

Signaling in H₂O₂-induced increase in cell proliferation In Barrett's Esophageal Adenocarcinoma Cells

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Abbreviations: BE: Barrett's esophagus; EA: esophageal adenocarcinoma; GERD: gastroesophageal reflux disease; MAPK: mitogen activated protein kinase; MEK: mitogen activated protein kinase kinase; NOX: NADPH oxidase; NF- κ B: nuclear factor kappa B; ROS: Reactive oxygen species.

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Abstract

Mechanisms whereby acid reflux may accelerate the progression from Barrett esophagus (BE) to esophageal adenocarcinoma (EA) are not fully understood. We have previously shown that NADPH oxidase NOX5-S generates reactive oxygen species (ROS) when Barrett's metaplastic cells are exposed to acid. Besides metaplastic cells, other H₂O₂-producing cells (e.g. inflammatory cells) present in BE mucosa may produce additional ROS which may also affect metaplastic cells contributing to esophageal tumorigenesis. In this study we investigate whether exogenous H₂O₂ stimulates cell proliferation by increasing NOX5-S expression. Low dose (10⁻¹³ M) of H₂O₂ significantly increased thymidine incorporation, NOX5-S mRNA and protein expression in a Barrett's EA cell line FLO. H₂O₂-induced increase in NOX5-S expression was significantly inhibited by knockdown of NF-κB1 p50 with p50 siRNA in EA cell lines FLO and OE33. H₂O₂ significantly increased p65 phosphorylation and the luciferase activity in FLO cells transfected with a NF-κB activation reporter plasmid pNF-κB-Luc. H₂O₂-induced increase in luciferase activity in FLO cells was significantly decreased by knockdown of ERK2 MAP kinase. Overexpression of p50 and p65 remarkably increased the luciferase activity in FLO cells transfected with a NOX5-S reporter plasmid NOX5-LP. In addition, H₂O₂-induced thymidine incorporation in FLO cells was significantly decreased by the MEK1/2 inhibitor PD98059 and ERK2 siRNA, but not by ERK1 siRNA. Similarly, H₂O₂-induced increase in NOX5-S expression was significantly decreased by ERK2 siRNA in FLO and OE33 cells. We conclude that low dose of H₂O₂ increases cell proliferation. H₂O₂-induced increase in cell proliferation may depend on sequential activation of ERK2 MAP kinase, NF-κB1 p50 and NOX5-S.

Introduction

Esophageal adenocarcinoma has increased in incidence at a rate exceeding that of any other cancer (Blot and McLaughlin, 1999; Howe et al., 2001; Pohl and Welch, 2005). The major risk factor for esophageal adenocarcinoma is gastroesophageal reflux disease (GERD) complicated by Barrett esophagus (BE) (Lagergren et al., 1999). 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 (Haggitt, 1994; Kim et al., 1997; Wild and Hardie, 2003). However, mechanisms of the progression from metaplasia (BE) to adenocarcinoma are not fully understood.

Reactive oxygen species (ROS) may be an important factor mediating this progression since 1) high levels of ROS are present in BE (Olyaei et al., 1995; Wetscher et al., 1997) and in esophageal adenocarcinoma (Farhadi et al., 2002; Sihvo et al., 2003); 2) 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) (Farhadi et al., 2002; Ohshima et al., 2003); Besides metaplastic cells, other cells (e.g. inflammatory cells) in BE mucosa may also produce ROS and affect metaplastic cells.

Lower levels of ROS, seen in non-phagocytic cells, were thought to be byproducts of aerobic metabolism. More recently, superoxide-generating homologues of phagocytic NADPH oxidase catalytic subunit gp91phox (NOX1, NOX3–NOX5, DUOX1, and DUOX2) and homologues of

other subunits (p41phox or NOXO1, p51phox, or NOXA1) have been found in several cell types (Suh et al., 1999; Banfi et al., 2000; Lambeth, 2004), suggesting that ROS generated in these cells may have distinctive cellular functions. We have shown that NOX5-S is the major isoform of NADPH oxidase in FLO EA cells (Hong et al., 2010b) and that the expression of NOX5-S is significantly higher in BE with high grade dysplasia than in BE without dysplasia (Fu et al., 2006). The expression of NOX5-S is also significantly higher in FLO cells than in esophageal squamous epithelial cells (Hong et al., 2011). We have also shown that acid-induced H₂O₂ production is mediated by the NADPH oxidase NOX5-S (Hong et al., 2010c). Overproduction of ROS, derived from up-regulation of NOX5-S, increases cyclooxygenase-2-derived prostaglandin E₂ production (Fu et al., 2006) and downregulates a tumor suppressor gene p16 (Hong et al., 2010c), thus increasing cell proliferation and decreasing apoptosis. These changes may contribute to progression from BE to dysplasia and to adenocarcinoma. However, whether exogenous ROS increase cell proliferation via upregulation of NOX5-S in EA cells is not known. In the present study, we find that H₂O₂ increases cell proliferation by sequential activation of mitogen activated protein kinase (MAPK), NF-κB, and NOX5-S.

Materials and Methods

Cell culture and H₂O₂ treatment

Human Barrett adenocarcinoma cell line FLO was derived from human Barrett's esophageal adenocarcinoma (Hughes et al., 1997) and generously provided by Dr. David Beer (University of Michigan). These cells were cultured in DMEM containing 10% fetal bovine serum and antibiotics

at 37°C in a 5% CO₂ humidified atmosphere. Human EA cell line OE33 was purchased from Sigma and cultured in DMEM containing 10% fetal bovine serum and antibiotics. The cell lines were cultured at 37°C in a 5% CO₂ humidified atmosphere.

For H₂O₂ treatment, FLO cells were incubated with different concentrations of H₂O₂ (10⁻⁵, 10⁻⁷, 10⁻⁹, 10⁻¹¹, 10⁻¹³, 10⁻¹⁴, 10⁻¹⁵M) for 48 h. For inhibitor treatment, FLO cells were exposed to DMEM medium plus H₂O₂ (10⁻¹³ M) in the absence or presence of MEK1 kinase inhibitor PD98059 (2'-Amino-3'-methoxyflavone, 10⁻⁵ M) or cell permeable NF-κB inhibitor SN50 (AAVALLPAVLLALLAPVQRKRQKLMP, 10⁻⁵ M) for 24 h. The culture medium and cells were then collected for measurement of H₂O₂ and NOX5-S mRNA level.

Small Interfering RNA (siRNA) and Plasmid Transfection

For siRNA 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 small interfering RNAs (siRNAs) was carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For each well, 60 pmol of siRNA duplex of NOX5, p50, ERK2, ERK1, or control siRNAs formulated into liposomes were applied; the final volume was 1.2 ml per well. Twenty-four hours later, the transfectants were exposed to H₂O₂ (10⁻¹³ M) in fresh medium for an additional 48 h. Finally, the culture medium and the transfectants were collected for measuring NOX5-S mRNA and protein level. Transfection efficiencies were determined by fluorescence microscopy after transfection of Block-it fluorescent oligo (Invitrogen) and were ~ 90% at 48 h. Control siRNA is a scrambled sequence that will not

lead to the specific degradation of any known cellular mRNA.

For plasmid transfection, 0.5µg plasmids (NOX5-LP, p-NF-κB-Luc, or pCDNA3.0) or 0.1µg renilla plasmid formulated into liposomes were applied. All other procedures were similar to those described above.

RT-PCR

Total RNA was extracted by TRIzol reagent (Invitrogen) according to the protocol of the manufacturer, 1.0 µg of total RNAs were reversely transcribed by using a kit SUPERSCRIPT First-Strand Synthesis System for RT-PCR (Invitrogen).

Quantitative real-time PCR

Gene expression and regulation were measured using real-time PCR analysis. Random hexamers were used for Real-time PCR analysis was performed in a 15 µl reaction on 96-well clear plate using Power SYBR Green RT-PCR Reagents Kit (Applied Biosystems, Foster City, CA). The primers used were: NOX5-S sense (5'-AAGACTCCATCACGGGGCTGCA-3'), NOX5-S antisense (5'-CCTTCAGCACCTTGGCCAGA-3'), 18S sense (5'-CGGACAGGATTGACAGATTGATAGC-3') and 18S antisense (5'-TGCCAGAGTCTCGTTCGTTATCG-3'). Reactions were carried out in an Applied Biosystems StepOnePlus™ Real-Time 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 a StepOne™ 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 18S amplification.

