Silencer-of-death- domain (SODD) mediates acid-induced decrease in cell apoptosis in Barrett’s associated esophageal adenocarcinoma cells

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Abstract

We have shown that NOX5-S may mediate the acid-induced decrease in cell apoptosis. However, mechanisms of NOX5-S-dependent decrease in cell apoptosis are not fully understood. In this study we found that silencer of death domain (SODD) was significantly increased in esophageal adenocarcinoma (EA) tissues, EA cell lines FLO and OE33, and a dysplastic cell line CP-B. Strong SODD immunostaining was significantly higher in low-grade dysplasia (66.7%), high-grade dysplasia (81.2%) and EA (71.2%) than in Barrett’s mucosa (10.5%). Acid treatment significantly increased SODD protein and mRNA expression and promoter activity in FLO cells, an increase which was significantly decreased by the knockdown of NOX5-S and NF-κB1 p50 with their siRNAs. Similarly, acid-induced increase of SODD mRNA was blocked by knockdown of NOX5-S and p50 in a BE cell line CP-A. Overexpression of NOX5-S significantly increased SODD protein expression in FLO cells. Moreover, overexpression of NOX5-S or p50 significantly increased the SODD promoter activity and decreased the caspase 9 activity or apoptosis. NOX5-S overexpression-induced increase in SODD promoter activity was significantly decreased by knockdown of p50. In addition, acid treatment significantly decreased the caspase 9 activity, a decrease which was significantly inhibited by knockdown of SODD. Furthermore, CHIP assay showed that NF-κB1 p50 bound to SODD genomic DNA containing an NF-κB binding element GGGGACACCCT. This binding element was further confirmed by a gel mobility shift assay. We conclude that acid-induced increase in SODD expression and decrease in cell apoptosis may depend on the activation of NOX5-S and NF-κB1 p50 in FLO cells.
Introduction

The major risk factor for esophageal adenocarcinoma (EA) is gastroesophageal reflux disease (GERD) complicated by Barrett’s esophagus (BE) (Lagergren et al., 1999; Kahrilas, 2011; Pohl et al., 2013). The mechanisms of the progression from BE to EA are not fully understood. Acid reflux is thought to contribute to this progression (Fitzgerald et al., 1996; El-Serag et al., 2004; Zhang et al., 2009; Das et al., 2011; Kastelein et al., 2013).

NOX5-S has been shown by us to mediate acid induced-increase in H$_2$O$_2$ production and cell proliferation in EA cells (Fu et al., 2006; Si et al., 2007) since acid exposure-induced increase in H$_2$O$_2$ production and cell proliferation is blocked by knockdown of NOX5-S. The mechanisms of NOX5-S-dependent increase in cell proliferation and decrease in apoptosis are not fully understood. We have shown that COX2 upregulation (Si et al., 2007) and p16 downregulation (Hong et al., 2010b) may partially mediate NOX5-S-dependent increase in cell proliferation.

Besides COX2 and p16, other proteins may also be involved in NOX5-S-dependent increase in cell proliferation and decrease in apoptosis. SODD was identified in 1999 and belongs to a family of anti-apoptotic proteins of the BAG family (Takayama et al., 1999). SODD contains a conserved region of approximately 45 amino acids near its C terminal end, the so-called BAG domain. A potential role of SODD in carcinogenesis has been proposed based on increased mRNA levels in cancer tissues; e.g. pancreatic cancers (Ozawa et al., 2000). The over-expression of SODD in various cancer cell lines is reported to suppress cell death (Jiang et al., 1999; Ozawa...
et al., 2000). We now show that acid increased SODD gene expression, which inhibited cell apoptosis in EA FLO cells. Acid-induced SODD expression may depend on activation of NOX5-S and NF-κB1 p50 in these cells.

Material and Methods

Cell culture and acid treatment- Cell culture and acid treatment were similar to those we described previously (Hong et al., 2010a; Hong et al., 2013; Hong et al., 2016). Briefly, human esophageal squamous HET-1A cells were purchased from ATCC, Manassas, VA in 2011 and cultured in the bronchial epithelial cell medium (BEGM BulletKit, Cambrex, East Rutherford, NJ). Human Barrett’s cell line CP-A and Barrett’s dysplastic cell line CP-B were bought from ATCC (Manassas, VA) 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 nM cholera 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 bought from Sigma in 2012 and cultured in DMEM containing 10 % fetal bovine serum and antibiotics. Human Barrett’s adenocarcinoma cell line FLO was obtained in 2004 from Dr. David Beer (Hughes et al., 1997) (University of Michigan Medical School). FLO cells were cultured in DMEM containing 10% fetal bovine serum and antibiotics. All the cell lines were cultured at 37 °C in a 5 % CO₂ humidified atmosphere.
For acid treatment, FLO cells were exposed to acidic DMEM (pH 4.0) or normal DMEM (pH 7.2, control) for 1 hours, washed, and cultured in fresh medium (pH 7.2, without phenol red) for an additional 24 hours. Finally, the culture medium and cells were collected for measurements. Acidic DMEM (pH 4.0, 300μl) was added to each well in a 12-well plate, and the final pH was about 4.9 after a 1-h incubation. CP-A cells were exposed to acidic culture medium (pH 6.5) or normal culture medium (pH 7.2, control) for 24 hours and then collected for measurements.

