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Original Paper

MAPK and JAK-STAT Signaling Pathways are Involved in the Oxidative Stress – Induced Decrease in Expression of Surfactant Protein Genes

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Key Words
Oxidative • Stress • Reactive oxygen species • Hydrogen peroxide • ERK • p38 MAPK • STAT3

Oxidative stress is generated by reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and superoxide anion (O^{2-}), which are produced as by-products of cellular metabolism. An imbalance in cellular redox status is a potent pathogenic factor that contributes to various chronic inflammatory diseases. In this study, we demonstrate that H_2O_2 decreases surfactant protein A, B and ABCA3 mRNA level, and increases SP-D mRNA level in human pulmonary lung epithelial cells. The decreased mRNA level of SP-A and SP-B were significant with a maximum inhibition of 79 and 87%, respectively by 150 μM H_2O_2 after 24 hrs of incubation. In addition, ABCA3 mRNA level was decreased with a maximum inhibition of 55% by 150 μM H_2O_2 after 12 hrs of incubation. In contrast, 150 μM H_2O_2 caused the SP-D mRNA level to increase to 200% of control after 8 hrs of incubation. The H_2O_2-induced gene repression or activation of SP-A, SP-B, SP-D and ABCA3 was blocked by pretreatment with the antioxidants N-acetyl-L-cysteine (NAC) and catalase. Furthermore, the inhibition of SP-A and SP-B was associated with reduced thyroid transcription factor -1 (TTF-1) DNA binding activity, and this reduced TTF-1 binding activity may be due to decreased TTF-1 protein expression level. The analyses of signal transduction pathways that may play a role in the regulation of gene expression by H_2O_2 using several specific inhibitors showed that U0126, an inhibitor of ERK1/2 upstream kinase MEK1/2, blocked both H_2O_2-induced inhibition of SP-A and SP-B gene expression, whereas SB203580, an inhibitor of p38 MAPK, partially blocked H_2O_2-mediated inhibition of SP-A gene expression but not SP-B expression. In contrast, AG-490, a specific inhibitor of JAK-STAT pathway, blocked H_2O_2-mediated inhibition of SP-B gene expression but not SP-A expression. Immunoblot analyses using specific phosphor-antibodies demonstrated that ERK1/2, p38 MAPK and STAT3 are phosphorylated by oxidative stress suggesting that H_2O_2-induced inhibition of SP-A and SP-B gene expression is associated with MAPK and JAK-STAT signaling pathway. These data, therefore, suggest that H_2O_2 affects SP-A and SP-B gene regulation by reducing TTF-1 DNA binding activity via MAPKs or STAT signaling pathways.

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Introduction

Pulmonary surfactant, a mixture of phospholipid and protein synthesized exclusively by alveolar type II cells, is important in reducing surface tension at the air-liquid interface of lung alveoli [1]. The protein component of surfactant is composed of four surfactant-associated proteins, designated surfactant protein (SP)-A, SP-B, SP-C and SP-D. SP-A and SP-D are hydrophilic proteins and are believed to contribute to immune defense in the lung, whereas SP-B and SP-C are hydrophobic proteins that are responsible for the surface tension lowering properties of surfactant [2, 3]. While SP-A, SP-B and SP-D are expressed in both alveolar type II and bronchiolar epithelial cells, the expression of SP-C is limited to alveolar type II cells. Decreased function of SP-A or SP-B or decreased expression in lung epithelial cells is associated with abnormal pulmonary function and fetal diseases in both humans and mice [4, 5]. Lung epithelial cells are exposed to oxidative stress due to oxidants from both the outside environment and from normal cellular processes. Oxidative stress refers to cellular damage caused by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and superoxide anion (O^{2-}). The level of oxidative stress in the lung also increases with inflammation as activated macrophages and neutrophils generate toxic oxygen products. ROS have been implicated in the onset and progression of a number of chronic and acute inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD), respiratory distress syndrome (RDS) and acute lung injury [6-9]. ROS activate multiple signaling pathways including mitogen-activated protein kinase (MAPK) [10] and Janus kinase (JAK)/signal transducers and activators of transcription (STAT) [11, 12] pathways.