Luciferase assay

FLO EA cells were seeded in 12-well plates for 24 h. 0.1 µg renilla and 0.5 µg of pCDNA3.0 (control) or NOX5-S luciferase reporter plasmid NOX5-LP containing the NOX5-S promoter fragment (-2501 to -1 from ATG) (Si et al., 2007), or p-NF-κ-Luc plasmid were transfected by using Lipofectamine 2000 (Invitrogen). Luciferase activity was assayed 24 h after transfection. Cell extracts were prepared by lysing the cells with lysis buffer (Promega). The lysate was centrifuged at 13,000 rpm for 10 min to pellet the cell debris. The luciferase activities in the cell lysates were measured by using Topcount-NXT™ Microplate Scintillation and Luminescence Counter (Packard Bioscience Company) according to the protocol (Promega) and normalized to renilla. The number of experiments was indicated in figure legends and each experiment was performed in triplicate.

Western blot analysis

Cells was lysed in Triton X lysis buffer containing 50 mM Tris_HCl (pH 7.5), 100 mM NaCl, 50mM NaF, 5mM EDTA, 1% (v/v) Triton X-100, 40mM β-glycerol phosphate, 40 mM *p*-nitrophenylphosphate, 200µM sodium orthovanadate, 100µM phenylmethylsulfonyl fluoride, 1µg/ml leupeptin, 1µg/ml pepstatin A, and 1µg/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 (Cao et al., 2003; Fu et al., 2006). Briefly, after these supernatants were subjected to SDS-PAGE, the separated proteins were electrophoretically transferred to a nitrocellulose membrane at 30 V overnight. The nitrocellulose membranes were blocked in 5% nonfat dry milk and then incubated with appropriate primary antibodies followed by 60-min incubation in horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnologies). Detection was achieved with an enhanced chemiluminescence agent (Amersham Biosciences).

Primary antibodies used were human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:1000), NOX5-S antibody (1:1000), p-ERK1/2 antibody (1:1000), ERK2 antibody (1:1000), p-p65 antibody (1:1000), and p65 antibody (1:1000). NOX5 antibody against a unique NOX5 peptide (NH₂-CLQTRTQPGRPDWSKV-COOH) was prepared by Sigma Genosys (The Woodlands, Texas) and used at a dilution of 1:1000.

Protein measurement

The amount of protein was determined by colorimetric analysis using the protein assay kit from Bio-Rad Laboratories (Richmond, CA) according to the method of Bradford (Bradford, 1976).

[³H] thymidine incorporation

For PD98059 treatment, cells were preincubated with PD98059 10⁻⁵M for 1 hour and then treated without (control) or with H₂O₂ (10⁻¹³ M) in the absence (control) or presence of PD98059 for 48h. For siRNA transfection, 24 hours after siRNAs of NOX5, p50, ERK2, ERK1 or control were introduced, cells were treated without or with H₂O₂ (10⁻¹³ M) 48h, and then incubated with methyl-[³H]thymidine (0.05 μCi/ml) for 4 h. After being washed three times with PBS to remove unincorporated radioactivity, cells were collected and homogenized with a lysis buffer containing

(pH7.4): 50 mM HEPES, 50 mM NaCl, 1% Triton X-100, 1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. Methyl-[³H] thymidine uptake was measured in a Topcount-NXT™ Microplate Scintillation and Luminescence Counter (Packard Bioscience Company). The level of protein in the homogenates was also determined and the level of methyl-[³H] thymidine incorporation was normalized to protein content.

Amplex red hydrogen peroxide fluorescent assay

Levels of H₂O₂ in culture medium were determined by the Amplex Red H₂O₂ assay kit (Molecular Probes, Eugene, Oregon, USA), according to the manufacturer's instruction. 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 540 nm and emission detection at 590 nm.

Materials

Human NOX5 siRNA was purchased from Applied Biosystems (Foster City, CA). PD98059 and SN50 were bought from Calbiochem (San Diego, CA); ERK2 antibody, GAPDH antibody, control siRNA, ERK2 siRNA, ERK1 siRNA, and p50 siRNA were from Santa Cruz Biotechnology; and phosphorylated MAP kinase antibody, phosphorylated p65 antibody and p65 antibody were from Cell Signaling Technology (Danvers, MA). pNF-κB-Luc Vector was purchased from Clontech Laboratories, Inc. (Mountain View, CA). P50 plasmid (Ballard et al., 1992) was from Addgene (Plasmid 21965, Cambridge, MA). Triton X-100, phenylmethylsulfonyl fluoride, DMEM, antibiotics, and other reagents were purchased from Sigma (St. Louis, MO).

Statistical analysis

Data were expressed as means \pm SE. Statistical differences between two groups were determined by Student's *t*-test. Differences among multiple groups were tested by ANOVA and checked for significance by Fisher's protected least significant difference test.

Results

H₂O₂ upregulates NOX5-S expression

To investigate whether H₂O₂ affects cell proliferation, we obtained a H₂O₂ dose-response curve. Figure 1A shows that 10⁻¹³ M H₂O₂ significantly increased thymidine incorporation by 39.3 \pm 6.6 % in FLO cells, while higher doses (10⁻⁵M and 10⁻⁷M) slightly decreased thymidine incorporation. The data suggest that low dose of H₂O₂ may increase cell proliferation in FLO cells.

We have shown that NOX5-S mediates acid-induced increase in cell proliferation. Therefore, we studied whether NOX5-S mediates exogenous H₂O₂-induced increase in cell proliferation. We found that knockdown of NOX5-S with NOX5 siRNA, which has been shown by us to effectively knock down NOX5-S (Fu et al., 2006), significantly decreased thymidine incorporation at basal condition as well as in response to H₂O₂ treatment in FLO cells (figure 1B), suggesting that NOX5-S may mediate H₂O₂-induced increase in cell proliferation.

Next we examined whether H₂O₂ upregulates NOX5-S. NOX5-S mRNA was measured by real time PCR. 10⁻¹³ M H₂O₂ (48h treatment) significantly increased NOX5-S mRNA level by 34.9 \pm 7.8% (ANOVA, P<0.05, figure 1C) and protein level by 42.5 \pm 11.6% (ANOVA, P<0.05, figure 1D-E) in FLO cells. Similarly, H₂O₂ significantly increased NOX5-S mRNA level by 93.9 \pm 13.5% in an EA cell line OE33 (ANOVA, P<0.01, figure 2D). To examine whether upregulation of

NOX5-S enhances H₂O₂ production, FLO cells were incubated with 10⁻¹³ M H₂O₂ for 48 hours, washed and then cultured in regular culture medium for additional 24 hours. Culture medium was collected for measurement. 48-hour H₂O₂ treatment significantly increased H₂O₂ production by 27.6±4.9% (t test, P<0.01, figure 1F).

Role of NF-κB in H₂O₂ induced NOX5-S expression

To examine the role of NF-κB in H₂O₂-induced NOX5-S expression, we used p50 siRNA which has been shown by us to effectively knock down p50 (Hong et al., 2010a). Figure 2A, 2B and 2C show that knockdown of p50 significantly decreased NOX5-S mRNA levels from 140.7±6.8% to 116.6±7.2 % (ANOVA, P<0.05) and NOX5-S protein expression from 158.0±2.2% to 85.0±5.8 % (ANOVA, P<0.05) in response to H₂O₂ treatment in FLO cells. Similarly, knockdown of p50 significantly decreased NOX5-S mRNA levels from 193.9±13.5% to 116.6±6.4 % in OE33 cells (ANOVA, P<0.01, figure 2D). In addition, knockdown of p50 significantly decreased H₂O₂-induced thymidine incorporation from 164.9±8.42 % to 112.3±9.2% (ANOVA, P<0.05, figure 2E).

The data suggest that NF-κB1 p50 may be involved in H₂O₂ induced NOX5-S expression. To further confirm this result, we transfected FLO cells with a NF-κB reporter plasmid p-NF-κB-luc. 10⁻¹³ M H₂O₂ significantly increased the luciferase activity by 36.0±10.0 % (t test, P<0.05, figure 3A). In addition, 10⁻¹³ M H₂O₂ (48h treatment) significantly increased p65 phosphorylation by 59.4±19.6% (ANOVA, P<0.05, figure 3D). These data suggest that H₂O₂ may activate NF-κB.

To examine whether NF-κB activates NOX5-S promoter, we transfected FLO cells with a NOX5-S reporter plasmid NOX5LP. Transfection with p65 or p50 expression plasmid significantly

increased luciferase activity by 124.1 ± 3.6 % (figure 3B), and by 140.2 ± 8.3 % (figure 3C), respectively, suggesting that NF- κ B may activate NOX5-S promoter.

