Role of Rac1 in regulation of NOX5-S function in Barrett’s esophageal adenocarcinoma cells

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Hong J, Resnick M, Behar J, Wands J, DeLellis RA, Cao W. Role of Rac1 in regulation of NOX5-S function in Barrett’s esophageal adenocarcinoma cells. Am J Physiol Cell Physiol 301: C413–C420, 2011. First published April 27, 2011; doi:10.1152/ajpcell.00027.2011.—We have shown that a novel NADPH oxidase isoform, NOX5-S, is the major isoform of NADPH oxidases in an esophageal adenocarcinoma (EA) cell line, FLO, and is overexpressed in Barrett’s mucosa with high-grade dysplasia. NOX5-S is responsible for acid-induced reactive oxygen species production. In this study, we found that mRNA levels of NOX5-S were significantly higher in FLO EA cells than in the normal human esophageal squamous cell line HET-1A or in a Barrett cell line, BAR-T. The mRNA levels of NOX5-S were also significantly increased in EA tissues. The data suggest that NOX5-S may be important in the development of EA. Mechanisms of functional regulation of NOX5-S are not fully understood. We show that small G protein Rac1 was present in HET-1A cells, BAR-T cells, and EA cell lines FLO and OE33. Rac1 protein levels were significantly higher in FLO and OE33 cells than in HET-1A or BAR-T cells. Knockdown of Rac1 with Rac1 small interfering RNA significantly decreased acid-induced increase in H2O2 production in FLO EA cells. Overexpression of constitutively active Rac1 significantly increased H2O2 production, an increase that was blocked by knockdown of NOX5-S. By immunofluorescence staining and immunoprecipitation, we found that NOX5-S was present in the cytosol of FLO EA cells and colocalized with Rac1 and SERCA1/2 Ca2+-ATPase which is located in the endoplasmic reticulum membrane. We conclude that Rac1 may be important in activation of NOX5-S in FLO EA cells.

Barrett’s esophagus; SERCA1/2 Ca2+-ATPase

ESOPHAGEAL ADENOCARCINOMA (EA) has increased in incidence over the past four decades (6, 39). It is characterized by a poor prognosis, with a median survival time following diagnosis of <18 mo, and a 5-yr survival rate of <20% in operable tumors (44). The major risk factor for EA is gastroesophageal reflux disease (GERD) complicated by Barrett’s esophagus (BE) (26). 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 is associated with a 30- to 125-fold increased risk for the development of esophageal adenocarcinoma, with an estimated cancer incidence of about 0.5–1.0% per year, i.e., one cancer per 100–200 patients for each year of observation (14, 16, 49). A middle-aged individual with BE for 20 yr 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 (49). However, the mechanisms responsible for the progression from metaplasia to adenocarcinoma are not known.

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 (21); 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 (32); 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 antagonists (10).

We have found that a novel NADPH oxidase isoform, NOX5-S, is the major isoform of NADPH oxidases in FLO EA cells and is overexpressed in Barrett’s mucosa with high-grade dysplasia. This NOX5-S isoform is responsible for the acid-induced reactive oxygen species (ROS) production (13). The carcinogenesis of ROS has been shown in an animal model of hepatocellular carcinoma (15) and in other types of cancer such as colon cancer (1, 35). The levels of ROS are increased in BE and EA (18, 31, 48).

We have also shown that pulsed acid exposure causes upregulation of NOX5-S in EA cells and increases ROS production through a cytosolic Ca2+ increase and activation of the cAMP response element-binding protein (CREB) (13). Overproduction of ROS, derived from upregulation of NOX5-S, increases cyclooxygenase-2-derived prostaglandin E2 production (40) and downregulates a tumor suppressor gene, p16 (19), thus increasing cell proliferation and decreasing apoptosis. These changes may contribute to progression from BE to dysplasia and to adenocarcinoma. However, the regulatory proteins required for NOX5-S function are not fully understood.

The small G protein, Rac1, is thought to play a role in activation of NOX2 (37). In this study, we show that Rac1 is present in esophageal adenocarcinoma cells and that acid-induced H2O2 production and NOX5-S activation depends on Rac1. NOX5-S is mainly expressed in the cytosol of FLO EA cells and coupled with SERCA1/2 Ca2+-ATPase, which is located in the endoplasmic reticulum (ER).

