-S was generated by ligating a NOX5-S promoter fragment (−1396 to −6 from ATG) into the pGL3-basic vector. Overexpression of CREB caused a 5.3-fold increase in luciferase activity (Fig. 10), indicating CREB induced activation of NOX5-S promoter.

NOX5-derived ROS in Cell Proliferation and Apoptosis—We next examined the role of NOX5-derived ROS in cell proliferation and apoptosis in esophageal adenocarcinoma cells. Knockdown of NOX5-S significantly decreased basal [³H]thymidine incorporation (Fig. 11A). In cultured biopsy specimens of intestinal metastatic cells, it has been shown that pulsed acid treatment increases cell proliferation (9). SEG1 cells were therefore exposed to acidic medium at pH 4.0 for 1 h and then cultured at pH 7.2 for an additional 24 h. Pulsed acid treatment significantly increased [³H]thymidine incorporation, and the increase was blocked by knockdown of NOX5-S (Fig. 11A), suggesting that NOX5-S may mediate increased cell proliferation in SEG1 cells. To test the role of H₂O₂ in cell proliferation, SEG1 EA cells were treated with different concentration of H₂O₂ for 24 h. Low doses of H₂O₂ significantly increased [³H]thymidine incorporation. However, high doses of H₂O₂ significantly decreased [³H]thymidine incorporation (Fig. 11B), suggesting that NOX5-S may mediate increased cell proliferation in SEG1 cells. To test the role of H₂O₂ in cell proliferation, SEG1 EA cells were treated with different concentration of H₂O₂ for 24 h. Low doses of H₂O₂ significantly increased [³H]thymidine incorporation. However, high doses of H₂O₂ significantly decreased [³H]thymidine incorporation (Fig. 11B),
suggesting that low doses of H_2O_2 stimulate cell proliferation, whereas high doses of H_2O_2 inhibit cell proliferation. In addition, the decrease in cell proliferation induced by knockdown of NOX5 was restored by 10^{-13} M H_2O_2 (Fig. 11C), suggesting that NADPH oxidase NOX5-S-derived H_2O_2 production may contribute to increased cell proliferation in esophageal adenocarcinoma cells. To further confirm the role of NOX5-S in cell proliferation, we examined the phosphorylation of Rb protein, a key protein in the regulation of a restriction point in the G_1 phase of the cell cycle. Similarly, knockdown of NOX5-S significantly decreased basal Rb phosphorylation and blocked acid-induced Rb phosphorylation (Fig. 11, D and E), further supporting a key role of NOX5-S in cell proliferation. Conversely, knockdown of NOX5-S by NOX5 siRNA significantly increased cell apoptosis (Fig. 12A) and the expression of caspase-9 (Fig. 12, B and C) in SEG1 esophageal adenocarcinoma cells, suggesting that NOX5 causes decreased apoptosis in esophageal adenocarcinoma cells.

DISCUSSION

The major risk factor for esophageal adenocarcinoma is GERD complicated by BE (5). Both GERD and Barrett esophagus are characterized by the presence of inflammatory cells. Recent data suggest that ROS may play an important role in reflux esophagitis. ROS include superoxide radical anions (O_2^-), H_2O_2, singlet oxygen (^1O_2), hydroxyl radical (OH^-), and hypochlorous acid (HOCl). O_2^- in aqueous solution is short lived and rapidly dismutated to the much more stable molecule H_2O_2 through either enzymatic or nonenzymatic mechanisms. Thus in most biological systems, generation of O_2^- usually results in the formation of H_2O_2, which diffuses across biological membranes, whereas O_2^- does not dismutate to H_2O_2 (40). H_2O_2, in turn, may generate more highly reactive oxygen species such as the free radicals OH^- and HOCl (12).

ROS levels are elevated in esophageal tissues of patients with esophagitis (14) and are correlated with the severity of esopha-
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A role of ROS in esophagitis is further supported by data from animal models (42, 43) indicating that ROS scavengers improve esophagitis. These data suggest that acid-induced inflammation induces production of ROS. Inflammation characterized by inflammatory cell infiltration is also present in BE (44), and levels of proinflammatory cytokines interleukin-1β and tumor necrosis factor-α are increased in BE (44, 45), suggesting that the inflammatory process persists despite the columnar epithelium being more resistant to the actions of gas-troduodenal reflux.