Role of MAP kinases in H₂O₂-induced NOX5-S expression

To examine the role of MAP kinases in H₂O₂-induced NOX5-S expression, we used the MAPK kinase 1 (MEK1) inhibitor PD98059. Figure 4A-C shows that PD98059 significantly decreased H₂O₂-induced NOX5 protein levels from 298.2 ± 33.4 % to 122.2 ± 35.83 % (ANOVA, $P < 0.05$, figure 4A & B) and NOX5 mRNA levels from 186.5 ± 26.8 to 28.3 ± 12.2 % control (ANOVA, $P < 0.05$, figure 4C). In addition, PD98059 significantly reduced thymidine incorporation from 163 ± 9.9 to 94.7 ± 2.1 % control (ANOVA, $P < 0.01$, figure 4D).

To determine which MAP kinases mediate H₂O₂-induced NOX5-S expression, we used ERK1 and ERK2 siRNAs which have been shown by us to effectively knockdown of ERK1 and ERK2 (Hong et al., 2010a), respectively. Figure 5A-C shows that knockdown of ERK2 with ERK2 siRNA significantly decreased NOX5 protein expression from 160.1 ± 19.4 to 122.9 ± 5.2 % control (ANOVA, $P < 0.01$, figure 5A & B) and NOX5 mRNA levels from 153.9 ± 12 to 116.2 ± 3.5 % control (ANOVA $P < 0.01$, figure 5C) in FLO cells. Similarly, in OE33 cells knockdown of ERK2 significantly reduced NOX5 mRNA levels from 169.4 ± 5.7 to 114.3 ± 4.6 % control (ANOVA $P < 0.01$, figure 5D). In addition, knockdown of ERK2 significantly reduced thymidine incorporation from 149.3 ± 12.4 to 99.8 ± 14.9 % control in FLO cells (ANOVA, $P < 0.01$, figure 5E). Conversely, knockdown of ERK1 with ERK1 siRNA had no significant effect on NOX5 mRNA levels (figure 6A) and thymidine incorporation (figure 6B) in FLO cells. The data suggest that ERK2 MAP kinase may be involved in H₂O₂-induced NOX5-S expression. To investigate whether H₂O₂ activates

ERK2 MAP kinase, we studied ERK2 MAP kinase phosphorylation. Figure 7A and 7B show that H₂O₂ significantly increased ERK2 MAP kinase phosphorylation, suggesting that H₂O₂ may activate ERK2 MAP kinase. P98059 significantly decreased ERK2 phosphorylation, suggesting that PD98059 effectively blocks MAP kinase pathway. However, NF-κB inhibitor SN50 had no significant effect on ERK2 phosphorylation. Knockdown of ERK2 with ERK2 siRNA significantly reduced H₂O₂-induced increase in luciferase activity in FLO cells transfected with p-NF-κB-luc (figure 7C), suggesting that ERK2 MAP kinase may mediate H₂O₂ induced activation of NF-κB.

Discussion

Gastro-esophageal reflux disease complicated by Barrett's esophagus (BE) is a major risk factor for esophageal adenocarcinoma (EA) (Lagergren et al., 1999). Mechanisms whereby acid reflux may accelerate the progression from BE to EA are not fully understood. Reactive oxygen species (ROS) have been reported to be increased both in BE (Olyaei et al., 1995; Wetscher et al., 1997) and esophageal adenocarcinoma (Farhadi et al., 2002; Sihvo et al., 2003). We have previously shown that Barrett's metaplastic cells may be a source of ROS and that NADPH oxidase NOX5-S is responsible for acid-induced H₂O₂ production in Barrett's cells and in EA cells (Hong et al., 2010c). Besides metaplastic cells, other cells in BE mucosa, e.g. inflammatory cells, may also produce ROS which may affect metaplastic cells and thereby contribute to esophageal tumorigenesis.

We have shown that H₂O₂ increases cell proliferation in Barrett's cell line BAR-T and an EA cell line OE33 (Hong et al., 2010c). Consistently with our previous findings, we found that low

dose (10^{-13} M) of H_2O_2 significantly increased thymidine incorporation, while higher doses (10^{-5} M and 10^{-7} M) slightly decreased thymidine incorporation in FLO cells. The data suggest that low dose of H_2O_2 may increase cell proliferation in FLO cells. However, mechanisms whereby ROS increase cell proliferation are not fully understood.

In this study we investigated whether exogenous H_2O_2 affects cell proliferation in a Barrett's EA cell line FLO by stimulating NOX5-S expression. We found that NOX5-S may mediate H_2O_2 -induced increase in cell proliferation because 10^{-13} M H_2O_2 significantly increased NOX5-S mRNA level in FLO and OE33 cells as well as protein level and H_2O_2 production in FLO cells, while knockdown of NOX5-S blocked H_2O_2 -induced increase in thymidine incorporation in FLO cells.

ROS have been reported to activate NF- κ B (Flohe et al., 1997). We have also shown that NOX5-S-derived ROS activates NF- κ B in BE cell line BAR-T (Si et al., 2007). NF- κ B, a transcription factor, is known to function as tumor promoter (Pikarsky et al., 2004; Karin, 2006) and plays a key role in the development of colitis associated cancer (Greten et al., 2004) and cholestatic hepatitis-associated hepatocellular carcinoma (Pikarsky et al., 2004). NF- κ B is thought to be a member of a family of Rel domain-containing proteins, including Rel A (also called p65), Rel B, c-Rel, NF- κ B1 (p105/p50), and NF- κ B2 (p100/p52). P105 and p100 are larger precursor proteins containing I κ B (an inhibitor of κ B)-like ankyrin repeat sequences in their carboxyl termini. Because of their I κ B-like ankyrin repeat sequences these precursors are retained in the cytoplasm and require proteolytic processing to generate their mature DNA-binding proteins, p50 and p52, respectively (Karin et al., 2002). In the cytoplasm NF- κ B is in an inactive state and its activity is

regulated by three pathways. In the first pathway a heterotrimer composed of p50, p65, and I κ B is degraded in a ubiquitin-dependent reaction leading to the translocation of the p65-p50 dimers to the nucleus (Karin et al., 2002). In the second pathway, the dimers consisting of p100 and Rel B undergo proteolytic removal of the I κ B-like C-terminal domain of p100, allowing Rel B-p52 dimers to translocate to nucleus. In the third pathway, p50 (or p52) homodimers enter the nucleus, where NF- κ B activates gene transcription (Karin et al., 2002; De Bosscher et al., 2006). P50 plays an important role in lymphoid organogenesis and inflammation, whereas p52 is mainly involved in lymphoid organogenesis (Shih et al., 2011). Therefore, we focused on the role of p50 in NOX5-S expression.

We found that knockdown of p50 with p50 siRNA significantly decreased cell proliferation in FLO cells and inhibited H₂O₂-induced increase in NOX5-S expression in OE33 and FLO cells, suggesting that NF- κ B1 p50 may be involved in H₂O₂-induced NOX5-S expression in FLO and OE33 cells. This result is further supported by the following findings: 1) H₂O₂ significantly increased p65 phosphorylation and the luciferase activity in FLO cells transfected with a reporter plasmid pNF- κ B-Luc, suggesting that H₂O₂ may activate NF- κ B; 2) transfection with p50 or p65 expression plasmid significantly increased a NOX5-S reporter plasmid NOX5-LP, suggesting that NF- κ B may activate NOX5-S promoter.

ROS have been shown to activate MAP kinases (Kumar et al., 2008). ERK1 and ERK2 are isoforms of the 'classical' MAPK. Both ERK1 and ERK2 are activated by MAP kinase kinase (MEK) 1/2. MEK1/2 phosphorylates threonine and tyrosine residues in the Thr-Glu-Tyr (TEY) sequence of ERK1/2, resulting in the activation of ERK1/2. The ERK1/2 MAP kinase pathway is

involved in various cellular functions, including cell proliferation, differentiation and migration (Nishimoto and Nishida, 2006). This pathway is constitutively active in human tumors (Gioeli et al., 1999; Hoshino et al., 1999; Tanaka et al., 2003) and is known to be involved in acid-induced increase in cell proliferation in EA cells (Souza et al., 2002; Souza et al., 2004). It is also known to be involved in acid-induced increase in cell proliferation and in MAPK phosphorylation in SEG1 cells (Souza et al., 2002; Souza et al., 2004), suggesting that ERK1/2 MAPKs are activated by acid treatment. However, whether MAP kinases contribute to H₂O₂-induced expression of NOX5-S by upregulation of NF-κB and increase in cell proliferation is not known. We found that MEK1 kinase inhibitor PD98059 and knockdown of ERK2 significantly decreased H₂O₂-induced increase in NOX5-S protein and mRNA levels and in thymidine incorporation in EA cells, whereas knockdown of ERK1 had no effect. In addition, H₂O₂ significantly increased ERK2 MAPK phosphorylation, suggesting that H₂O₂ may activate ERK2 MAP kinase. These data suggest that ERK2 MAP kinase, but not ERK1, may be involved in H₂O₂-induced NOX5-S expression.