**Human esophageal tissues**- Fresh normal esophageal mucosa and esophageal adenocarcinoma tissues were obtained from patients with esophageal adenocarcinoma undergoing esophagogastrectomy. Formalin-fixed and paraffin-embedded samples were collected between the years of 2011 and 2016 from the archives of the Department of Pathology at the Rhode Island Hospital (RIH). Twenty-one cases were included: 19 male patients and 2 female patients. The age ranged from 34 to 89 (average 63.8). The experimental protocols were approved by the Human Research Institutional Review Committee at Rhode Island Hospital. Informed consents were obtained from the participants.

**Immunohistochemistry**- Immunohistochemistry for SODD was performed on 4-μm paraffin sections. Slides were stained with SODD antibody (1:200, Sigma-Aldrich Co., St. Louis, MO) using the DAKO Envision + Dual Link System and the DAKO Liquid 3,3’-diaminobenzidine (DAB+) Substrate Chromagen System (DAKO North America, Inc., Carpinteria, CA). Fallopian tubes were used as positive controls. Negative controls included replacement of the primary antibody with non-reacting antibodies of the same species.
**Immunohistochemistry assessment**- Cancers, dysplasia and non-neoplastic mucosa that displayed a strong staining pattern for TGR5 were scored as 3+, moderately intense staining as 2+, and weak staining as 1+. The extent of staining (percentage of cells staining) was scored as follows: 1+ 1-30%, 2+ 31-60%, 3+ 61-100%. A combined score of intensity and extent was calculated and categorized as follows: weak staining 1-2, moderate staining 3-4, strong staining 5-6.

**Construction of pGL3-SODDP reporter plasmid**- The DNA fragment containing part of the promoter region (-988 to 19 from ATG) of SODD gene (GenBank® accession number NM_004874.3) was amplified by PCR from human genomic DNA. The primers used were: SODDP-sense: 5’-GGGGTACCTAGGTATTCGATCCATCCAC-3’ (the introduced Kpn I is underlined) and SODDP -antisense: 5’-GAAGATCTGAGCGCTCAGGGCCGACATGG-3’ (the introduced BglII is underlined). The obtained cDNA fragment was then cloned into pGL3-basic (Promega) between Kpn I and BglII.

**Small interfering RNA (siRNA) and plasmid transfection**- The transfection protocol has been described previously by us (Hong et al., 2010a; Hong et al., 2013; Hong et al., 2016). Briefly, transfection of siRNAs was achieved by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). 75 pmol of NOX5-S siRNA (Ambion Inc., Austin, TX), NF-κB p50 siRNA (Santa Cruz biotechnologies) or control siRNA formulated into liposomes were added to each well. 24 hours after transfection, cells were treated with acidic culture medium as described above.

The pCMV-tag5a-NOX5-S plasmid was obtained from Dr. David Lambeth (Emory University School of Medicine, Atlanta, GA). Cells were transfected with 2 μg NOX5-S plasmid
or control plasmid by using Amaxa-Nucleofector-System (Lonza).

**Reverse Transcription-PCR**- RT-PCR was performed as we previously described (Hong et al., 2010a; Hong et al., 2013; Hong et al., 2016). Total RNA was extracted by TRIzol reagent and 1.5 μg of total RNA was reversely transcribed by using a kit SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA).

**Quantitative Real Time PCR**- Real time PCR was performed as we previously described (Hong et al., 2010a; Hong et al., 2013; Hong et al., 2016) and carried out on a StepOne™ Real-Time PCR System (Thermo Fisher Scientific Inc.). The primers used were: SODD F: 5’-5’-GGGGTACCCTGATCTCGGCTCACTG-3’, SODD R: 5’-GAAGATCTCTCGAGGGGATCCGCTGCCCTGAAGCGCT-3’, 18S F: 5’-CGGACAGGATTGACAGATTGATAGC -3’, and 18S R: 5’-TGCCAGAGTGCTCGTTATCG -3’. PCR reactions were performed as follows: 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. The transcript level of each specific gene was normalized to 18s amplification.

**Western Blot Analysis**- Western blot analysis was done as described previously (Cao et al., 2003; Hong et al., 2010a; Hong et al., 2013; Hong et al., 2016). Primary antibodies used were as follows: SODD antibody (1:1000, Santa Cruz biotechnologies) and GAPDH antibody (1:2000, Santa Cruz biotechnologies).

