STAT5 mediates PAF-induced NADPH oxidase NOX5-S expression in Barrett’s esophageal adenocarcinoma cells

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Si J, Behar J, Wands J, Beer DG, Lambeth D, Chin YE, Cao W. STAT5 mediates PAF-induced NADPH oxidase NOX5-S expression in Barrett’s esophageal adenocarcinoma cells. Am J Physiol Gastrointest Liver Physiol 294: G174–G183, 2008. First published October 18, 2007; doi:10.1152/ajpgi.00291.2007.—We have shown that NADPH oxidase NOX5-S is overexpressed in Barrett’s esophageal adenocarcinoma (EA) cells and may contribute to the progression from Barrett’s esophagus (BE) to EA presumably by increasing cell proliferation and decreasing apoptosis (Fu X; Beer DG, Behar J, Wands J, Lambeth D, Cao W. J Biol Chem 281: 20368–20382, 2006). The mechanism(s) of NOX5-S overexpression in EA, however, is not fully understood. In SEG1 EA cells we found that acid treatment significantly increased platelet-activating factor (PAF) production, which in turn markedly increased NOX5-S expression and hydrogen peroxide (H2O2) production. Knockdown of NOX5-S by NOX5-S small interfering RNA (siRNA) blocked PAF-dependent H2O2 production. PAF-dependent induction of NOX5-S expression and H2O2 production were significantly decreased by the MAPK kinase 1 inhibitor PD-98059, by the cytosolic phospholipase A2 (cPLA2) inhibitor AACOCF3, and by STAT5 downregulation with STAT5 siRNA. PAF significantly increased the phosphorylation of ERK1/2 MAPK, cPLA2, and STAT5. Using inhibitors, we demonstrated that PAF-induced STAT5 phosphorylation depends on activation of ERK1/2 MAPK and cPLA2, whereas PAF-induced cPLA2 phosphorylation was associated with activation of ERK1/2 MAPK. Given that STAT5 bound to the c-sis-inducible element (TTCTGGTAA) of the NOX5-S promoter, overexpression of STAT5 significantly increased NOX5-S promoter activity. We conclude that acid-induced NOX5-S expression and H2O2 production is mediated in part by production of PAF in SEG1 EA cells, and that PAF-induced increase in NOX5-S expression depends on sequential activation of ERK MAP kinases, cPLA2, and STAT5 in these cells.

platelet-activating factor; signaling; MAPK; cPLA2; reactive oxygen species

THE MAJOR RISK FACTOR FOR esophageal adenocarcinoma (EA) is gastroesophageal reflux disease complicated by Barrett’s esophagus (BE) (29), where esophageal squamous epithelium damaged by acid reflux is replaced by a metaplastic, intestinal-type epithelium. However, the mechanisms of progression from Barrett’s esophagus to esophageal adenocarcinoma are not fully understood. Acid reflux may contribute to this progression since 1) cultured biopsy specimens of intestinal metaplastic cells demonstrate a significant increase in [3H]thymidine uptake when explants are briefly exposed to acid (19); 2) long-term inhibition of esophageal acid exposure by administration of proton pump inhibitors (PPI) to patients with BE has been shown to inhibit metaplastic cell proliferation (35); and 3) a prospective study has shown that PPI treatment significantly reduces the incidence of dysplasia in BE patients, compared with no therapy or treatment with H2 receptor antagonist (17).

Reactive oxygen species (ROS) may be an important factor mediating acid reflux-induced damage. ROS may damage DNA, RNA, lipids, and proteins, leading to increased mutation and altered functions of enzymes and proteins (e.g., activation of oncogene products and/or inhibition of tumor suppressor proteins) (18, 33). A high level of ROS is generated through activation of NADPH oxidase in phagocytes. Phagocytic NADPH oxidase consists of two membrane subunits gp91phox and p22phox and three cytosolic subunits p40phox, p47phox, and p67phox (2, 30). Recently superoxide-generating homologues of gp91phox (NOX1, NOX3-NOX5, DUOX1, DUOX2) and homologues of other subunits (p41phox or NOXO1, p51phox or NOXA1) have been found in nonphagocytic cells (3, 30, 39). Two types of NOX5 have been described: NOX5-S and NOX5-L (41). All the four isoforms of NOX5-L (4, 14). Elevated ROS level have been reported in BE (34, 42) and in EA (18, 36). We have shown that the NADPH oxidase isoforms NOX1 and NOX5-S are present in EA cells and that levels of NOX5-S are significantly increased in EA cells and in Barrett’s esophageal mucosa with high-grade dysplasia (20). We have shown that in SEG1 EA cells, the NADPH oxidase NOX5-S is responsible for acid-induced H2O2 production. Overproduction of ROS, derived from acid-induced up-regulation of NOX5-S, increases cell proliferation and decreases apoptosis, possibly contributing to progression from intestinal metaplasia (BE) to dysplasia and to adenocarcinoma (20).