It has been reported that levels of ROS are increased in BE (14, 15) and in adenocarcinoma (13, 16). In addition, ROS may cause damage to DNA, RNA, lipids, and proteins, which may result in increased mutation and altered functions of enzyme and proteins (e.g. activation of oncogene products and/or inhibition of tumor suppressor proteins) (12, 13). Therefore, ROS may play an important role in the development of esophageal adenocarcinoma. However, the sources of ROS in these conditions have not been defined so far. In this study, we showed that NADPH oxidase isoforms NOX1 and NOX5 were present in esophageal adenocarcinoma cells (Fig. 2A) and that NOX5 was shorthand NOX5 (NOX5-S; Fig. 2, B–D). NOX5-S has much higher levels in esophageal adenocarcinoma cells than in normal esophageal squamous epithelial cells (Fig. 2E). In addition, knockdown of NOX5 by NOX5 small interfering RNA significantly reduced H2O2 production in SEG1 EA cells (Fig. 3E), suggesting that NOX5-S may contribute to the basal production of ROS in esophageal adenocarcinoma cells. Our finding that esophageal adenocarcinoma cells contained NOX1 and NOX5-S indicates that one cell type may contain more than one NOX, which is consistent with the literature showing that DU145 prostate cancer cells contain NOX2 and NOX5 (29).

NOX5 was also detectable in esophageal mucosal biopsies from Barrett patients with dysplasia by RT-PCR using primers common to NOX5-L and NOX5-S (1F and 1R) or specific to NOX5-L (4F and 4R). Real-time PCR using primers common to NOX5-S and NOX5-L showed that levels of NOX5 mRNA were significantly higher in Barrett esophageal mucosa with high grade dysplasia than in normal mucosa and Barrett mucosa without dysplasia (Fig. 2F). However, the levels of NOX5-L were not different in these tissue biopsies. These data suggest that both NOX5-L and NOX5-S may be present in Barrett mucosa with high grade dysplasia and that NOX5-S might be overexpressed in mucosal biopsies from Barrett patients with high grade dysplasia. However, the sources of NOX5 in Barrett mucosal tissues need to be further explored.

Acid reflux has been suggested as a risk factor in the progression from Barrett metaplasia to adenocarcinoma (9, 35). It has been shown that luminal acid penetrates into the most superficial portion of the esophageal squamous epithelium (46). Acid reduces extracellular pH on the surface of the esophageal epithelium and reduces the intracellular pH of esophageal epithelial cells (47). Acid exposure may play an important role in this progression in patients with BE as discussed in the Introduction. Therefore, we examined whether acid treatment induces production of ROS and whether NADPH oxidases contribute to acid-induced H2O2 production.