**Luciferase Assay**- Luciferase assay was done as we previously described (Fu et al., 2006; Hong et al., 2013). Briefly, 0.1 μg renilla and 1.0 μg reporter plasmids pSODDP in combination
with NOX5 siRNA, p50 siRNA, control siRNA, pcDNA3.1 or NOX5-S plasmid were transiently transfected in duplicate with Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were collected for the measurement of luciferase activity by using the Dual-Luciferase Reporter Assay System (Promega). For acid treatment, 12 hours after transfection FLO cells were treated with acidic DMEM (pH 4.0) for 1 hours, washed, and cultured in fresh medium (pH 7.2, without phenol red) for an additional 24 hours. Finally, the cells were collected for measurements. Luciferase activity was measured for 10 seconds after a 2-second delay using a BD Monolight 3010 luminometer (BD Biosciences, San Jose, CA). Variation in transfection efficiency was normalized by dividing the construct luciferase activity by the corresponding renilla luciferase activity.

**Chromatin Immunoprecipitation (ChIP) Assay**—ChIP assay was performed using the ChIP assay kit (Upstate, Charlottesville, VA) following manufacturer’s protocol as we previously described (Fu et al., 2006; Hong et al., 2013). PCR was carried with the primer pairs that targeted the -1147 to -1019 region of the human SODD promoter (sense 5’-CACTTCTCTGAACACGTGG-3’ and antisense 5’-GAGCGCCTCAGGGCCGACATGG-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.

**Gel Mobility Shift Assay**—Gel shift assay was performed using the gel shift assay kit (Licor, Nebraska USA) following the manufacturer’s protocol as we previously described (Fu et al., 2006; Hong et al., 2013). Briefly, human SODD promoter oligonucleotides (-310 to -300)
were synthesized and labeled with IRDye 700 by Integrated DNA Technologies (IDT), IA USA). 1 μl of 50 nM IRDye 700-labeled oligonucleotides were incubated with 5 μg of HeLa nuclear extract or 0.25 μg recombinant NF-κB p50 protein (Cayman Inc., Ann Arbor, MI). The wild type competitor (-317 to -307) is 5’-CGTGGGGGACACCCTTTCC- 3’ and the mutant competitor (-317 to -307) is 5’- CGTTGGTTGACACTTTTCC- 3’. For a supershift assay, an NF-κB p50 antibody (1 μl Upstate) was preincubated with the NF-κB p50 protein for 20 min at room temperature before addition of the IRDye700 labeled probes. The DNA-protein complexes were resolved by electrophoresis and the gel was imaged immediately by Licor Odyssey® imager system.

**ELISA apoptosis detection assay**-The levels of apoptosis are measured by using ApoStrand™ ELISA apoptosis detection kit (Biomol, Plymouth Meeting, PA). This assay is based on the sensitivity of DNA in apoptotic cells to formamide denaturation and the denatured DNA is detected with an antibody against single-stranded DNA (ssDNA). We have successfully used this assay in our published work (Fu et al., 2006).

**Caspase-Glo® 9 Assay**: pcDNA3.1, pCMV, p50 or NOX5-S plasmids were transiently transfected in duplicate with Lipofectamine 2000 (Invitrogen) according to manufacturer’s instruction. Twenty-four hours after transfection, caspase-9 activity was measured by using a Caspase-Glo® 9 assay kit (Promega Corporation, Madison, WI). For acid treatment, FLO cells were first transfected with SODD siRNA or control siRNA with Lipofectamine 2000. 12 hours after transfection FLO cells were treated with acidic DMEM (pH 4.0) for 1 hours, washed, and cultured in fresh medium (pH 7.2, without phenol red) for an additional 24 hours. Finally, the
caspase 9 activity was measured.

The Caspase-Glo® 9 assay is a homogeneous luminescent assay that measures caspase-9 activity. The assay provides a luminogenic substrate selective for caspase-9 in a buffer system optimized for caspase activity. The addition of a single Caspase-Glo® 9 Reagent in an “add-mix-measure” format results in cell lysis, followed by caspase cleavage of the substrate, and generation of a “glow-type” luminescent signal. The signal generated is proportional to the amount of caspase-9 activity present.

Materials: Other reagents were purchased from Sigma.

Statistical Analysis - Data is expressed as mean ± S.E. 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. For immunohistochemical data, statistical differences were determined by Chi square test.

Results

Expression of SODD in different esophageal tissues and cells

We found that SODD mRNA were significantly increased by 345.8% control in human EA tissue (figure 1A), when compared with normal mucosa. Real-time PCR showed that SODD mRNA was significantly higher in a dysplastic cell line CP-B and EA cells FLO than in CP-A cells (figure 1B). In addition, SODD mRNA was significantly increased in FLO cells, when compared with CP-B cells (figure 1B). Western blot analysis and summarized data showed that the protein levels of SODD were significantly higher in EA cell line FLO and OE33 than normal
esophageal squamous epithelial cell line HET-1A or CP-A cells (figure 1C & 1D). The data suggest that SODD may be important in the development of esophageal adenocarcinoma.