MATERIALS AND METHODS

Cell culture and acid treatment. Human Barrett’s adenocarcinoma cell line FLO was derived from human Barrett’s esophageal adenocarcinoma (23) and generously provided by Dr. David Beer (Univer-

sity of Michigan, Ann Arbor). These cells were cultured in DMEM containing 10% fetal bovine serum and antibiotics. For acid treatment, cells were exposed to acidic DMEM (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 ~4.9 after 1-h incubation.

Human Barrett’s cell line BAR-T was derived from esophageal mucosal biopsies of patients with BE (intestinal metaplasia) and generously provided by Dr. Rhonda Souza and Dr. Stuart J. Spechler (VA North Texas Health Care System and University of Texas Southwestern Medical Center, Dallas, TX). BAR-T cells were immortalized with telomerase, as described previously (24), and 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 mM chola toxin, 20 μg/ml adenine, 5 μg/ml insulin, 70 μg/ml bovine pituitary extract, and antibiotics.

Human Barrett’s adenocarcinoma cell line OE33 was cultured in DMEM containing 10% fetal bovine serum and antibiotics. All of the cell lines were cultured at 37°C in a 5% CO₂ humidified atmosphere.

Esophageal tissues. Mucosal tissues were obtained from patients with documented BE undergoing endoscopy for cancer surveillance or from patients with EA undergoing esophagogastrectomy. Patients enrolled were informed of the study and written consent forms to use patients’ biopsies were obtained. The experimental protocols and consent form were approved by the Human Research Institutional Review Committee at Rhode Island Hospital.

Construction of pCDNA3.1-green fluorescent protein-NOX5-S plasmid. The NOX5-S expression plasmid (pCMV-tag5a-NOX5-S) was generously provided to us by David Lambeth (Emory University School of Medicine, Atlanta, GA). The full cDNA fragment of NOX5-S was digested from pCMV-tag5a-NOX5-S and then subcloned into pCDNA3.1-enhanced green fluorescent protein (EGFP)-RhOa (42) (plasmid no. 12965, Addgene, Cambridge, MA) to replace RhOa between EcoRI and Xhol. The final recombinant plasmid pCDNA3.1-EGFP-NOX5-S was verified by sequencing.

Human immunodeficiency virus and plasmid transfection. Twenty-four hours before transfection at 70–80% 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 small interfering RNAs (siRNAs) and plasmid transfections were conducted in a 2 μl volume containing 1× transfection reagent (Invitrogen) and without phenol red for an additional 24 h. Finally, cells were exposed to acidic medium, 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 ~4.9 after 1-h incubation.

For transfection of pCDNA3.1-GFP-NOX5-S plasmid, FLO cells were transfected with pCMV-tag5a-NOX5-S, using Amaxa-Nucleofector-System (Lonza) according to the manufacturer’s instructions. Amaxa-Nucleofector-System (Lonza) according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were prepared for immunofluorescence experiments. Transfection efficiencies were determined by fluorescence microscopy after transfection of pmax-GFP (Lonza).

Reverse transcription-PCR. Total RNA was extracted by TRIzol reagent (Invitrogen) for the cultured cells and purified by the total RNA purification system (Invitrogen). According to the protocol of the manufacturer, 1.5 μg of total RNAs from cultured cells was reversely transcribed by using a SUPERSCRIPT kit first-strand synthesis system for reverse transcription-PCR (Invitrogen).

Quantitative real-time PCR. Quantitative real-time PCR was carried out on a Stratagene MX4000 multiplex quantitative PCR system. The primers used were the following: NOX5 sense (5′-AAGACTCATCACGGGGGCTGCA-3′), NOX5 antisense (5′-CTTCCAGCACCTGGCGGAGCA-3′), GAPDH sense (5′-ATGACCAGTGTCGCAATC-3′), and GAPDH antisense (5′-AGGTCCACACCCCTGTTGGTGTGA-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), and the concentrations of each sense and antisense primer were 100 nM, 1 μl cDNA, and 30 nM reference dyes. 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.