In addition to acid, inflammatory mediators present in acid and/or bile acid reflux-induced inflammation in BE may also cause production of ROS. Platelet-activating factor (PAF), a potent proinflammatory lipid mediator, is increased in a cat model of esophagitis (16). PAF increases ROS production in macrophages, tracheal epithelial cells, and esophageal smooth muscles (15, 21, 28). PAF may also promote migration and proliferation of tumor cells (11), enhance adhesiveness of tumor cells to vascular endothelia (11), increase oncogene expression (40), and stimulate the angiogenic response (32). However, it is not known whether acid increases production of

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PAF and whether PAF upregulates NADPH oxidase NOX5-S. In this work, we provide evidence that in SEG1 EA cells acid increases production of PAF, which in turn upregulates NOX5-S expression level. To our knowledge we are the first to report that STAT5 activation via sequential activation of ERK1/2 MAP kinases and cytosolic phospholipase A2 (cPLA2) is responsible for PAF-induced NOX5-S expression.

MATERIALS AND METHODS

Cell culture and acid treatment. Human Barrett’s adenocarcinoma cell line SEG1 was derived from human esophageal Barrett’s adenocarcinomas (26). These cells were cultured in DMEM containing 10% fetal bovine serum and antibiotics at 37°C in a 5% CO2 humidified atmosphere.

For acid treatment, SEG1 cells were exposed to acidic DMEM medium (pH 4.0), acidic medium plus CV-3988 (10^{-5} M), or normal DMEM medium (control) for 1 h, washed, and cultured in fresh medium (pH 7.2, without phenol red) for an additional 24 h. For the CV-3988 group, CV-3988 was added to the culture medium in this additional 24-h culture. Finally the culture medium and cells were collected for measurements. Acidic DMEM medium (pH 4.0, 250 μl) was added to each well in a 12-well plate and the final pH was −4.9 after 1-h incubation. For PAF treatment, SEG1 cells were incubated with different concentrations of PAF (from 10^{-10} to 10^{-7} M) or with PAF 10^{-7} M in the absence or presence of PD-98059 (10^{-5} M) or AACOCF3 (10^{-5} M) for 24 h. The culture medium and cells were then collected for measuring H2O2 and NOX5-S mRNA level. When the culture medium was used to measure H2O2, RPMI 1640 (without phenol red) was used to avoid the interferences of DMEM medium on H2O2 assay.

siRNA transfection. At 24 h before transfection and when reaching 40–50% confluency, cells were trypsinized and diluted 1:5 with fresh medium without antibiotics (1–3 × 10^5 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, 75 pmol siRNA duplex of NOX5, STAT5 or control siRNA formulated into liposomes were applied; the final volume was 1.2 ml per well. After 4-h transfection, the transfection medium was replaced with regular medium. Twenty-four hours later, the transfectants were exposed to PAF (10^{-7} M) in fresh medium for an additional 24 h. Finally, the culture medium and the transfectants were collected for measuring H2O2 and/or NOX5-S mRNA level. Transfection efficiencies were determined by fluorescence microscopy after transfection of Block-it fluorescent oligo (Invitrogen) and were ~90% at 48 h. Control siRNA for NOX5 is the scrambled siRNA of the same target gene. Control siRNA for STAT5 is a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA.

RT-PCR. Total RNA was extracted by TRizol reagent (Invitrogen) according to the protocol of the manufacturer; 1.5 μg of total RNAs were reversely transcribed by using a kit SUPERSCRIPT First-Strand Synthesis System for RT-PCR (Invitrogen).

Quantitative real-time PCR. Quantitative real-time PCR was carried out on a Stratagene Mx4000 multiplex quantitative PCR system (Stratagene, La Jolla, CA). The primers used were specific for NOX5:

![Fig. 1. Effect of platelet-activating factor (PAF) on acid-induced H2O2 production in SEG1 cells. A: total RNA was prepared from SEG1 cells that were treated with culture medium (control), acid, or acid plus PAF receptor antagonist CV3988 for 1 h, washed, and then cultured for additional 24 h in the absence or presence of CV-3988 (10^{-5} M), NOX5-S mRNA expression was analyzed with quantitative real-time PCR and normalized to GAPDH (N = 4). B: levels of H2O2 in the culture medium were determined by using an Amplex Red H2O2 fluorescent assay kit and normalized to protein content of cells in each well (N = 3). Culture medium was collected 24 h after pulsed acid treatment as described above. C: PAF was measured in the culture medium collected 24 h after pulsed acid treatment (pH 4, 1 h) and normalized to protein content of cells in each well (N = 3). D: levels of H2O2 were measured in the culture medium collected after SEG1 cells were incubated with PAF for 24 h (N = 3). ANOVA †P < 0.02, **P < 0.001, ***P < 0.0001, compared with control group; #P < 0.05, ##P < 0.02, compared with acid group. *P < 0.05, unpaired t-test.](http://ajpgi.physiology.org/)

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sense 5'-AAAGCTTCATCAAGGGCTGCA-3', antisense 5'-CCT-TCAGCCACCTTGCCAGA-3'; NOX1: sense 5'-GGTGCTGACGAG- CATTAAAACIT-3', antisense 5'-AAAAACTCTAGTTTACCACAT-TGC-3'; GAPDH: sense 5'-CATGCCACACAGTTTACGGCAT- CAC-3', antisense 5'-AGTTCCACCACCTGTTTGC-TGA-3'.