To further confirm these data, we did immunohistochemical staining with SODD antibody. We found that strong SODD immunostaining was significantly higher in low-grade dysplasia (66.7%), high-grade dysplasia (81.2%) and esophageal adenocarcinoma (71.2%) than in Barrett’s mucosa (figure 2 and table 1). Although the strong immunostaining was slightly higher in high-grade dysplasia and EA than in low-grade dysplasia, the difference did not reach the statistical significance. The data suggest that SODD may be a potential marker for dysplasia and EA.

**SODD is involved in acid-induced decrease in cell apoptosis in EA cells.**

Since acid reflux may play an important role in the progression from BE to dysplasia and to adenocarcinoma (Fitzgerald et al., 1996; Ouatu-Lascar et al., 1999), we examined whether acid treatment affects levels of SODD protein. Acid treatment significantly increased SODD protein level in FLO cells (figure 3A & 3B) and mRNA levels in CP-A cells (figure 3C). In addition, acid treatment significantly increased the luciferase activity in FLO cells transfected with SODD reporter plasmid pGL3-SODDP (figure 3D). The data suggest that acid treatment may increase SODD promoter activity and protein expression in FLO EA cells and mRNA expression in CP-A cells. Moreover, acid treatment significantly decreased caspase 9 activity in FLO EA cells (figure 3E), a decrease which was blocked by knockdown of SODD (figure 3F). At the basal condition, SODD siRNA significantly increased caspase 9 activity by 32.5% (N=3, p<0.02). Furthermore, acid-induced decrease in cell apoptosis was reversed by knockdown of SODD (figure 3G). These
data indicate that SODD may contribute to acid-induced decrease in cell apoptosis in FLO cells.

**NOX5-S may contribute to acid-induced SODD expression in FLO cells.**

We have shown that NADPH oxidases may mediate acid-induced H$_2$O$_2$ production in Barrett’s mucosal biopsies (Fu et al., 2006) and that NADPH oxidase NOX5-S is the major isoform of NADPH oxidases in FLO EA cells (Hong et al., 2010a). Therefore, we examined whether NOX5-S participates in acid-induced increase in SODD expression. Western Blot analysis and summarized data showed that knockdown of NOX5-S with its siRNA, which has been shown by us to effectively knock down NOX5-S expression (Fu et al., 2006), significantly decreased SODD expression in response to acid treatment in FLO cells (figure 3A & 3B). Knockdown of NOX5-S also significantly decreased SODD mRNA expression in CP-A cells (figure 3C) and remarkably inhibited acid-induced increase in luciferase activity in FLO cells transfected with SODD reporter plasmid pGL3-SODDP (figure 3D). These data suggest that acid-induced SODD expression may be mediated by activation of NOX5-S. To further confirm this result, we transfected NOX5-S expression plasmid into FLO EA cells, which has been shown by us to overexpress NOX5-S protein (Si et al., 2007), and found that overexpression of NOX5-S significantly increased the protein expression of SODD (figure 4A &4B). Moreover, overexpression of NOX5-S dramatically increased the luciferase activity in FLO cells transfected with SODD reporter plasmid pGL3-SODDP (figure 4C) and significantly decreased caspase 9 activity (figure 4D) and cell apoptosis (figure 4E) at the basal condition in FLO EA cells. The data suggest that NOX5-S may be involved in SODD expression in FLO EA cells.

**Role of NF-κB in acid-induced SODD expression**
We have shown that NADPH oxidase NOX5-S mediates acid-induced cyclooxygenase-2 expression via activation of NF-κB in Barrett’s esophageal adenocarcinoma cells (Si et al., 2007). Next we examined whether NF-κB participates in acid-induced SODD expression in FLO cells. We found that knockdown of p50 with its siRNA, which has been shown by us to effectively knock down p50 expression (Si et al., 2007), significantly decreased acid-induced increase in SODD protein expression in FLO cells (figure 5A & 5B) and in SODD mRNA expression in CP-A cells (figure 5C). In addition, knockdown of p50 significantly decreased NOX5-S-overexpression- or acid-induced increase in luciferase activity in FLO cells transfected with SODD reporter plasmid pGL3-SODDP (figure 4C and 5D). Moreover, overexpression of NF-κB p50 significantly increased the luciferase activity in FLO cells transfected with SODD reporter plasmid pGL3-SODDP (figure 5E) and remarkably decreased the cell apoptosis in FLO cells (figure 5F). The data suggest that NF-κB p50 may be involved in acid-induced increase in SODD expression.