Next we examined the relationship between ERK2 MAPK and NF-κB. We found that ERK2 MAPK activates NF-κB since 1) knockdown of ERK2 significantly reduced H₂O₂-induced increase in luciferase activity in FLO cells transfected with p-NF-κB-luc; 2) NF-κB inhibitor SN50 had no significant effect on ERK2 phosphorylation. This result is consistent with the findings in a citrobacter rodentium-induced transmissible murine colonic hyperplasia model (Chandrakesan et al., 2010).

Mechanisms of NOX5-S-mediated increase in cell proliferation are not fully understood. We have shown two possible mechanisms: 1) NOX5-S-derived ROS activate cyclooxygenase 2,

increase PGE₂ production and thereby enhance cell proliferation(Si et al., 2007); 2)

NOX5-S-derived ROS also cause p16 promoter hypermethylation, downregulate p16 expression and thus stimulate cell proliferation (Hong et al., 2010c).

In conclusion, low doses of H₂O₂ increase cell proliferation. H₂O₂-induced increase in cell proliferation may depend on sequential activation of ERK2 MAP kinase and NF-κB and thus upregulate NOX5-S expression (figure 8). It is possible that in Barrett's esophagus ROS produced by Barrett's cells, inflammatory cells and other parenchymal cells may activate ERK2 MAP kinase and NF-κB, and cause upregulation of NOX5-S, which further enhances production of ROS (a positive feedback), increases cell proliferation and DNA damage, thereby contributing to the esophageal tumorigenesis.

Authorship contribution:

Participated in research design: *WC, XZ, JB and DL.*

Conducted experiments: *XZ and DL.*

Contributed new reagents or analytic tools: *WC, MBR and JW.*

Performed data analysis: *XZ and WC.*

Wrote or contributed to the writing of the manuscript: *WC, XZ, MBR, and JB*

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Footnote

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Figure Legends

Figure 1. H₂O₂ upregulates NOX5-S expression in FLO cells. **A)** FLO cells were incubated with different concentrations of H₂O₂ (10⁻⁵, 10⁻⁷, 10⁻⁹, 10⁻¹¹, 10⁻¹³, 10⁻¹⁴, 10⁻¹⁵ M) for 48 h and then incubated with methyl-[³H]thymidine (0.05 μCi/ml) for 4 h. 10⁻¹³ M H₂O₂ significantly increased thymidine incorporation, while higher doses (10⁻⁵ M and 10⁻⁷ M) slightly decreased thymidine incorporation. **B)** FLO cells were treated with H₂O₂ (10⁻¹³ M, 48h) 24 hours after NOX5 siRNA and control siRNA were introduced into FLO cells by Lipofectamine 2000. Knockdown of NOX5-S significantly decreased thymidine incorporation at basal condition as well as in response to H₂O₂ treatment. **C)** FLO cells were treated with 10⁻¹³ M H₂O₂ for 48h, and then NOX5-S mRNA levels were measured by real-time PCR. 10⁻¹³ M H₂O₂ significantly increased NOX5-S mRNA levels. **D)** A typical image of three Western blot analysis and **E)** summarized data showed that 10⁻¹³ M H₂O₂ significantly increased NOX5-S protein level. FLO cells were treated with 10⁻¹³ M H₂O₂ for 48h. **F)** FLO cells were treated with 10⁻¹³ M H₂O₂ for 48h, washed and cultured for additional 24 h. H₂O₂ levels in culture medium were measured by using an Amplex Red H₂O₂ assay kit. 48-hour H₂O₂ treatment significantly increased H₂O₂ production (n=3). t test, * P < 0.05, # P < 0.01; ANOVA, **, P < 0.01, compared with control or control siRNA group; ANOVA, ##, P < 0.01, compared with control siRNA plus H₂O₂ group.

Figure 2. Role of NF-κB in H₂O₂-induced NOX5-S expression. Transfection of p50 siRNA and control siRNA was carried out with Lipofectamine 2000. After 4-h transfection, the transfection medium was replaced with regular medium. 24 hours later, the transfectants were exposed to H₂O₂

(10^{-13} M) in fresh medium for an additional 48 h. **A)** A typical image of three Western blot analysis and **B)** summarized data show that knockdown of p50 significantly decreased NOX5-S protein expression at basal condition as well as in response to H_2O_2 treatment in FLO cells. **C)** Knockdown of p50 significantly decreased NOX5 mRNA levels in response to H_2O_2 treatment in FLO cells. **D)** Knockdown of p50 significantly decreased NOX5 mRNA levels in response to H_2O_2 treatment in OE33 cells. **E)** Knockdown of p50 significantly decreased not only thymidine incorporation at basal condition but also H_2O_2 -induced increase in thymidine incorporation in FLO cells. The data suggest that p50 mediates H_2O_2 -induced increase in NOX5-S expression and in cell proliferation. N=3, ANOVA, * $P < 0.05$, ** $P < 0.01$, compared with control siRNA group; ANOVA, $^{##} P < 0.01$, compared with control siRNA group plus H_2O_2 group.

Figure 3. NF- κ B1 p50 may be involved in H_2O_2 -induced NOX5-S. **A)** 24 h after transfection with p-NF- κ B-Luc plasmid and renilla plasmid, FLO cells were treated with 10^{-13} M H_2O_2 for 48h and then luciferase activity was measured. 10^{-13} M H_2O_2 significantly increased the luciferase activity in FLO cells, suggesting that H_2O_2 may activate NF- κ B. **B)** 24 h after transfection with NOX5-LP and renilla plasmid plus p65 or pCDNA3.0 (control), luciferase activity was measured. Transfection with p65 expression plasmid significantly increased luciferase activity, suggesting that p65 may activate NOX5-S promoter. **C)** 24 h after transfection with NOX5-LP and renilla plasmid plus p50 or pCMV4 (control), luciferase activity was measured. Transfection with p50 expression plasmid significantly increased luciferase activity, suggesting that p50 may activate NOX5-S promoter. **D)** A typical image of three Western blot analysis and **E)** summarized data showed that 10^{-13} M H_2O_2 significantly increased p65 phosphorylation. FLO cells were treated with 10^{-13} M

H₂O₂ for 48h. N=3, t test, * P < 0.05, compared with p-NF-κ-luc group or NOX5-LP plus pCDNA 3.0 group; ** P < 0.01, compared with NOX5-LP or NOX5-LP+pCDNA3.0.

Figure 4. Role of MAP kinases in H₂O₂-induced NOX5-S expression. **A)** A typical image of three Western blot analysis and **B)** summarized data show H₂O₂ significantly increased NOX5-S protein expression in FLO cells, an increase which was significantly decreased by PD98059. **C)** H₂O₂ significantly increased NOX5-S mRNA levels in FLO cells, an increase which was significantly decreased by PD98059, suggesting that MAPK may be involved in H₂O₂-induced NOX5-S expression. **D)** H₂O₂ significantly increased thymidine incorporation in FLO cells, an increase which was significantly decreased by PD98059, suggesting that MAPK may be involved in H₂O₂-induced increase in cell proliferation. N=3, ANOVA, * P < 0.05, ** P < 0.01, compared with control group; ### P < 0.01, compared with H₂O₂ group.

Figure 5. ERK2 MAP kinase may be involved in H₂O₂-induced NOX5-S expression.