All reactions of each experiment were performed in triplicate in 25 μl total volume containing a 1× concentration of Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA), 100 nM of each sense and antisense primer, 1 μl cDNA, and 30 nM reference dye. Reactions were carried out in a Stratagene MX4000 multiplex quantitative PCR system (Stratagene, La Jolla, CA) for one cycle at 94°C for 5 min; 40 cycles at 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s; one cycle at 94°C for 1 min; and one cycle at 55°C for 30 s. Fluorescence values of SYBR Green I dye, representing the amount of product amplified at that point in the reaction, were recorded in real time at both the annealing step and the extension step of each cycle. The threshold cycle, defined as the point at which the fluorescence signal was statistically significant above background, was calculated for each amplicon in each experimental sample by use of Stratagene MX4000 software. This value was then used to determine the relative amount of amplification in each sample by interpolating from the standard curve. Transcript level of each specific gene was normalized to GAPDH amplification. The number of experiments was indicated for each experiment.

ChIP assay. Chromatin immunoprecipitation (ChIP) assay was performed using the ChIP assay kit (Upstate, Charlottesville, VA) as described previously (20). Briefly, SEG1 cells grown in plastic dishes for 2 days (~1 × 10^6 cells) were treated with PAF 10^{-7} M for 1 h to see more binding and then treated with 1% formaldehyde for 10 min to cross-link STAT5 to DNA. After removal of the formaldehyde, the cells were washed with ice-cold PBS containing 0.1% EDTA and protease inhibitors (1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor mixture) and gently scraped into a conical tube, centrifuged for 5 min at 700 g at 4°C. Pelleted cells were resuspended in 400 μl of lysis buffer [10 mM HEPES, pH 7.9, 60 mM KCl, 0.5% (vol/vol) NP-40] with protease inhibitors and incubated on ice for 10 min. Nuclei were recovered by centrifugation at 100 g for 10 min and resuspended in 400 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing protease inhibitors. The mixture was incubated on ice for 10 min and the lysate was sonicated eight times for 10 s each time on ice to shear the genomic DNA to lengths of 0.2–1 kb. The debris was removed by centrifugation and the supernatant was then diluted 10 times with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 16.7 mM NaCl, and protease inhibitors, pH 8.0). Five hundred microliters of the diluted lysate was kept for input control. The chromatin solution was precleared with salmon sperm DNA-protein A agarose for 1 h at 4°C. Polyclonal antibody against STAT5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the supernatant fraction and incubated overnight at 4°C with rotation. The mixture was then incubated with 60 μl of salmon sperm DNA-protein A agarose slurry for 1 h at 4°C with rotation. c-Myc antibody (SeroTec, Raleigh, NC) and IgG were used as the negative control. The protein A agarose-antibody-histone complex was pelleted by gentle centrifugation (1,000 g at 4°C for 1 min). The pellet was washed sequentially (3–5 min per wash) on a rotating platform with 1 ml each of low-salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl, pH 8.0, 0.05, Student’s t-test. NTC, no template control. Standards: 364 pg, 36.4 pg, 3.64 pg, 0.364 pg (from left to right).