We identified one possible NF-κB binding element, GGGACACCCT (positions -310 to -300), in the SODD promoter. To test whether NF-κB binds to SODD promoter, we did chromatin immunoprecipitation (ChIP) assay. Figure 6A showed that SODD genomic DNA was identifiable in the immunoprecipitate of FLO EA cell lysate with p50 antibody. The pair of primers used in the PCR targeted the -367 to +17 (position from ATG) region of the SODD promoter and covered the possible NF-κB-binding site as described above. The PCR products were sequenced and proved to be SODD genomic DNA. The data suggest that NF-κB may bind to the SODD promoter. A gel mobility shift assay was also done to confirm the above result. In
the gel shift assay, we identified one prominent complex by using Hela nuclear extracts and IRdye 700-labelled SODD oligonucleotide, containing the NF-κB binding site GGGACACCCT (figure 6B). A high concentration of unlabeled SODD oligonucleotide significantly reduced the binding, whereas the mutant SODD oligonucleotide had less effect on the binding (figure 6B). In addition, one prominent complex was also identifiable with IRdye 700-labelled SODD oligonucleotide and recombinant p50 protein (figure 6C). The bands of these prominent complex were super-shifted with a p50 antibody (figure 6C). These data further suggest that NF-κB p50 may bind to the potential binding site GGGACACCCT.

Discussion

Gastroesophageal reflux disease (GERD) complicated by Barrett’s esophagus (BE) is a major risk factor for EA (Lagergren et al., 1999). However, mechanisms of the progression from BE (intestinal metaplasia) to EA are not fully understood. We have previously shown that acid, a major refluxate in patients with Barrett’s esophagus, increases ROS production in Barrett’s mucosal biopsies (Fu et al., 2006). This increase is blocked by NADPH oxidase inhibitor apocynin, suggesting that NADPH oxidases may mediate acid induced increase in H2O2 production (Fu et al., 2006). NADPH oxidase has seven isoforms NOX1-5, DUOX1 and DUOX2 (Suh et al., 1999; Banfi et al., 2000; Lambeth, 2004), where NOX5 has five isoforms: α, β, δ and γ, and NOX5-S (Banfi et al., 2000; Vignais, 2002). NOX5 α, β, δ and γ have EF-hand motifs at its N-terminal (Banfi et al., 2000), whereas NOX5-S does not (Cheng et al., 2001). We have previously 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) (Fu et al., 2006). Overproduction of ROS derived from upregulation of NOX5-S increases cell proliferation and decreases apoptosis. However, the mechanism of acid-induced decrease in cell apoptosis is still not fully understood in EA cells.

In this study, we found that acid treatment remarkably increased SODD protein expression and promoter activity in FLO EA cells and SODD mRNA expression in CP-A cells, suggesting that acid treatment may upregulate SODD. Moreover, acid significantly decreased cell apoptosis, a decrease which was inhibited by knockdown of SODD, indicating that SODD may contribute to acid-induced decrease in cell apoptosis in FLO cells. It has been reported that pulsed acid treatment (pH 4) increased cell proliferation in mucosal tissues (Fitzgerald et al., 1996). We also found that pulsed acid treatment with pH 4.0 increased cell proliferation in FLO cells. Therefore, we used pH 4 in FLO cells. CP-A cells are less resistant to in vitro acid treatment. In our preliminary studies, we found that pH 4 and 5 caused cell apoptosis in CP-A cells, whereas pH 6.5 increased cell proliferation as seen in FLO cells and in Barrett’s mucosal tissues. To mimic the findings obtained in mucosal tissues which are much closer to the in vivo condition, we used pH 6.5 in CP-A cells.

SODD belongs to a family of anti-apoptotic proteins of the BAG family (Takayama et al., 1999) and contains a conserved region of approximately 45 amino acids near its C terminal end, the so-called BAG domain. Over-expression of SODD has been reported to suppress cell death in various cancer cell lines (Jiang et al., 1999; Ozawa et al., 2000). SODD interacts with the ATPase domain of Hsc70/Hsp70 (Takayama et al., 1999) through its BAG domain and is associated with
the cytoplasmic domain of the tumor necrosis factor receptor 1 (TNFR1) and death receptor-3, another member of the TNFR superfamily. TNF treatment releases SODD from TNFR1, permitting the recruitment of proteins such as TNFR-associated death domain and TNFR-associated factor 2 to the active TNFR1 signaling complex (Jiang et al., 1999). Under un-stimulated condition, SODD binds TNFR1 and prevents the ligand-independent oligomerization and spontaneous activation of TNFR1, thus inhibiting the cell apoptosis (Doong et al., 2002).

We also found that SODD expression was significantly increased in EA cells and tissues. By immunohistochemistry, strong SODD immunostaining was significantly higher in dysplasia and esophageal adenocarcinoma than in Barrett’s metaplastic cells, suggesting that SODD might be a potential marker for dysplasia and EA. Although the strong immunostaining was slightly higher in high-grade dysplasia and EA than in low-grade dysplasia, the difference did not reach the statistical significance, which might be due to our small sample size.