Immunoprecipitation. SERCA1 or SERCA2 Ca²⁺-ATPase body (5 μg) was incubated with 500 μl phosphate-buffered saline (PBS) containing 40–50 μl of suspended IP matrix (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C on a rotator overnight as recommended by the manufacturer. IP matrix was collected by centrifugation.

FLO EA cells were lysed in Triton X-100 lysis buffer containing 50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (vol/vol) Triton X-100, 20 mM β-mercaptoethanol, 70 μg/ml aprotinin, and 1 μg/ml leupeptin. The supernatants were mixed with SERCA1 or SERCA2 antibody-IP matrix complex and incubated at 4°C on a rotator for 5 h. The immunoprecipitates were recovered by centrifugation for 5 min at 700 g, washed three times with cell lysis buffer, mixed with SDS loading buffer (Sigma), and heated at 100°C for 5 min.

Western blot analysis. Cells were lysed in Triton X-100 lysis buffer. The suspension was centrifuged at 15,000 g for 5 min, and the protein concentration in the supernatant was determined. Western blot analysis was done as described previously (9). Briefly, after these supernatants were subjected to SDS-PAGE, the separated proteins were electrophoretically transferred to a nitrocellulose membrane at 100 V, 1 h. 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 (Amersham Biosciences).

Primary antibodies used were as follows: Rac1 antibody (1:1,000, Santa Cruz Biotechnology) and NOX5 antibody (1:1,000). NOX5 antibody prepared against a mixture of unique NOX5 peptides (NH2-YESFKASDPLGRGSKRC-COOH and NH2-YRHQKRKHTCPS-COOH) was generously provided by Dr. David Lambeth (45).
Immunofluorescence staining and image analysis. FLO EA cells were grown to 50% confluence on glass coverslips. The coverslips were removed from medium and washed in PBS before fixation in 4% paraformaldehyde for 20 min at room temperature (RT). Antigen retrieval was performed by submerging the coverslips in 100 mM Tris, pH 9.5, 5% urea for 10 min at 95°C. The coverslips were rinsed in PBS and placed in 0.1% Triton X-100 for 10 min at RT. Nonspecific antibody binding was blocked by placing the coverslips in 10% normal goat serum for 30 min at RT. Coverslips from each cell line were then exposed for 1 h at RT to buffer (control) or primary antibody consisting of anti-SERCA1 or anti-Rac1. Coverslips were washed three times for 2 min each in PBS. Coverslips were then exposed for 45 min at RT to a secondary antibody cocktail containing Alexa Fluor 594 goat anti-mouse IgG2b-specific antibody (Molecular Probes). Coverslips were washed three times for 2 min in PBS before mounting in Vectashield Mounting Medium with DAPI (Vector Laboratories; Burlingame, CA). Cell images were recorded using a Nikon C1si confocal microscope equipped with three diode lasers that excite at 402, 488, and 561 nm (Nikon Instrument Group; Melville, NY).

Amplex red hydrogen peroxide fluorescent assay. Levels of H2O2 in culture medium were determined by using the Amplex Red H2O2 assay kit (Molecular Probes, Eugene, OR). This assay uses the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to detect H2O2. In the presence of peroxidase, the Amplex Red reagent reacts with H2O2 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 550 nm and emission detection at 590 nm.

Protein measurement. The amount of protein was determined by colorimetric analysis (Bio-Rad) according to the method of Bradford (8).

Materials. Human Rac1 siRNA was purchased from Dharmacon (Lafayette, CO). Human NOX5 siRNA was purchased from Ambion (Austin, TX). Rac1 antibody and GAPDH antibody were bought from Santa Cruz Biotechnology. SERCA1 antibody was purchased from BIOMOL (Plymouth Meeting, PA). Triton X-100, α-dithiothreitol, HEPES sodium, basal medium Eagle’s amino acid supplement, and other reagents were purchased from Sigma.