Transfection of ERK2 siRNA and control siRNA was carried out with Lipofectamine 2000. After 4-h transfection, the transfection medium was replaced with regular medium. 24 hours later, the transfectants were exposed to H₂O₂ (10⁻¹³ M) in fresh medium for an additional 48 h. **A)** A typical image of three Western blot analysis and **B)** summarized data showed that knockdown of ERK2 significantly decreased NOX5-S protein expression at basal condition as well as in response to H₂O₂ treatment in FLO cells. **C)** Knockdown of ERK2 significantly decreased NOX5 mRNA levels at basal condition as well as in response to H₂O₂ treatment in FLO cells. **D)** Knockdown of ERK2

significantly decreased NOX5 mRNA levels in response to H₂O₂ treatment in OE33 cells. **E)**

Knockdown of ERK2 significantly decreased thymidine incorporation at basal condition as well as

in response to H₂O₂ treatment in FLO cells. The data suggest that ERK2 may mediate

H₂O₂-induced increase in NOX5-S expression and in cell proliferation. N=3, ANOVA, * P < 0.05,

** P < 0.01, compared with control siRNA group; # P < 0.05, ##, P < 0.01, compared with control

siRNA plus H₂O₂ group.

Figure 6. ERK1 MAP kinase may be not involved in H₂O₂-induced NOX5-S expression.

Transfection of ERK1 siRNA and control siRNA was carried out with Lipofectamine 2000. After

4-h transfection, the transfection medium was replaced with regular medium. 24 hours later, the

transfectants were exposed to H₂O₂ (10⁻¹³ M) in fresh medium for an additional 48 h. **A)**

Knockdown of ERK1 had no effect on H₂O₂-induced NOX5 mRNA levels. **B)** Knockdown of

ERK1 had no effect on H₂O₂-induced increase in thymidine incorporation. The data suggest that

ERK1 may not be involved in H₂O₂-induced increase in NOX5-S expression and in cell

proliferation. N=3, ANOVA, ** P < 0.01, compared with control siRNA group; #, P < 0.05, ##, P <

0.01, compared with control siRNA plus H₂O₂ group.

Figure 7. H₂O₂ may activate ERK2 MAP kinase. A) A typical image of three Western blot

analysis and **B)** summarized data showed H₂O₂ significantly increased ERK2 phosphorylation, an

increase which was significantly decreased by PD98059 (10⁻⁵ M), but not by SN50 (10⁻⁵ M).

FLO cells were exposed to H₂O₂ 10⁻⁵ M for 15 min in the absence or presence of PD98059 (10⁻⁵ M)

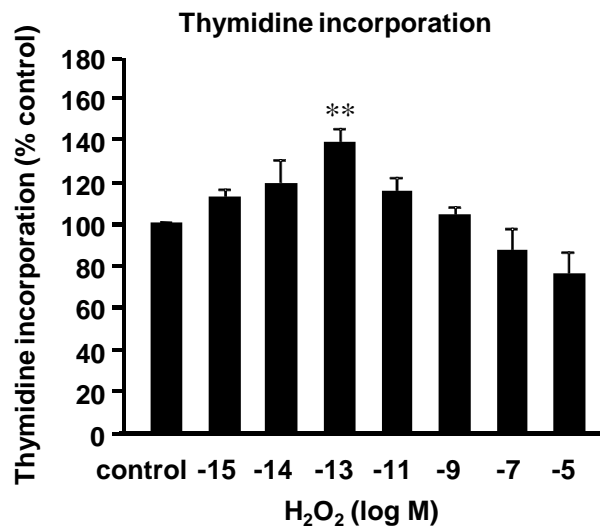
or SN50 (10⁻⁵ M). **C)** FLO cells were transfected with 0.1 µg renilla and 0.5 µg p-NF-κB-luc

plasmid by using Lipofectamine 2000. 24h after transfection with plasmids, 60 pmol ERK2 or control siRNA per well were transfected. 24 h later, FLO cells were treated with 10^{-13} M H_2O_2 for additional 48h. Finally the luciferase activity was measured. H_2O_2 significantly increased luciferase activity in FLO cells, an increase which was significantly reduced by knockdown of ERK2, suggesting that activation of NF- κ B may depend on activation of ERK2. N=3. **, P < 0.01, ANOVA, compared with control siRNA group; * P<0.05, ANOVA, compared with control siRNA group; #, P < 0.05, ANOVA, compared with control siRNA plus H_2O_2 group; ###, P < 0.01, ANOVA, compared with H_2O_2 group.

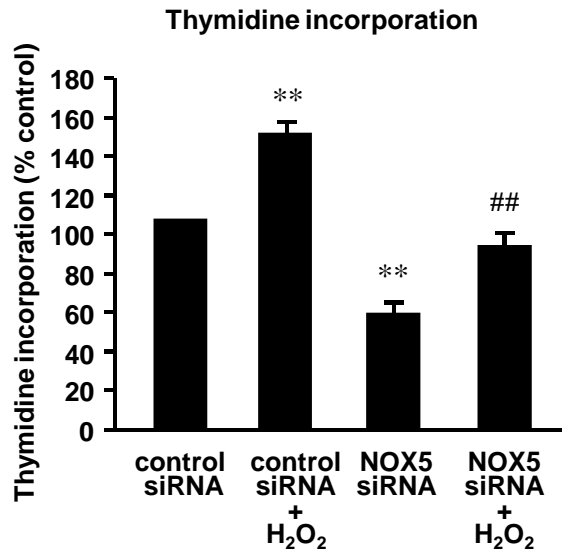
Figure 8. Signaling in H_2O_2 -induced increase in cell proliferation. Low doses of H_2O_2 increase cell proliferation. H_2O_2 -induced increase in cell proliferation may depend on sequential activation of ERK2 MAP kinase and NF- κ B and thus upregulate NOX5-S expression, which further enhances production of ROS (a positive feedback).

Figure 1

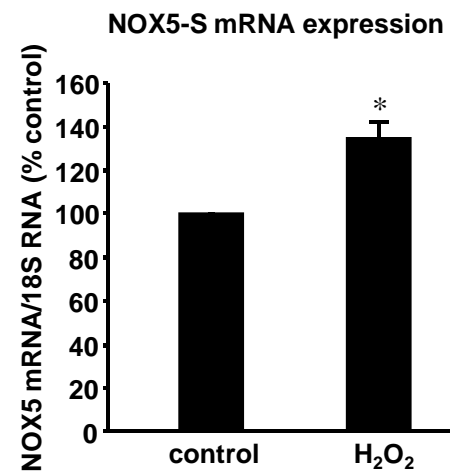
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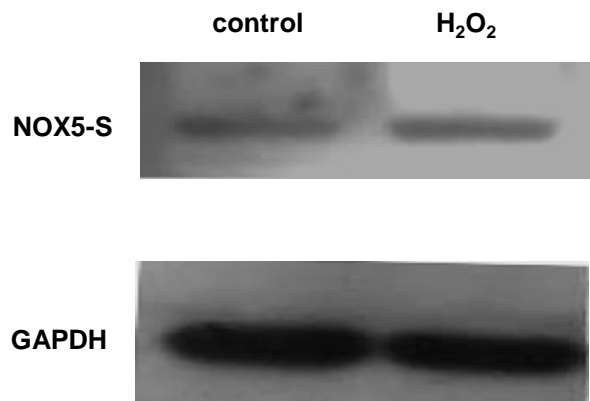
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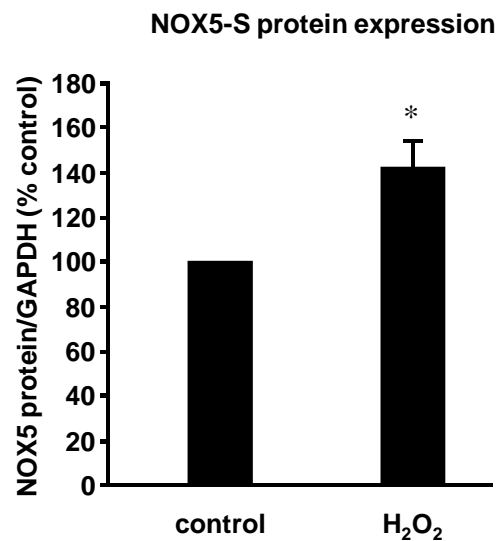
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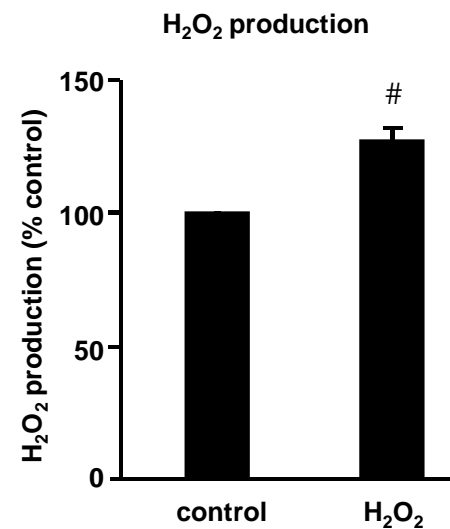