Acid-induced increase in SODD expression may depend on activation of NOX5-S since 1) knockdown of NOX5-S blocked acid-induced upregulation of SODD protein and mRNA; 2) knockdown of NOX5-S remarkably inhibited acid-induced increase in luciferase activity in FLO cells transfected with SODD reporter plasmid pGL3-SODDP; and 3) overexpression of NOX5-S significantly increased SODD promoter activity.

We have previously shown that NF-κB plays an important role in acid-induced cyclooxygenase-2 expression in Barrett’s esophageal adenocarcinoma cells (Si et al., 2007) and that acid treatment significantly decreased IκBα protein levels and increased luciferase activity.
in cells transfected with NF-κB reporter plasmid pNFκB-Luc, which contains five repeats of
NF-κB binding element GGGGACTTTCC in the enhancer element of the plasmid, suggesting
that acid may activate NF-κB (Si et al., 2007). In addition, knockdown of NOX5-S significantly
decreased acid-induced increase in luciferase activity in cells transfected with pNFκB-Luc,
suggesting that activation of NF-κB may depend on activation of NOX5-S (Hong et al., 2013).
Therefore, we examined the role of NF-κB in acid-induced SODD expression.

NF-κB has two isoforms p50 and p52 (Karin et al., 2002). P50 is involved in lymphoid
organogenesis and inflammation, whereas p52 mainly contributes to lymphoid
organogenesis (Shih et al., 2011). Therefore, we focused on the role of p50 in acid-induced
SODD expression.

We found that knockdown of p50 significantly decreased acid-induced increase in SODD
protein expression in FLO cells and in SODD mRNA expression in CP-A cells. In addition,
knockdown of p50 significantly decreased NOX5-S overexpression- or acid-induced increase in
luciferase activity in FLO cells transfected with SODD reporter plasmid pGL3-SODDP.
Moreover, overexpression of NF-κB p50 significantly increased SODD reporter activity and
remarkably decreased the cell apoptosis in FLO cells. These data suggest that NF-κB1 p50 may
be responsible for acid-induced SODD expression in EA cells.

One NF-κB binding element GGGACACCCT (positions -310 to -300) was identified in the
SODD promoter. Our data indicate that NF-κB may bind to this binding element since 1) SODD
genomic DNA was identifiable in the immunoprecipitate of EA cell lysate with p50 antibody; 2)
In the gel shift assay, IRdye 700-labelled SODD oligonucleotide containing the NF-κB binding
site GGGACACCCT formed one prominent complex with Hela nuclear extracts or recombinant p50 protein; 3) A high concentration of unlabeled SODD oligonucleotide significantly decreased the binding, whereas the mutant SODD oligonucleotide had less effect on the binding; 4) In the supershift assays, p50 antibody caused the supershift of the bands.

We conclude that acid-induced increase in SODD expression and decrease in cell apoptosis may depend on activation of NOX5-S and NF-κB1 p50. It is possible that acid reflux present in patients with Barrett’s esophagus may activate NOX5-S. High levels of ROS derived from NOX5-S may activate NF-κB1 p50 and upregulate SODD, which in turn decreases cell apoptosis, thereby contributing to the progression from BE to EA.
Authorship Contributions:

Participated in research design: Hong, Li, and Cao.

Conducted experiments: Hong and Li

Contributed new reagents or analytic tools: Cao

Performed data analysis: Hong, Li, and Cao

Wrote or contributed to the writing of the manuscript: Hong and Cao
Reference:


Footnotes:

¶ These two authors contributed equally.

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Figure legends

Figure 1. **SODD expression in different cells and tissues.** (A) Real time RT-PCR showed that the levels of SODD mRNA were significantly increased in tumor tissues by 345.8% control, when compared with normal esophageal (ESO) mucosa (N=6, paired student t test P<0.01). (B) Levels of SODD mRNA were significantly higher in CP-B and FLO cells than in CP-A cells and markedly higher in FLO cells than in CP-B cells. (C) A typical image of three Western Blot analyses and (D) summarized data showed that the levels of SODD protein expression were significantly increased in FLO and OE33 EA cells (N=3), when compared with normal squamous epithelial cells HET-1A and Barrett’s cells. The data suggest that SODD may be important in the development of EA. ** P<0.02, *** P<0.001, compared with HET-1A cells or CP-A cells.