Fig. 2. NOX5-S mediates PAF-induced H2O2 production in SEG1 cells. A: SEG1 cells were transfected with control or NOX5 small interfering RNA (siRNA) by using Lipofectamine 2000; 24 h later, the transfectants were exposed to PAF (10^{-7} M) in fresh medium for an additional 24 h. The culture medium was used to measure H2O2 and cells to measure protein content. Percent increase was calculated by the equation (PAF group – control) × 100/control. N = 3. B: total RNA was prepared from SEG1 cells that were treated with PAF (10^{-7} M) for 24 h and analyzed for NOX1 mRNA expression with quantitative real-time PCR (N = 3). C: example of NOX5 amplification curve in real-time PCR. D, summarized data. Total RNA was prepared from SEG1 cells that were treated with PAF (10^{-7} M) for 24 h and analyzed for NOX5-S mRNA expression with quantitative real-time PCR (N = 3). E: SEG1 cell were transfected with NOX5-S promoter reporter plasmid and treated without or with PAF 10^{-7} M for 24 h (N = 3). *P < 0.05, Student’s t-test. NTC, no template control. Standards: 364 pg, 36.4 pg, 3.64 pg, 0.364 pg (from left to right).
8.0), high-salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl, pH 8.0), LiCl washing buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After the final wash, the pellet was eluted by two 15-min incubations with 250 μl of freshly made elution buffer (1% SDS and 50 mM NaHCO₃). Two fractions of elutes were combined and 20 μl of 5 M NaCl was added to the supernatant. Cross-linking was reversed by heating at 65°C for 4 h, followed by the addition of 10 μl of 0.5 mM EDTA, 20 μl of 1 M Tris-HCl, pH 6.5, and 2 μl of 10 mg/ml protease K. The sample was incubated at 45°C for 2 h, and DNA was then extracted by phenol chloroform extraction followed by ethanol precipitation. The DNA pellet was resuspended in 50 μl of H₂O, and 5 μl was used for PCR analysis. PCR was carried with the primer pairs that targeted the −2281 to −2076 region (position from ATG) of the human NOX5 promoter (sense: 5′-TCCAGAAGGGGAATTCCTTG-3′, antisense: 5′-ACAGAGTTGAGAATTCATCTC-3′) at 94°C 5 min; 94°C 30 s-62°C 30 s-72°C 30 s for 35 cycles followed 7-min extension at 72°C. Results were visualized on 2% agarose gels stained with ethidium bromide.

**Gel mobility shift assay:** Gel mobility shift assay was performed using the gel mobility shift assay kit (Promega, Madison, WI) following the manufacturer’s protocol. Radiolabeled probe oligonucleotide derived from human NOX5 promoter (−2255 to −2236) was prepared via end labeling by phosphorylation with [γ-³²P]ATP and T4 polynucleotide kinase. Probes were purified by centrifugation through Sephadex G-25 columns (Roche, Indianapolis, IN). Gel shift assay was performed by incubating 2 μg of Hela nuclear extract with 0.07 pmol of radiolabeled probe in a 10-μl reaction buffer containing 10 mM Tris (pH 7.5), 0.05 mg/ml poly(dI-dC)/poly(dI-dC), 4% glycerol, 0.5 mM EDTA, 0.1 M KCl, 0.5 mM PMSF, 0.5 mM DTT, 1 mM magnesium chloride, and 1 mM sodium chloride for 20 min at room temperature. The main purpose of this assay is to examine whether STAT5 binds to NOX5-S promoter. Therefore, we used Hela nuclear extracts instead of SEG1 cell nuclear extracts for competition experiments and supershift assay with rabbit STAT5 antibody, the competing unlabeled probes or antibodies were preincubated for 20 min at room temperature with the nuclear extracts before the addition of unlabeled probes or antibodies were preincubated for 20 min at room temperature. The main purpose of this assay is to examine whether STAT5 binds to NOX5-S promoter. Therefore, we used Hela nuclear extracts instead of SEG1 cell nuclear extracts for competition experiments and supershift assay with rabbit STAT5 antibody, the competing unlabeled probes or antibodies were preincubated for 20 min at room temperature with the nuclear extracts before the addition of unlabeled probes. The wild-type competitor (NOX5-pWT) (5′-TTGTAATTTTTCACCAGTTTCCA-3′) or mutant competitor (NOX5-pMUT) (5′-TTTGGAACTGTATCTTCCA-3′) and 5′-TAGAAATACCAAGACTCTACATTTACCA-3′). The DNA-protein complexes were resolved on a 6% nondenaturing polyacrylamide gel with 2.5% glycerol for 2 h at 150 V in 0.5× Tris-Borate-EDTA (TBE) buffer. After electrophoresis, the gel was dried and exposed to autoradiography film at −80°C overnight.

**Construction of NOX5-s promoter reporter (NOX5-S-LP):** The fragment (−2501 to −1 from ATG) of NOX5-S promoter was amplified by PCR using SEG1-EA cell genomic DNA as template with the primers containing a Mlu I restriction site at the 5′end and an Bgl II restriction site at the 3′end. The primers used for the PCR were 5′-CGACCCGCTCCTCAAGCTTTCATGTGGTACTG-3′ and 5′-TGTAGCTGTGGCTCCTCGGAGGAAATCCATCG-3′. The PCR products were digested with Mlu I and Bgl II and cloned into the luciferase reporter plasmid, pGL3-Basic (Promega). The constructed plasmid was verified by sequencing.

**Luciferase assay:** SEG1-EA cells were seeded in 24-well plates for 24 h, and 0.2 μg of either pGL3-Basic (as no-promoter control) or luciferase reporter plasmid containing the NOX5-S promoter fragment was transfected by using Lipofectamine 2000 (Invitrogen). In cotransfection experiments, 0.6 μg of STAT5B expression vector or pcDNA 3.1 vector (Invitrogen) was used.