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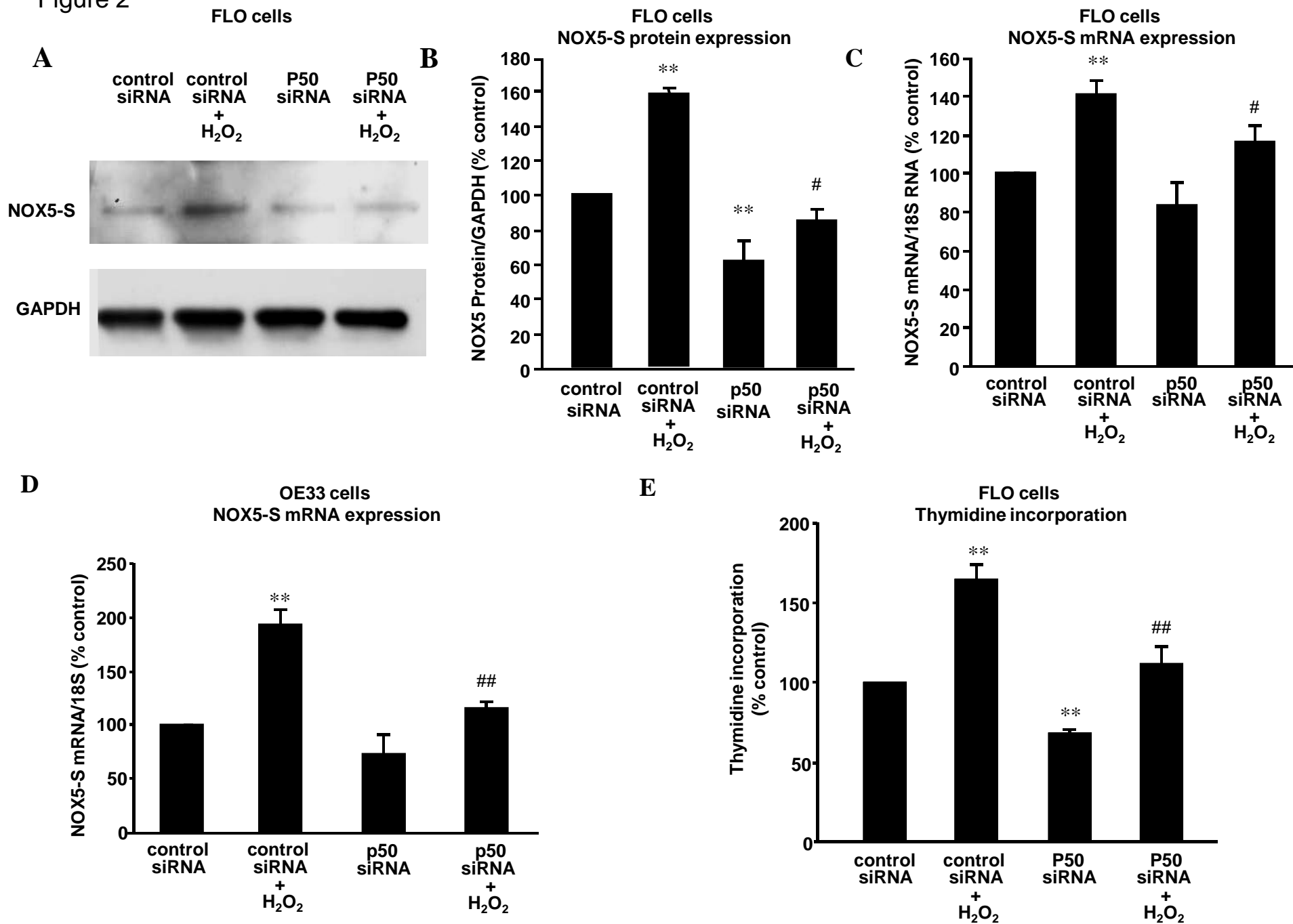


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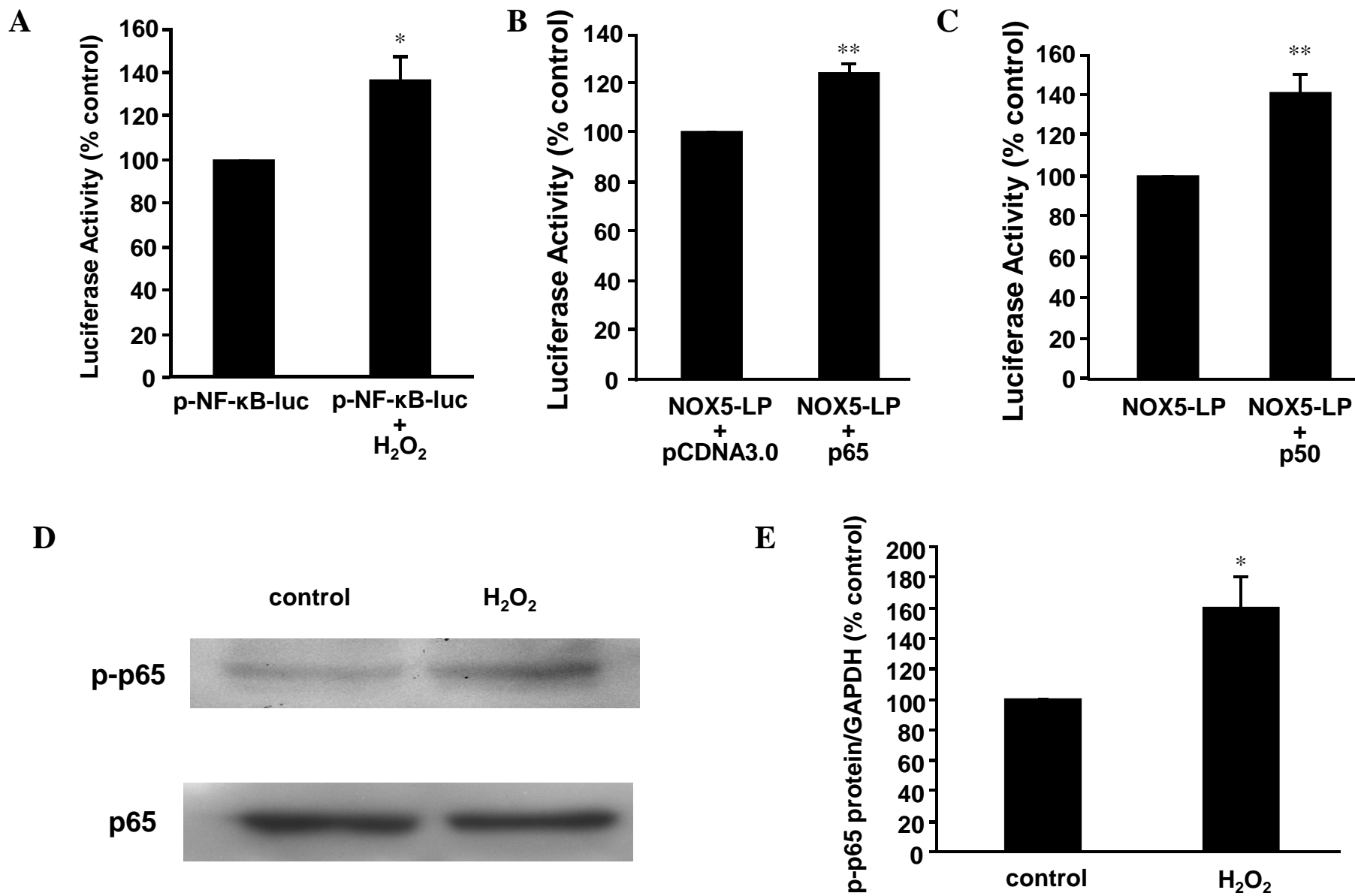
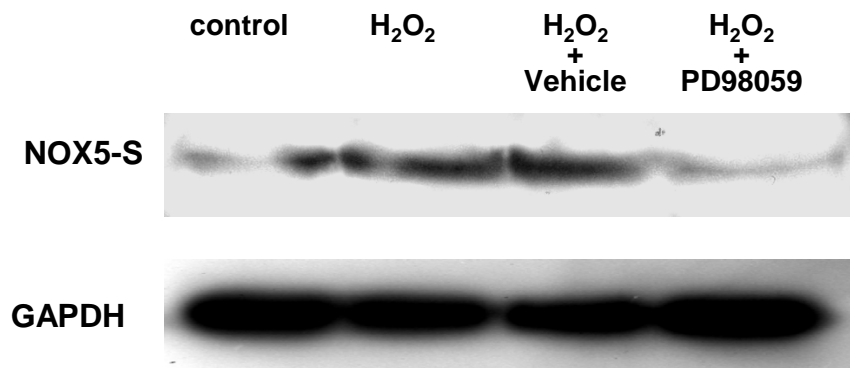
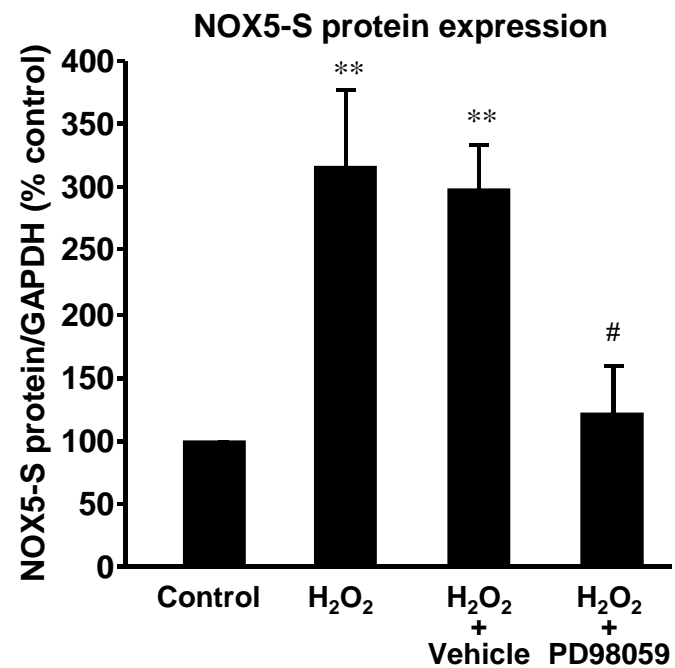