Figure 2. Representative images of BE mucosa, low-grade dysplasia, high-grade dysplasia and EA with SODD immunostaining. 200X

Figure 3. **The role of NOX5-S in acid-induced SODD expression.** (A) A typical image of three Western Blot analyses and (B) summarized data showed that acid treatment significantly increased SODD protein expression in FLO cells, an increase which was significantly decreased by knockdown of NOX5 with its siRNA (N=3). (C) Acid treatment significantly increased SODD mRNA expression in a Barrett’s cell line CP-A cells, an increase which was significantly decreased by knockdown of NOX5 with its siRNA (N=3). D) Acid treatment remarkably increased the luciferase activity of SODD promoter in FLO cells transfected with SODD reporter.
plasmid pGL3-SODDP (N=3), which was generated by ligating a SODD promoter fragment (-1008 to 20 from ATG) into the pGL3-basic vector, indicating that acid treatment may activate SODD promoter. Acid-induced increase in the luciferase activity was significantly decreased by knockdown of NOX5 (N=3), suggesting that acid-induced activation of SODD promoter may depend on the activation of NOX5-S. E) Acid treatment significantly decreased cell apoptosis in FLO EA cells (N=3-5). F) Acid-induced decrease in caspase 9 activity was significantly inhibited by knockdown of SODD, suggesting that SODD may mediate acid-induced decrease in cell apoptosis in FLO cells. G) Acid-induced decrease in cell apoptosis was significantly inhibited by knockdown of SODD, suggesting that SODD may mediate acid-induced decrease in cell apoptosis in FLO cells. % decrease was calculated as follows: % decrease = (control-acid) *100/control. ANOVA * P<0.02, ## P<0.01 and *** P<0.0001, compared with control siRNA group; ** P<0.01, compared with control siRNA + Acid group; ▲▲ P<0.001, compared with control siRNA + Acid group. t test # P<0.05; ▲ P< 0.0001.

Figure 4. The role of NOX5-S in SODD expression. (A) A typical image of three Western Blot analyses and (B) summarized data showed that overexpression of NOX5-S significantly increased SODD expression in FLO EA cells, indicating that NOX5-S may contribute to the SODD expression (N=3). (C) Overexpression of NOX5-S by transfection cells with NOX5-S plasmid remarkably increased the luciferase activity of SODD promoter in FLO cells (N=3), indicating that NOX5-S-derived reactive oxygen species may activate SODD promoter. NOX5-S overexpression-induced increase in SODD promoter activity was significantly decreased by the
knockdown of NF-κB1 p50 with its siRNA, suggesting that NOX5-S-dependent activation of SODD promoter may be mediated by NF-κB1 p50. (D) In FLO cells, overexpression of NOX5-S significantly decreased caspase-9 activity (N=6), indicating that NOX5-S-derived reactive oxygen species may inhibit cell apoptosis. (E) In FLO cells, overexpression of NOX5-S significantly decreased cell apoptosis (N=3), indicating that NOX5-S-derived reactive oxygen species may inhibit cell apoptosis. ANOVA *** P<0.0001, compared with control siRNA + pCMV group; ** P<0.01, compared with control siRNA + NOX5p group; T test, ▲ P< 0.05; * P<0.0001.

**Figure 5. The role of NF-κB in acid-induced SODD expression** (A) A typical image of three Western Blot analyses and (B) summarized data showed that knockdown of NF-κB1 p50 abolished acid-induced increase in SODD expression in FLO EA cells (N=3), indicating that acid-induced expression of SODD may depend on activation of NF-κB in FLO EA cells. (C) Acid treatment significantly increased SODD mRNA expression in a Barrett’s cell line CP-A cells, an increase which was significantly decreased by knockdown of NF-κB1 p50 with its siRNA (N=3). D) Acid treatment remarkably increased the luciferase activity of SODD promoter in FLO cells transfected with SODD reporter plasmid pGL3-SODDP (N=3), which was generated by ligating a SODD promoter fragment (-1008 to 20 from ATG) into the pGL3-basic vector, indicating that acid treatment may activate SODD promoter. Acid-induced increase in the luciferase activity was significantly decreased by knockdown of NF-κB1 p50 (N=3), suggesting that acid-induced activation of SODD promoter may depend on the activation of NF-κB1 p50. (E)
In FLO EA cells, overexpression of NF-κB1 p50 remarkably increased the luciferase activity in FLO cells transfected with SODD reporter plasmid pGL3-SODDP (N=3), indicating that NF-κB1 p50 may activate SODD promoter. (F) In FLO cells, overexpression of NF-κB1 p50 significantly decreased cell apoptosis (N=6), indicating that NF-κB1 p50 may inhibit cell apoptosis. ANOVA, * P<0.05, *** P<0.01 and ## P<0.0001, compared with control siRNA group; ** P<0.01 and # P<0.05, compared with control siRNA + acid group. t test ▲ P<0.01, ▲▲ P<0.0001.