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

Fig. 1. Expression of NOX5-S in different cell lines and tissues. A: real-time RT-PCR shows that levels of NOX5-S mRNA were significantly higher in FLO esophageal adenocarcinoma (EA) cells than in the human esophageal squamous cell line HET-1A or in a Barrett cell line, BAR-T. B: NOX5 mRNA expression was significantly increased in EA tissues from patients undergoing esophagectomy (n = 11), when compared with normal esophageal (ESO) mucosa and BE mucosa, supporting the importance of NOX5-S in the development of EA. Real-time PCR was done with a Brilliant SYBR Green QPCR master mix kit (Stratagene, La Jolla, CA). ANOVA, *P < 0.01, compared with HET-1A or BE cell line; **P < 0.05, compared with normal ESO or Barrett’s esophagus (BE) group.

Fig. 2. Expression of Rac1 in different cell lines. A and B: typical example of Western blot analysis (A) and summarized data (B) show that Rac1 proteins are present in the human esophageal squamous cell line HET-1A, Barrett cell line BAR-T, FLO, and OE33 EA cells. Rac1 levels were significantly higher in EA cells (FLO and OE33) than in HET-1A or BAR-T cells. n = 3; ANOVA, **P < 0.02, compared with HET-1A and BAR-T cells.
RESULTS

Rac1 is involved in acid-induced hydrogen peroxide production. We have previously shown that acid increases H$_2$O$_2$ production in EA cells, an increase that is abolished by knockdown of NADPH oxidase NOX5-S expression. Knockdown of NOX5-S also decreases H$_2$O$_2$ production in basal conditions (13). In the present study, we show that the mRNA levels of NOX5-S are significantly higher in an EA cell line FLO than in normal human esophageal squamous cell line HET-1A or in a Barrett’s cell line BAR-T (Fig. 1A). The mRNA levels of NOX5-S are significantly increased in EA tissues, when compared with normal esophageal (ESO) or BE...
mucosal tissues (Fig. 1B). The data suggest that NOX5-S may be important in the development of EA.

Functional regulation of NOX5-S by its subunits is not known. Rac1 belongs to small GTPase of the Rho family and plays an important role in the organization of the actin cytoskeleton (17). It is an important subunit in activation of other NADPH oxidases (14, 20, 29). To determine whether Rac1 mediates acid-induced H₂O₂ production, we first examined the expression of Rac1 in HET-1A, BAR-T, and FLO cells and in another EA cell line, OE33. We found that Rac1 was present in these cell lines (Fig. 2, A and B). In addition, Rac1 protein levels were significantly higher in FLO and OE33 cells than in HET-1A or BAR-T cells. Next we used Rac1 siRNA to reduce expression of this protein in FLO EA cells. Rac1 siRNA significantly decreased Rac1 protein expression 48 h after transfection (Fig. 3, A and B), indicating that Rac1 siRNA effectively downregulates Rac1. More importantly, knockdown of Rac1 significantly reduced acid-induced increase in H₂O₂ production in FLO EA cells (Fig. 3C). The data suggest that Rac1 may mediate acid-induced H₂O₂ production.

To examine whether Rac1 is involved in activation of NOX5-S, we transfected FLO cells with constitutively active Rac1 plasmid pcDNA3.1-myc-Rac1-v12. Overexpression of constitutively active Rac1 significantly increased H₂O₂ production in FLO EA cells, an increase that was blocked by knockdown of NOX5-S (Fig. 4A). Next we used a Rac1 antibody to immunoprecipitate Rac1 and then detected NOX5-S in the immunoprecipitate by Western blot analysis. We found that NOX5-S proteins coimmunoprecipitated with Rac1 from FLO cell lysate (Fig. 4B). The data suggest that Rac1 may be coupled with NOX5-S and contribute to activation of NOX5-S in FLO EA cells.

In addition, we examined the colocalization of NOX5-S and Rac1. We constructed a NOX5-S expression recombination plasmid with GFP reporter, pCDNA-NOX5S-GFP, and transfected this plasmid into FLO cells. Then we stained FLO cells by using Rac1 antibody and Alexa Fluor 594 secondary antibody. Figure 5A shows that NOX5-S proteins were mainly present in the cytosol of FLO cells. Immunofluorescence staining with Rac1 antibody shows that Rac1 was mainly present in the cytosol of FLO cells (Fig. 5A). Coimmunofluorescence data showed that NOX5-S proteins were colocalized with Rac1 in the cytosol in FLO EA cells (Fig. 5A).