Figure 4

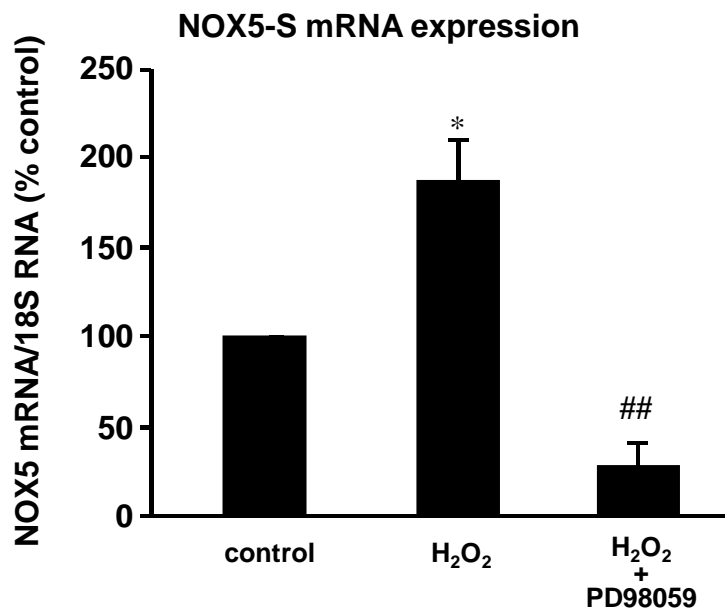
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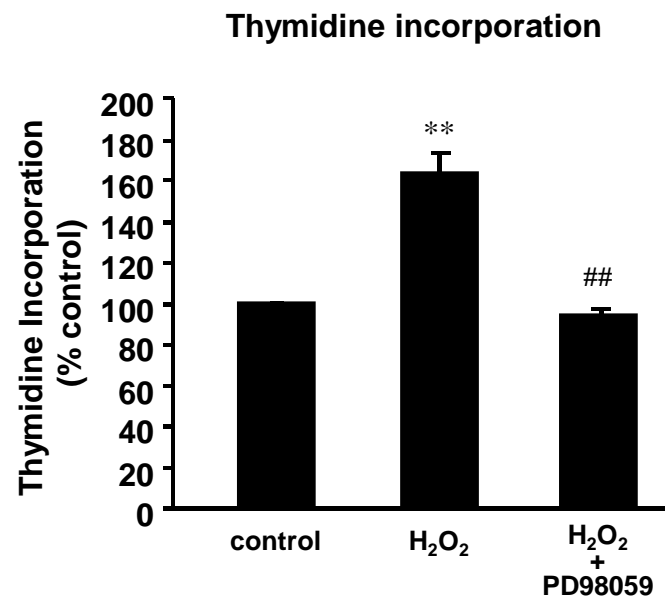