**Figure 6.** A) A typical example of three experiments showed that SODD DNA was detectable in the immunoprecipitated chromatin sample of FLO cells by using an antibody against NF-κB P50, suggesting that NF-κB binds to SODD promoter. Positive control, genomic DNA used as a positive control; rabbit IgG and c-Myc antibody (Ab) were used as negative controls. B) In gel mobility assay, a double-stranded IRdye® 700-labeled oligonucleotide containing the sequence CGTTGGGGGACACCCTTTCC (SODD-pWT1) and HeLa nuclear extract provided by the kit were used. One prominent complex was detected (lane 2). Competition experiments with unlabeled DNMT1-pWT1 oligonucleotide significantly reduced binding (lane 3); however, the addition of the mutant oligonucleotide SODD-pMUT1 CGTTGGTTGACACTTTTTCC (lane 4) had no effect on binding. These data suggest that NF-κB binds to the site GGGGACACCCT at the SODD promoter.
Table 1. SODD expression in Barrett’s esophagus, low-grade dysplasia, high-grade dysplasia and esophageal adenocarcinoma

<table>
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<tr>
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<th>Negative</th>
<th>Mild</th>
<th>Moderate</th>
<th>Strong</th>
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<tbody>
<tr>
<td>Barrett’s cells (N=19)</td>
<td>1 (5.3%)</td>
<td>2 (10.5%)</td>
<td>14 (73.7%)</td>
<td>2 (10.5%)</td>
</tr>
<tr>
<td>Low-grade dysplasia (N=6)</td>
<td>0</td>
<td>0</td>
<td>2 (33.3%)</td>
<td>4 (66.7%)*</td>
</tr>
<tr>
<td>High-grade dysplasia (N=16)</td>
<td>0</td>
<td>0</td>
<td>3 (18.8%)</td>
<td>13 (81.2%)*</td>
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<tr>
<td>EA (N=7)</td>
<td>0</td>
<td>0</td>
<td>2 (28.6%)</td>
<td>5 (71.4%)*</td>
</tr>
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* P<0.01, compared with Barrett’s cells
Figure 1

A

B

C

D

Relative value of SODD mRNA/18S

Ratio of SODD mRNA/18S (% control)

SODD

GAPDH

HET-1A  CP-A  FLO  OE33

SODD/GAPDH (% control)

HET-1A  CP-A  FLO  OE33

P<0.01

P<0.05

***

**
Figure 2

Barrett’s mucosa

Low-grade dysplasia

High-grade dysplasia

Esophageal adenocarcinoma
Figure 3

A

B

C

D

E

F

G

**Figure 3:**

A. Western blot analysis showing SODD and GAPDH expression in control siRNA, Control siRNA + Acid, NOX5 siRNA, and NOX5 siRNA + Acid groups.

B. Graph showing SODD protein/GAPDH (%) control for FLO cells in control siRNA, Control siRNA + Acid, NOX5 siRNA, and NOX5 siRNA + Acid groups.

C. Graph showing SODD mRNA/18S (% control) for CP-A cells in control siRNA, Control siRNA + Acid, NOX5 siRNA, and NOX5 siRNA + Acid groups.

D. Graph showing luciferase activity (% control) for FLO cells in control siRNA, Control siRNA + Acid, NOX5 siRNA, and NOX5 siRNA + Acid groups.

E. Graph showing Caspase 9 activity (% control) for FLO cells in Control and Acid groups.

F. Graph showing Caspase 9 activity (% decrease) for FLO cells in Control, SODD siRNA, and SODD siRNA + Acid groups.

G. Graph showing apoptosis (% decrease) for FLO cells in Control siRNA, Control siRNA + Acid, NOX5 siRNA, and NOX5 siRNA + Acid groups.
Figure 4

A

control

NOX5 overexpression

SODD

GAPDH

B

FLO cells

SODD/GAPDH (% control)

500

450

400

350

300

250

200

150

100

50

0

Control plasmid

NOX5-S overexpression

C

FLO cells

Luciferase activity (% control)

1000

800

600

400

200

0

Control siRNA + pCMV

Control siRNA + NOX5p

p50 siRNA + pCMV

p50 siRNA + NOX5p

D

FLO cells

Caspase 9 activity (% control)

100

80

60

40

20

0

Control Plasmid

NOX5-S Overexpression

E

FLO cells

Apoptosis (% control)

100

80

60

40

20

0

Control Plasmid

NOX5-S Overexpression
**Figure 6**

<table>
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<tr>
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<th>Positive Control</th>
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<td>SODD</td>
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**B**

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<td>IRdye® 700 oligo (50 nM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Hela cell nuclear extract (5 μg)</td>
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<td>+</td>
<td>+</td>
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<td>Consensus competitor oligo (100 nM)</td>
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<td>−</td>
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<tr>
<td>Mutant competitor oligo (100 nM)</td>
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<td>−</td>
<td>−</td>
<td>+</td>
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**C**

<table>
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<th>Lane</th>
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<td>P50 protein (0.25 μg)</td>
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<tr>
<td>P50 antibody (1 μl)</td>
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→ Supershifted band