We found that acid treatment significantly increased ROS production in human Barrett biopsies (Fig. 1A) and in the esophageal adenocarcinoma cell line SEG1 (Fig. 1, B and C). Acid-induced ROS production in Barrett mucosal biopsies (Fig. 1A) and in SEG1 cells (Fig. 1, B and C) was significantly decreased by apocynin, an inhibitor of NADPH oxidases. In addition, acid treatment significantly increased NOX5 expression in SEG1 cells (Fig. 4A) but not NOX1 (Fig. 4B). Knockdown of NOX5-S with NOX5-S siRNA significantly inhibited acid-induced production of H2O2 in SEG1 EA cells (Fig. 4D). These data suggest that in SEG1 EA cells acid-induced H2O2 production may be mediated by NADPH oxidase NOX5-S. The data that apocynin blocked acid-induced ROS production in SEG1 cells suggest that NOX5-S might need other regulatory subunits in order to function. In this respect, NOX5-S is...
The mechanisms of acid-induced NOX5-S up-regulation and H2O2 production are not known. We found that acid treatment significantly increased the intracellular Ca2+/H11001, and this Ca2+/H11001 increase was significantly reduced by removal of extracellular calcium with a Ca2+/H11001-free medium plus 200 μM BAPTA (Fig. 5A and B). In addition, removal of extracellular Ca2+ significantly reduced acid-induced up-regulation of NOX5 and production of H2O2 (Fig. 5, C and D) in SEG1 EA cells, suggesting that increased intracellular Ca2+ mediates acid-induced NOX5-S expression and H2O2 production. How acid increases intracellular Ca2+ in SEG1 cells is not clear. In pulmonary smooth muscle cells reduction of extracellular pH reduces the membrane potential, causing membrane depolarization (48). In cortical neurons reduction of extracellular pH increased intracellular Ca2+ through Ca2+ influx (49). In rat aorta sustained contraction induced by acidic pH was significantly reduced by verapamil, an inhibitor of voltage-gated Ca2+ channels, suggesting that acidic pH may cause Ca2+ influx through activation of these Ca2+ channels (50). Therefore, it is possible that reduction of extracellular pH may activate Ca2+ channels in the cell membrane, causing Ca2+ influx. Because removal of extracellular Ca2+ did not completely block intracellular Ca2+ increase, Ca2+ release from intracellular stores might also be involved in the increased Ca2+ signal.

The transcription factors regulating expression of NOX5 are not known. Because exposure to acid increases the levels of different from NOX5-L, which has been shown to be activated by calcium directly (22).
intracellular calcium, it is likely that calcium-dependent transcription factors may be involved in NOX5 expression. After analyzing the genomic DNA sequence of NOX5, two possible cAMP-response elements in the NOX5-S promoter were found: TGACGAGA and TGACGCTG. Therefore, we focused on CREB. CREB is a Ca\(^{2+}\)-dependent and ubiquitous transcription factor, which binds the consensus CRE DNA sequence TGACGTCA (27, 51). CREB may also bind similar DNA sequences at the promoter region. For example, TGACGTTT is the c-Fos CRE (52). CREB binds DNA through its basic region and homodimerizes or heterodimerizes with closely related family members through its leucine zipper motif (53). The ability of CREB to activate transcription requires its phosphorylation on serine 133 (27).

We found that knockdown of CREB with CREB siRNA blocked acid-induced NOX5-S expression (Fig. 6D) and \(\text{H}_2\text{O}_2\) production (Fig. 6E), and that acid treatment significantly increased CREB phosphorylation in SEG1 EA cells (Fig. 7A), suggesting that CREB may be responsible for acid-induced NOX5-S expression in SEG1 EA cells. In the ChIP assay, the promoter region of NOX5-S DNA was detectable in the immunoprecipitated chromatin sample of the SEG1 cell lysate (Fig. 8), indicating that CREB binds to the NOX5-S promoter. Gel mobility shift assay showed that the prominent complexes were formed between nuclear extracts and oligonucleotides containing the above two binding sites (Fig. 9, A and B). In supershift assay, the supershifted band was observed with a CREB antibody but not with immunoglobulin G (Fig. 9C), suggesting that the complex may be formed by CREB and oligonucleotides. Competition experiments with increasing concentrations of

**FIGURE 10.** A reporter plasmid of NOX5-S (NOX5P) was generated by ligating a NOX5-S promoter fragment (−1396 to −6 from ATG) into the pGL3-basic vector. Overexpression of CREB caused a 5.3-fold increase in luciferase activity in SEG1 EA cells, indicating CREB induced activation of NOX5-S promoter. \(n = 3\). ***, \(p < 0.0001\); t-test.