Figure 5

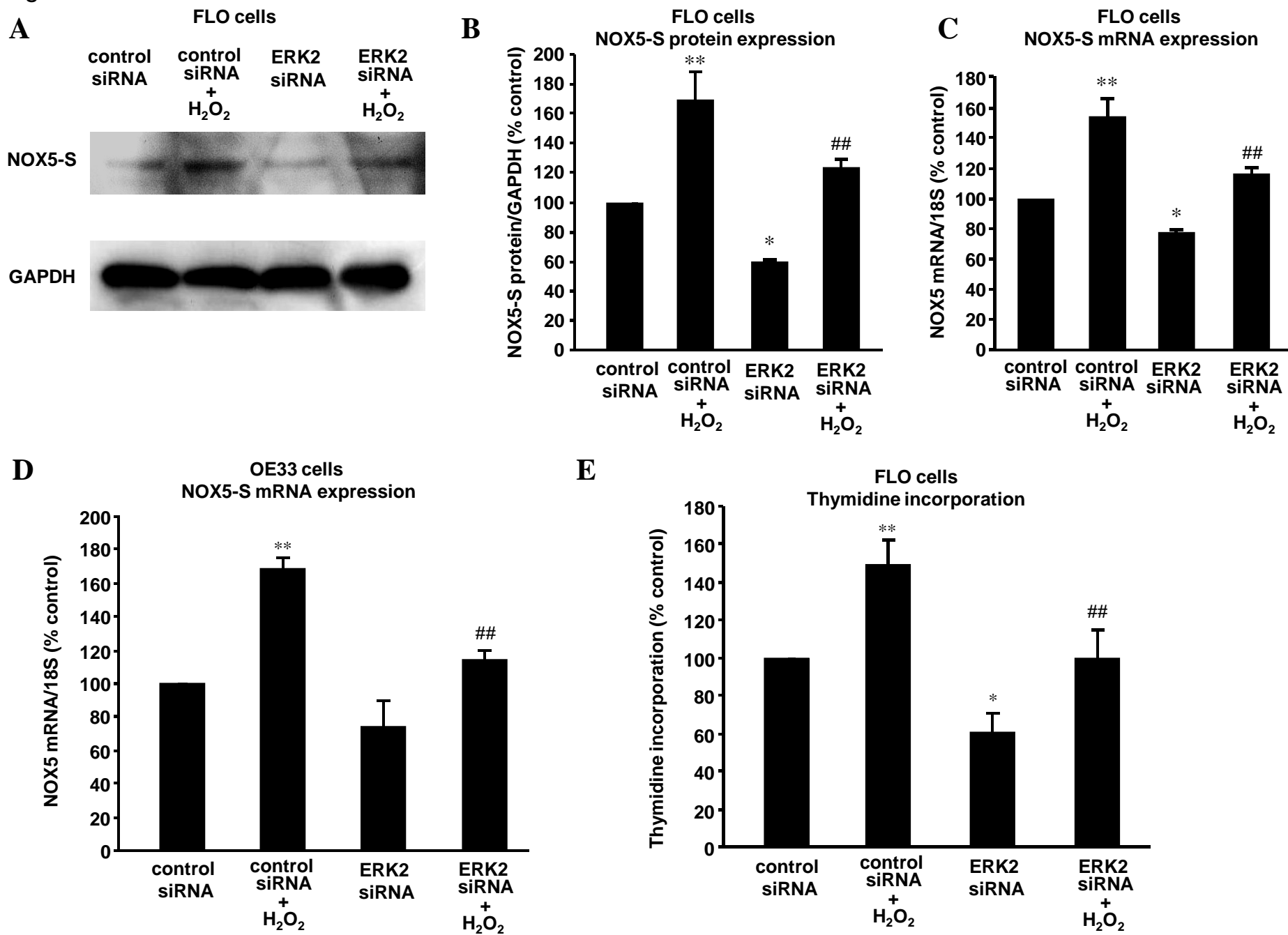


Figure 6

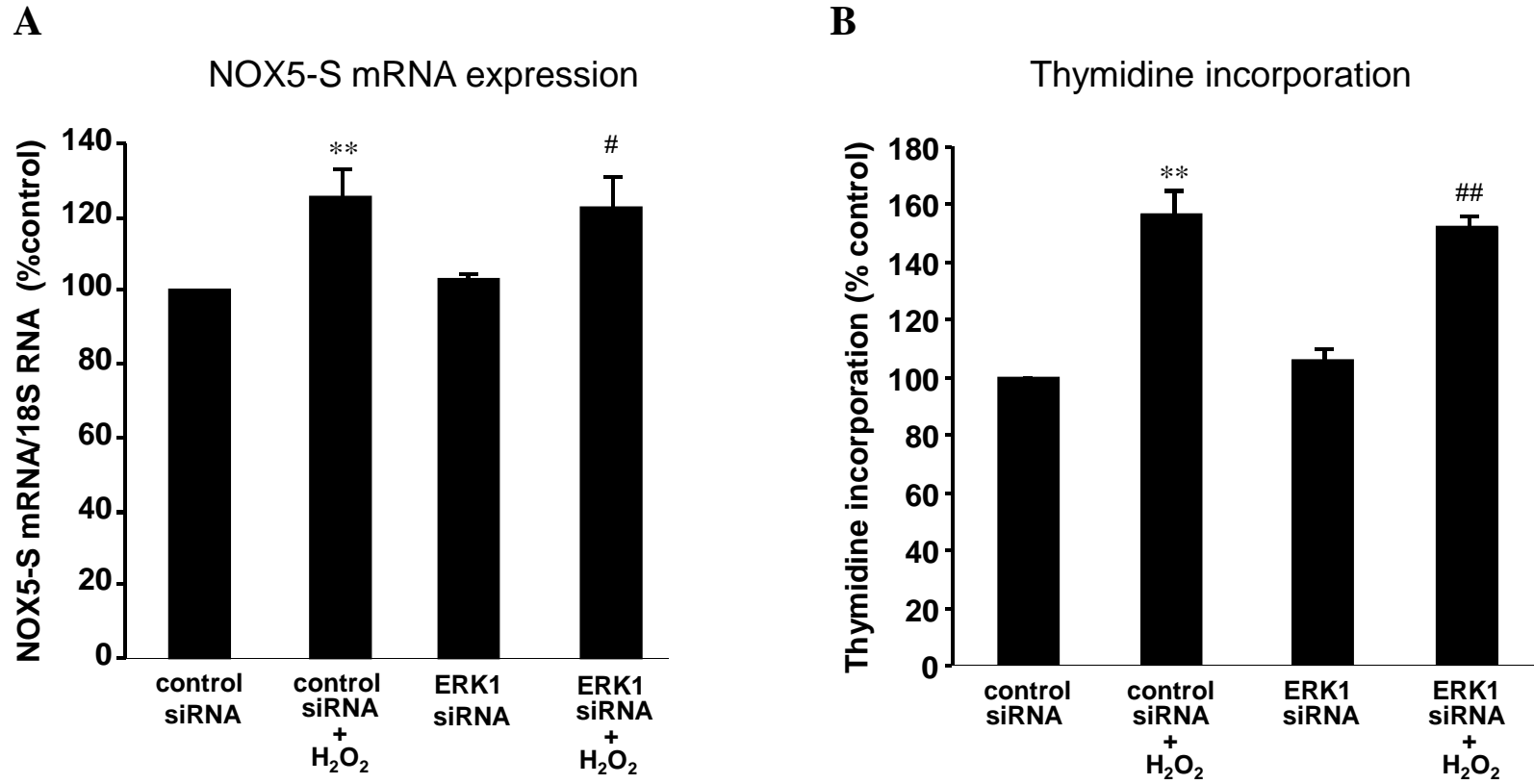
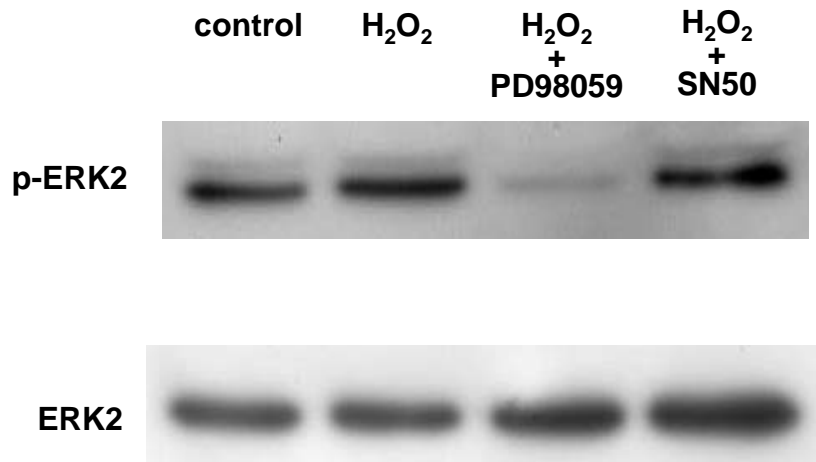
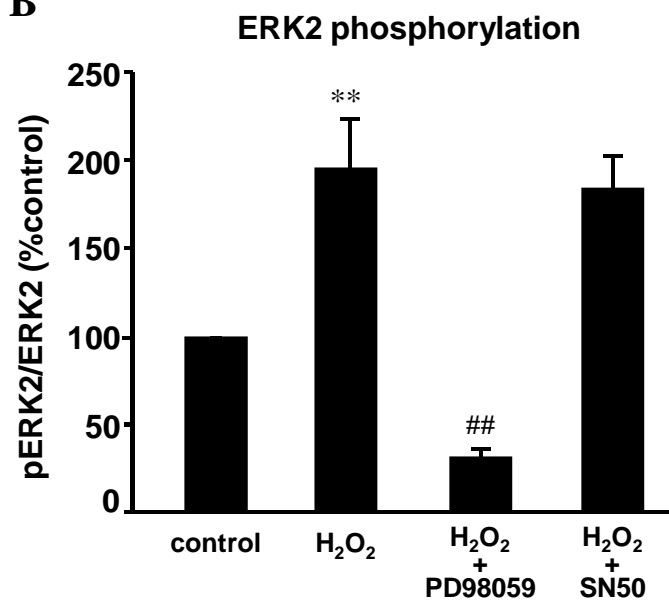


Figure 7

A



B



C

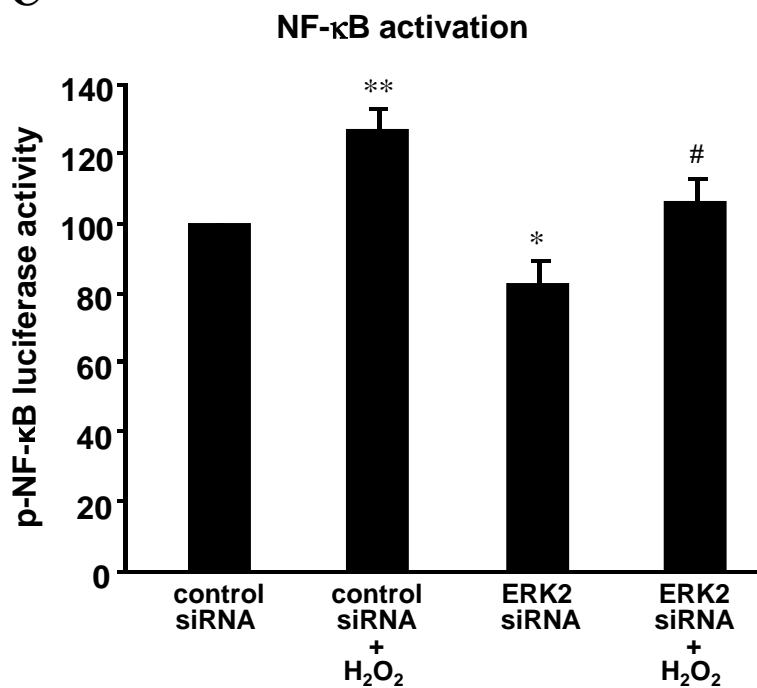


Figure 8