**Luciferase activity** was assayed 24 h after transfection. Cell extracts were prepared by lysing the cells with lysis buffer (Roche). The lysate was centrifuged at 13,000 rpm for 10 min to pellet the cell debris. The protein concentration in the supernatants was determined.

The luciferase activities in the cell lysates were measured by using Luciferase assay substrate (Roche) and normalized to protein content. The number of experiments was indicated in figure legends and each experiment was performed in triplicate.

**Western blot analysis:** Cells were pretreated with or without PD-98059 10⁻⁵ M or AACOCF3 10⁻⁵ M for 30 min and then treated with
PAF $10^{-5}$ M in the absence or presence of the above inhibitors for additional 30 min. Cells were lysed in Triton X-100 lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (vol/vol) Triton X-100, 40 mM β-glycerol-phosphate, 40 mM p-nitrophenylphosphate, 200 μM sodium orthovanadate, 100 μM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin. The suspension was centrifuged at 15,000 g for 5 min, and the protein concentration in the supernatant was determined. Western blot was done as described previously (12, 20). Briefly, after these supernatants were subjected to SDS-PAGE the separated proteins were electrophoretically transferred to a nitrocellulose (NC) membrane at 30 V overnight. The NC membranes were blocked in 5% nonfat dry milk and then incubated with appropriate primary antibodies followed by 60 min of incubation in horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Piscataway, NJ). Detection was achieved with an enhanced chemiluminescence agent (GE Healthcare).

Primary antibodies used were phosphorylated MAP kinase antibody (1:1,000), ERK2 antibody (1:5,000), phosphorylated cPLA$_2$ antibody (1:1,000), cPLA$_2$ antibody (1:1,000), phosphorylated STAT5 antibody (1:1,000), and STAT5 antibody (1:1,000).

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

PAF measurement. PAF in the culture medium was measured by using the PAF-3H-labeled scintillation proximity assay (SPA) system (TRK 990; GE Healthcare). In this assay the antibody-bound PAF was reacted with the SPA reagent, which contains protein A bound to fluomicrospheres. Any $[^3]$H]PAF that was bound to the primary rabbit antibody will therefore be immobilized on the protein A fluomicrosphere, which would produce light. Measurement in a β-scintillation counter enabled the amount of labeled PAF bound to be calculated. The concentration of unlabeled PAF in a sample was then determined by interpolation from a standard curve.

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 (9).

Materials. Human NOX5 siRNA was purchased from Ambion (Austin, TX). PD-98059 and AACOCF3 were bought from Calbiochem (San Diego, CA), ERK2 antibody, STAT5 antibody, control siRNA, and STAT5 siRNA were from Santa Cruz Biotechnology, and phosphorylated STAT5 antibody, phosphorylated MAP kinase antibody, and phosphorylated and unphosphorylated cPLA2 antibodies were from Cell Signaling Technology.

Fig. 4. PAF-induced phosphorylation of MAPK and cPLA$_2$. A: whole cell extracts were obtained from SEG1 cells treated without (control) or with PAF (10$^{-5}$ M) for 30 min in the absence or presence of inhibitors and then subjected to Western blotting. Proteins were probed with an antibody against phosphorylated MAPK and reprobed with an antibody against ERK2 MAPK. B: summarized data of PAF-induced phosphorylation of ERK1/2 MAPK (N = 4). Relative density was calculated by the ratio of phosphorylated over unphosphorylated ERK2. C: whole cell extracts were obtained from SEG1 cells treated without (control) or with PAF (10$^{-5}$ M) for 30 min in the absence or presence of PD-98059, and then subjected to Western blotting. Proteins were probed with an antibody against phosphorylated cPLA$_2$ and reprobed with an antibody against unphosphorylated cPLA$_2$. D: summarized data of PAF-induced phosphorylation of cPLA$_2$ (N = 4). Relative density was calculated by the ratio of phosphorylated over unphosphorylated cPLA$_2$. ANOVA *$p < 0.05$, †$p < 0.02$, compared with control group; #$p < 0.05$, ***$p < 0.01$, compared with PAF group.
were from Cell Signaling Technology (Danvers, MA). CV3988 and PAF C-16 were purchased from Biomol (Plymouth Meeting, PA). Triton X-100, PMSF, DMEM, antibiotics, and other reagents were purchased from Sigma (St. Louis, MO).

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

RESULTS

Effect of PAF on acid-induced H$_2$O$_2$ production. Consistently with our previous findings (20), acid treatment significantly increased NOX5-S expression and H$_2$O$_2$ production in SEG1 cells. This increase was partially reduced by the PAF antagonist CV3988 (Fig. 1, A and B). In addition, acid treatment significantly increased PAF production (Fig. 1C) and PAF dose dependently increased H$_2$O$_2$ production in SEG1 cells (Fig. 1D). The data suggest that acid-induced H$_2$O$_2$ production may be partially due to production of PAF.