**FIGURE 11.** A, in SEG1 Barrett adenocarcinoma cells, knockdown of NOX5 by NOX5 siRNA for 36 h significantly decreased \[^3\]H\]thymidine incorporation at the basal condition. Acid treatment significantly increased \[^3\]H\]thymidine incorporation, an increase that was blocked by knockdown of NOX5, suggesting that NOX5 may mediate increased cell proliferation in SEG1 cells. \(n = 4\). B, SEG1 EA cells were treated with different concentrations of \(\text{H}_2\text{O}_2\) for 24 h. Low doses of \(\text{H}_2\text{O}_2\) significantly increased \[^3\]H\]thymidine incorporation. However, high doses of \(\text{H}_2\text{O}_2\) significantly decreased \[^3\]H\]thymidine incorporation, suggesting that low doses of \(\text{H}_2\text{O}_2\) stimulate cell proliferation, whereas high doses of \(\text{H}_2\text{O}_2\) inhibit cell proliferation. \(n = 3\). C, in SEG1 EA cells, the decrease in cell proliferation induced by knockdown of NOX5 was restored by 10\(^{-13}\) \(\text{M}\) \(\text{H}_2\text{O}_2\) (\(n = 4\)), suggesting that NADPH oxidase NOX5-S-derived \(\text{H}_2\text{O}_2\) production contributes to increased cell proliferation in esophageal adenocarcinoma cells. D, in SEG1 cells, a typical Western blot analysis; E, summarized data (\(n = 4\)) showed that, in the basal condition, pH 7.2, knockdown of NOX5 significantly decreased Rb phosphorylation in SEG1 cells. Acid treatment at pH 4 significantly increased Rb phosphorylation, and this increase was blocked by knockdown of NOX5, suggesting that NOX5 may mediate Rb phosphorylation in esophageal adenocarcinoma cells. ANOVA: ***, \(p < 0.0001\); **, \(p < 0.01\); *, \(p < 0.05\), compared with pH 7.2 scrambled group; &, \(p < 0.01\), compared with pH 4 scrambled group; #, \(p < 0.001\), compared with untreated (control) group. The percentage of change was calculated by the ratio of the difference between untreated (control) and treated group over control group.
unlabeled (cold) oligonucleotides significantly reduced binding, whereas addition of the mutant oligonucleotides had less effect on binding (Fig. 9, A and B). These data suggest that CREB binds to the sites TGACGAGA and TGACGCTG on the NOX5-S promoter region. Overexpression of CREB significantly increased NOX5-S promoter activity (Fig. 10), indicating CREB induced activation of the NOX5-S promoter.

NOX5-derived production of ROS may contribute to the increased cell proliferation and decreased apoptosis in esophageal adenocarcinoma cells because of the following. 1) Knockdown of NOX5 significantly decreased thymidine incorporation (Fig. 11A) and retinoblastoma protein phosphorylation (Fig. 11, B and C). 2) Low doses of H$_2$O$_2$ significantly increased thymidine incorporation (Fig. 11B) and restored the reduction in thymidine incorporation induced by knockdown of NOX5 (Fig. 11C). 3) Knockdown of NOX5 significantly increased apoptosis (Fig. 12A) and caspase-9 expression (Fig. 12, B and C). Our data are consistent with other reports showing that blockade of NOX5 inhibits cell proliferation and increases apoptosis in the prostate cancer cell line DU145 (41) and that blockade of NOX4 by transfection of NOX4 antisense oligonucleotide decreases melanoma cell proliferation (49).

We conclude that NADPH oxidase NOX5-S is overexpressed in esophageal adenocarcinoma cells and mediates acid-induced H$_2$O$_2$ production. Acid-induced NOX5-S expression depends on an increase in intracellular calcium and activation of CREB in SEG1 EA cells. NOX5-S contributes to increased cell proliferation and decreased apoptosis in these cells. It is possible that acid reflux present in patients with Barrett esophagus may cause an increase in intracellular Ca$^{2+}$ in metaplastic cells and activation of CREB, causing up-regulation of NOX5-S. Overproduction of ROS derived from up-regulation of NOX5-S may increase cell proliferation and decrease apoptosis contributing to progression from intestinal metaplasia (Barrett esophagus) to dysplasia and to adenocarcinoma. Our data suggest that complete acid suppression using high dose proton pump inhibitors in BE patients with or without symptoms may be important for prevention of this progression. Elucidating the pathways leading from acid exposure to increased ROS production, increased proliferation, and decreased apoptosis may provide a number of potentially useful therapeutic targets.

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