Colocalization of NOX5-S protein and calcium ATPases in FLO EA cells. To investigate the cellular localization of NOX5-S in FLO cells, we stained SERCA protein with SERCA1 antibody and Alexa Fluor 594 secondary antibody in

![Fig. 5. Colocalization of NOX5-S with Rac1 or SERCA in FLO EA cells. A: FLO cells were transfected with NOX5-S plasmid with green fluorescent protein (GFP) and then stained immunohistochemically with Rac1 antibody (red). The data suggest that NOX5-S is mainly present in the cytoplasm of FLO cells and is colocalized with Rac1. Images are representative samples of three individual experiments. B: FLO cells were transfected with NOX5-S plasmid with GFP and then stained immunohistochemically with SERCA1 antibody (red). The data suggest that NOX5-S may be colocalized with SERCA1 Ca²⁺-ATPase in FLO cells. Images are representative samples of three individual experiments.](http://ajpcell.physiology.org/
FLO EA cells transfected with pCDNA-NOX5S-GFP. Calcium ATPase SERCA is known to be present in endoplasmic reticulum. Coimmunofluorescence data (Fig. 5B) show that NOX5-S proteins were colocalized with SERCA1 calcium ATPase, suggesting that NOX5-S may be present in endoplasmic reticulum of FLO cells.

Finally, we used the SERCA1 or SERCA2 Ca\(^{2+}\)-ATPase antibodies to immunoprecipitate SERCA1 or SERCA2 respectively and then detected NOX5-S expression in the immunoprecipitate by using Western blot analysis. We found that NOX5-S proteins were coimmunoprecipitated with SERCA1 or SERCA2 Ca\(^{2+}\)-ATPases from FLO cell lysate (Fig. 6), indicating that NOX5-S proteins are coupled with SERCA1/2 Ca\(^{2+}\)-ATPase. These data further confirm that NOX5-S may be located in endoplasmic reticulum in FLO EA cells.

DISCUSSION

Increased levels of ROS have been reported both in BE (31, 48) and in esophageal adenocarcinoma (11, 41). ROS include superoxide radical anions (O\(_2^−\)), \(H_2O_2\), singlet oxygen\((^1O_2)\), hydroxyl radical (OH\(-\)), and hypochlorous acid (HOCl). ROS may cause damage to DNA, RNA, lipids, and proteins, possibly resulting in increased mutation and altered functions of enzyme and proteins (e.g., activation of oncogene products and/or inhibition of tumor suppressor proteins) (11, 30). Therefore, ROS may play an important role in the development of esophageal adenocarcinoma.

The source of ROS in Barrett’s cells is not fully understood. Low levels of ROS, seen in nonphagocytic cells, were thought to be byproducts of aerobic metabolism. More recently, however, superoxide-generating homologues of phagocytic NADPH oxidase catalytic subunit gp91\(^{phox}\) (NOX1, NOX3-NOX5, DUOX1, DUOX2) and homologues of other subunits (p41\(^{phox}\) or NOX1, p51\(^{phox}\) or NOX1A) have been found in several cell types (2, 13, 43), suggesting that ROS generated in these cells may have distinctive cellular functions related to immunity, signal transduction, and modification of the extracellular matrix. NOX5 has five isoforms: α, β, δ, and γ, and NOX5-S (3, 47). NOX5-α, -β, -δ, and -γ have EF-hand motifs at their NH\(_2\) terminus (3), whereas NOX5-S does not (12). We have found that acid treatment significantly increased ROS production in EA cells and in human Barrett biopsies (13) and that NOX5-S mediates acid-induced ROS production in BAR-T and OE33 cells (19).

In this study, we found that mRNA levels of NOX5-S were significantly higher in EA cells than in HET-1A cells and BAR-T cells. NOX5-S mRNA levels were also significantly increased in EA tissues (Fig. 1).