NOX5-S mediates PAF-induced H$_2$O$_2$ production. We have previously shown that only NOX5-S and NOX1 are detectable in SEG1 EA cells (20). Therefore, we examined the role of NADPH oxidases in PAF-induced H$_2$O$_2$ production.

Role of MAP kinases and cPLA2 in PAF-induced NOX5-S expression and H$_2$O$_2$ production. In SEG1 EA cells, PAF-induced increase in NOX5-S expression and H$_2$O$_2$ production were significantly decreased by MAPK kinase 1 (MEK1) inhibitor PD-98059 (1) and cytosolic PLA2 inhibitor AA-COCF3 (38) (Fig. 3, A and B). PD-98059 and AACOCF3 were dissolved in ethanol and the final concentration of ethanol was 0.1%. This amount of ethanol had no effect on H$_2$O$_2$ production (data not shown). PAF-induced increase in NOX5-S promoter activity was also significantly reduced by PD-98059 and AACOCF3 (Fig. 3C). PD-98059 or AACOCF3 alone had no effect on NOX5-S promoter activity (data not shown). The data

Fig. 5. STAT5 mediates PAF-induced NOX5-S mRNA expression and H$_2$O$_2$ production. A: whole cell extracts were obtained from SEG1 cells transfected with control siRNA or STAT5 siRNA for 48 h, and then subjected to Western blotting. Proteins were probed with a STAT5 antibody and reprobed with a GAPDH antibody. B: summarized data of A (N = 4). Relative density was calculated by the ratio of STAT5 over GAPDH. C: SEG1 cells were transfected with control or STAT5 siRNA by using Lipofectamine 2000; 24 h later, the transfectants were exposed to PAF (10$^{-7}$ M) in fresh medium for an additional 24 h. NOX5-S mRNA expression was analyzed with quantitative real-time PCR and normalized to GAPDH (N = 3). D: H$_2$O$_2$ was measured in the culture medium collected after PAF treatment (N = 3). The experimental procedure was the same as in C. *P < 0.05, paired t-test. ANOVA **P < 0.01, ***P < 0.001, compared with control group transfected with control siRNA; ANOVA ***P < 0.01, ***P < 0.0001, compared with PAF group transfected with control siRNA.
suggest that PAF-induced NOX5-S expression may depend on activation of ERK1/2 MAP kinases and cPLA2. To further confirm this conclusion, we examined the phosphorylation of ERK1/ERK2 MAPKs and cPLA2 at 30 min after PAF treatment. We found that PAF significantly increased the phosphorylation of ERK1/2 MAPK (Fig. 4, A and B) and of cPLA2 (Fig. 4, C and D), suggesting that PAF may activate ERK1/2 MAPK and cPLA2. PAF-induced ERK1/ERK2 MAPK phosphorylation was blocked by PD-98059, but not by AACOCF3 (Fig. 4, A and B), suggesting that MAPK phosphorylation may not depend on activation of cPLA2. The same concentration of PD-98059 that effectively blocked ERK1/ERK2 MAPK activation blocked PAF-induced cPLA2 phosphorylation (Fig. 4, C and D), suggesting that PAF-induced cPLA2 phosphorylation may depend on activation of ERK1/ERK2 MAPKs.

STAT5-mediated upregulation of NOX5-S. The transcription factors regulating PAF-induced expression of NOX5-S are not known. After analyzing the genomic DNA sequence of NOX5, we found one STAT binding element in the NOX5-S promoter: TTCTGGTAA, located from −2249 to −2240 (position from ATG). To estimate a possible role of STAT5 on NOX5-S promoter regulation, we transfected SEG1 cells with STAT5 siRNA, which significantly decreased levels of STAT5 protein (Fig. 5, A and B). Control siRNA, however, had no effect on the levels of STAT5 (data not shown). Knockdown of STAT5 with STAT5 siRNA blocked PAF-induced NOX5-S expression (Fig. 5C) and H2O2 production (Fig. 5D). Furthermore, PAF significantly increased STAT5 phosphorylation, which was detected by a phospho-STAT5 antibody recognizing both phosphorylated tyrosine 694 of STAT5A and phosphorylated tyrosine 699 of STAT5B. This increased phosphorylation was reduced by PD-98059 and AACOCF3 (Fig. 6, A and B). These data suggest that STAT5 may be involved in PAF-induced NOX5-S expression and that activation of STAT5 may depend on activation of ERK1/2 MAP kinases and cPLA2.

In addition, we examined STAT5 binding to the possible binding element in the NOX5-S promoter by ChIP assay. NOX5 DNA was detectable in the immunoprecipitated chromatin sample of SEG1 cell lysate by PCR using the primers targeting the −2281 to −2076 (position from ATG) region of NOX5-S promoter (Fig. 7A). This pair of primers covers the possible STAT5 binding site as described above. The PCR product was sequenced and is specific for NOX5-S promoter. The data suggest that STAT5 binds to the NOX5-S promoter. This result was confirmed by gel mobility shift assay. In the gel mobility shift assay one prominent complex was detectable with the oligonucleotide NOX5-pWT (containing TTCTGGTAA, Fig. 7B). Competition experiments with unlabeled (cold) NOX5-pWT oligonucleotide significantly reduced binding. The addition of the mutant oligonucleotide NOX5-pMUT had less effect on binding (Fig. 7B). The supershifted band was detected when a STAT5 antibody was added (Fig. 7B). These data suggest that STAT5 binds to the site TTCTGGTAA on the NOX5-S promoter region. To confirm the role of STAT5 in the NOX5-S expression, SEG1 cells were transfected with NOX5-S promoter reporter plasmid NOX5-5p-LP. Overexpression of STAT5 caused 3.5-fold increase in luciferase activity (Fig. 8A). In addition, knockdown of STAT5 with STAT5 siRNA blocked PAF-induced increase in NOX5-S promoter activity (Fig. 8B). The data indicate STAT5-induced activation of NOX5-S promoter.

DISCUSSION

Esophageal adenocarcinoma has increased in incidence over the past three decades (7), at a rate exceeding that of any other cancer in the last 10 years (8, 24). The major risk factor for EA is gastroesophageal reflux disease complicated by BE (29). However, the mechanisms of the progression from metaplasia to adenocarcinoma are not fully understood.

We have shown that NOX1 and NOX5-S are the major isoforms of NADPH oxidases in esophageal adenocarcinoma cells and levels of NOX5-S are significantly increased in esophageal adenocarcinoma cells and in Barrett’s esophageal mucosa with high-grade dysplasia. NOX5-L is not detected in these cells (20). We have also shown that acid-induced H2O2 production is mediated by the NADPH oxidase NOX5-S in SEG1 EA cells. Overproduction of ROS, derived from acid-induced upregulation of NOX5-S, increases cell proliferation and decreases apoptosis, possibly contributing to progression from intestinal metaplasia (BE) to dysplasia and to adenocarcinoma (20).

Mechanisms of NOX5-S overexpression in EA are not fully understood. We have shown that acid treatment upregulates NOX5-S through intracellular calcium increase and activation of cyclic AMP response element binding protein in SEG1 cells...
In the present study we also found that acid-induced H$_2$O$_2$ production may be partially due to production of PAF in SEG1 cells because 1) acid-induced increase in NOX5-S expression and H$_2$O$_2$ production was partially inhibited by PAF antagonist CV3988 (Fig. 1, A and B); and 2) acid treatment significantly increased PAF production (Fig. 1 C); and 3) PAF dose dependently increased H$_2$O$_2$ production in SEG1 cells (Fig. 1 D). PAF has also been shown to increase ROS in macrophages, guinea pig tracheal epithelial cells, esophageal smooth muscles, and others (15, 21, 28).

It is not known whether PAF upregulates NADPH oxidases. It is also not clear whether NADPH oxidases are involved in PAF-induced ROS production in SEG1 cells. We found that knockdown of NOX5-S by NOX5 siRNA blocked PAF-induced H$_2$O$_2$ production in SEG1 EA cells (Fig. 2A), suggesting that NADPH oxidase NOX5-S may contribute to PAF-induced H$_2$O$_2$ production in SEG1 cells. This conclusion was further supported by our finding that PAF significantly increased NOX5-S expression (Fig. 2, C and D) and NOX5-S promoter activity (Fig. 2E) but did not affect NOX1 expression in SEG1 cells (Fig. 2B).

In SEG1 EA cells, PAF-induced increase in NOX5-S expression and H$_2$O$_2$ production may depend on sequential activation of ERK1/2 MAP kinases and cPLA$_2$ since 1) PAF-induced increase in NOX5-S expression, H$_2$O$_2$ production, and NOX5-S promoter activity were significantly decreased by MEK1 inhibitor PD-98059 (1) and cytosolic PLA$_2$ inhibitor AACOCF3 (38) (Fig. 3, A–C); 2) PAF significantly increased the phosphorylation of ERK1/2 MAPK (Fig. 4, A and B) and of cPLA$_2$ (Fig. 4, C and D); and 3) PAF-induced ERK1/ERK2 MAPK phosphorylation was not affected by AACOCF3 (Fig. 4, A and B), whereas PAF-induced cPLA$_2$ phosphorylation was blocked by PD-98059 (Fig. 4, C and D). The link between ERK1/ERK2 MAP kinases and cPLA$_2$ is consistent with other studies (13, 31, 37, 43). Our data are also in agreement with PAF-induced activation of MAPKs (6, 23, 25) and cPLA$_2$ (27) in variety of cells. PAF is reported to activate ERK1/ERK2 MAP kinases through a specific PAF receptor coupled to pertussis toxin-sensitive G proteins in Chinese hamster ovary cells (23). It is known that activation of phospholipase A2 causes the hydrolysis of 1-alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine to lyso-PAF and arachidonic acid. Lyso-PAF...
is then converted to PAF by an acetyltransferase. Therefore, PAF-induced activation of cPLA2 may further enhance PAF production as a positive feedback.