Mechanisms responsible for the functional regulation of NOX5-S are not known. Activation of some NOXs, such as NOX1, NOX2, and NOX4, is regulated by guanine nucleotide exchange on the monomeric G protein Rac, by lipid metabolism, or by protein phosphorylation. Several isoforms of Rac proteins are present: Rac1, Rac1b, Rac2, and Rac3. Rac2 is only expressed in myeloid cells and is required for activation of NOX2 in human neutrophils. Rac3 is predominantly found in the central nervous system. Conversely, Rac1 is ubiquitously distributed and is possibly the main Rac GTPase for NOX activation in nonphagocytic cells. Rac1b is a Rac1 splice variant that is constitutively active (28). Rac1b has recently been linked to mitochondria-derived production of ROS in an in vitro model of epithelial-mesenchymal transition (36). Rac1 binds to the COOH-terminal domain of NOX1 and contributes to activation of NOX1 (20, 29). NOX2 may be activated by Rac1 or Rac2. The role of Rac1 in activation of NOX3 is still controversial (4, 7, 46). NOX4 activity appears to be Rac independent upon heterologous expression (27); however, some data from cells endogenously expressing NOX4 show that NOX4 activation may require Rac1 activation (14). Therefore, we examined the role of Rac1 in activation of NOX5-S.

![Image](http://ajpcell.physiology.org/)

Fig. 6. Immunoprecipitation with SERCA1 or SERCA2 antibody. A: FLO cell extracts were immunoprecipitated with a SERCA1 antibody, and NOX5-S was then determined in the immunoprecipitate by using Western blot analysis. A typical example of three experiments shows that SERCA1 Ca\(^{2+}\)-ATPase was identified in the FLO cell lysate (top lane). NOX5-S protein was detectable in the immunoprecipitate of FLO cell lysate with SERCA1 antibody (bottom lane), indicating that NOX5-S protein may be located at the endoplasmic reticulum membrane and coupled with SERCA1. B: FLO cell extracts were immunoprecipitated with a SERCA2 antibody and NOX5-S was then determined in the immunoprecipitate by using Western blot analysis. A typical example of three experiments shows that SERCA2 Ca\(^{2+}\)-ATPase was identified in the FLO cell lysate (top lane). NOX5-S protein was detectable in the immunoprecipitate of FLO cell lysate with a SERCA2 antibody (bottom lane), indicating that NOX5-S protein may be located at endoplasmic reticulum membrane and coupled with SERCA2.
We first studied the expression of Rac1 in different esophageal cell lines. Figure 2, A and B, shows that Rac1 protein levels were significantly higher in EA cells than in HET-1A and BAR-T cells. Knockdown of Rac1 with Rac1 siRNA significantly decreased acid-induced increase in H2O2 production in FLO EA cells, indicating that Rac1 may mediate acid-induced H2O2 production. In addition, we found that Rac1 may be involved in NOX5-S activation since 1) overexpression of constitutively active Rac1 significantly increased H2O2 production in FLO EA cells (Fig. 4A); 2) knockdown of NOX5-S abolished the increase in H2O2 production induced by overexpression of constitutively active Rac1; 3) Rac1 was coupled with NOX5-S in FLO EA cells (Fig. 4B). Mechanisms whereby Rac1 activates NOX5-S are not known and need to be further explored.

FLO cells are an appropriate cell line to study functional regulation of NOX5-S, because we have shown that NOX5-S is the only isoform of NADPH oxidases present in these cells. NOX5-S is also present in BAR-T cells, but whether other isoforms of NADPH oxidases are present in BAR-T cells is not known. Therefore, we used FLO cells in most of our studies.

To further elucidate the relationship between Rac1 and NOX5-S, we examined the colocalization of NOX5-S and Rac1 using immunofluorescence staining. We found that Rac1 was present at the plasma membrane and in the cytosol and that NOX5-S protein was mainly detected in cytosol. This result is consistent with the literature showing that NOX1, NOX2, and NOX4 are present at the plasma membrane and in the cytosol (33). We also found that NOX5-S was colocalized with Rac1 in the cytosol in FLO EA cells (Fig. 5A).

Different NOXs may be present in different intracellular compartments. For example, NOX2 is present not only at the membrane of endoplasmic reticulum (ER) but also at the plasma membrane (33). NOX4 has been identified in the membrane of endoplasmic reticulum (ER) but also at the compartments. For example, NOX2 is present not only at

in the endoplasmic reticulum membrane. We speculate that NOX5-S’s colocalization with SERCA1/2 Ca2+-ATPases may be important in regulation of stored calcium, thus modulating calcium release.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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