The transcription factors regulating PAF-induced expression of NOX5-S are not known. PAF has been shown to activate STAT5 (10). After analyzing the genomic DNA sequence of NOX5, we found one STAT5 binding elements in the NOX5-S promoter: TTCTGGTAA, located from -2249 to -2240 (position from ATG). Therefore, we examined the role of STAT5 in PAF-induced NOX5-S expression. STAT5 belongs to a family of signal transducers and activators of transcription and binds to a specific DNA motif [TT(C/A)YNR(G/T)AA] in the promoter region of target genes. STAT5 consists of two variants: STAT5A and STAT5B. The ability of STAT5 to activate transcription requires its phosphorylation on tyrosine 694 (STAT5A) or tyrosine 699 (STAT5B). Phosphorylated STAT5 forms homo- or heterodimers and then translocates into the nucleus, where it initiates gene transcription (5).

We found that PAF significantly increased STAT5 phosphorylation (Fig. 6, A and B) and that knockdown of STAT5 with STAT5 siRNA blocked PAF-induced NOX5-S expression (Fig. 5C) and H2O2 production (Fig. 5D), suggesting that STAT5 may be involved in PAF-induced NOX5-S expression in SEG1 cells. To further investigate the role of STAT5 in NOX5-S expression, we examined whether STAT5 binds to the possible binding element in the NOX5-S promoter by ChIP assay and gel mobility shift assay. In ChIP assay, NOX5 DNA was detectable in the immunoprecipitated chromatin sample of SEG1 cell lysate by PCR using the primers targeting the -2281 to -2076 (position from ATG) region of NOX5-S promoter (Fig. 7A). This pair of primers covers the possible STAT5 binding site as described above. In the gel mobility shift assay one prominent complex was detectable with the oligonucleotide NOX5-pWT (containing TTCTGGTAA, Fig. 7B). Competition experiments with unleveled (cold) NOX5-pWT oligonucleotide significantly reduced binding. The addition of the mutant oligonucleotides NOX5-pMUT had less effect on binding (Fig. 7B). The supershifted band was detected with the STAT5 antibody (Fig. 7B). In addition, overexpression of STAT5 caused 3.5-fold increase in luciferase activity in SEG1 cells cotransfected with a reporter plasmid of NOX5-S, which was generated by ligating a NOX5-S promoter fragment (-2501 to -1 from ATG) into the pGL3-basic vector (Fig. 8A). Knockdown of STAT5 with STAT5 siRNA blocked PAF-induced activation of NOX5-S promoter (Fig. 8B). These data indicate STAT5 induced activation of NOX5-S promoter via binding to the site TTCTGGTAA.

We also found that PAF-induced increase in STAT5 phosphorylation was significantly decreased by PD-98059 and AACOCF3 (Fig. 6, A and B), suggesting that PAF-induced activation of STAT5 may depend on activation of ERK1/2 MAP kinases and cPLA2. The mechanisms of cPLA2-mediated STAT5 activation are not known. It has been reported that cPLA2-dependent activation of STAT3 depends on production of prostaglandin E2 and activation of EP1 receptor, c-Src, and epidermal growth factor receptor (22). Whether this pathway applies to activation of STAT5 needs to be further explored.

At basal condition, knockdown of STAT5 with STAT5 siRNA slightly decreased NOX5-S expression, H2O2 production (Fig. 5, C and D) and NOX5-S promoter activity (Fig. 8), but the changes were not statistically significant. Our data cannot exclude the possibility that STAT5 might also be involved in NOX5-S expression at the basal condition, where STAT5 might be activated by factors present in culture medium.

We conclude that in SEG1 cells acid-induced NOX5-S expression and H2O2 production are mediated in part by production of PAF. PAF-induced increase in NOX5-S expression depends on sequential activation of ERK1/2 MAP kinases, cPLA2 and STAT5. It is possible that acid reflux present in patients with Barrett’s esophagus may induce production of PAF, causing upregulation of NOX5-S in metaplastic cells. Overproduction of ROS derived from upregulation of NOX5-S may increase cell proliferation and decrease apoptosis contributing to progression from intestinal metaplasia (BE) to dysplasia and to adenocarcinoma. PAF and NOX5-S may be potential therapeutic target to prevent this progression.

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