ROS can inactivate surfactant proteins [13, 14] and cellular oxidation has been reported to decrease SP-A and SP-B mRNA levels [15]. The molecular mechanism by which oxidative stress affects SP-A and SP-B-mRNA levels has not been explored. To address this issue, we exposed human pulmonary adenocarcinoma NCI-H441 cells to H_2O_2 mediated oxidative stress, examined effects on the expression of surfactant related proteins, and performed studies to elucidate the molecular mechanism of this effect.

Materials and Methods

Materials

Human pulmonary adenocarcinoma NCI-H441 cells were purchased from American Type Culture Collection (Rockville, MD). Cell culture media, antibiotics, HEPES, fetal bovine serum (FBS), SuperScript first-strand synthesis system for cDNA synthesis and Trizol reagent were purchased from Invitrogen (Carlsbad, CA). H_2O_2, N-Acetyl-L-cysteine (NAC), and catalase was obtained from Sigma (St Louis, MO). RNeasy mini kit and Qiaquick PCR purification kit were purchased from Qiagen (Valencia, CA). iQ SYBR Green supermix kit and 96-well plates for Quantitative Real-Time (qRT-PCR), SDS-polyacrylamide electrophoresis gel, nitrocellulose membrane and DC protein assay kits were purchased from Bio-Rad (Hercules, CA). BCA protein assay kits were purchased from Pierce (Rockford, IL). U0126 was purchased from Promega (Madison, WI).

SP600125 and AG490 were purchased from Calbiochem (San Diego, CA). Complete mini protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail were purchased from Roche Applied Science (Indianapolis, IN). SB203580 was purchased from BioMol (Plymouth Meeting, PA). Antibody for detection of actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-TTF-1 antibody was purchased from Seven Hills Bioreagents (Cincinnati, OH). Antibodies for detection of phosphorylated protein kinases, ERK1/2, p38 MAPK, STAT3 (Tyr705), as well as total ERK1/2, p38 MAPK, STAT3, and HRP-conjugated secondary antibody were purchased from Cell Signaling Technology (Beverly, MA). The enhanced chemiluminescence Western blot detection system was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Methods

Cell Culture and Treatment. Human pulmonary adenocarcinoma NCI-H441 cells with characteristics of bronchiolar (Clara) epithelial cells were maintained in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 25 mM HEPES, amphotericin B (0.25 µ/ml) and 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37°C. For experiments, cells (passage 3-10) were seeded

Table 1. Primers used in quantitative real-time PCR (qPCR)

Genes name	Accession number	Forward primer	Reverse primer
SP-A	NM006926	5′-TGAAGGACGTTTGTGTTGGAAGCC-3′	5′-TTCCCAGGAGGACATGGTGTTTCT-3′
SP-B	NM198843	5′-TAGGGCATTGCCTACAGGAAGTCT-3′	5′-ATGGCCTCCTTGGCCATCTTGTTA-3′
SP-D	NM003019	5′-AAAGGGAGAAAGTGGGCTTCCAGA-3′	5′-ACACTTTGGCCATTTGGGAAGAGC-3′
ABCA3	AB070929	5′-AGTGCGCAGGGCACTTGT-3′	5′-GGACGAGCAGTTGTCGTACCTA-3′
GAPDH	AB062273	5′-CATGTTCGTCATGGGTGTGAACCA-3′	5′-AGTGATGGCATGGACTGTGGTCAT-3′
TTF-1	NM001079668	5′-GCGACGCTTCAAGCAACAGAAGTA-3′	5′-TTGTCCTTGGCCTGGCGCTTCATTT-3′

into 60 mm dishes at a density of 5×10^5 or 100 mm dishes at a density of $1.5x10^6$ per dish, cultured overnight, washed with warm PBS and then cultured overnight in serum-free and phenol red-free RPMI 1640. The medium was then changed to fresh serum-free and phenol red-free RPMI 1640 that contained various concentrations of H_2O_2. In some experiments, cells were pretreated with the antioxidants N-acetyl-L-cysteine (NAC) or catalase, or with specific protein kinase inhibitors (U0126, SB203580, SP600125, AG490), for 1h at 37°C before treatment with H_2O_2.

Cell Viability. Cell viability was examined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) according to the manufacturer's instructions (Promega). The MTS reagent is reduced by metabolically active cells into a colored formazan product whose absorbance is then measured. In brief, MTS solution was added to wells of a 96-well microtiter plate, and the cells were incubated for 2h. The absorbance at 490 nm was then measured.

RNA isolation and quantitative real time RT-PCR. Total RNA was isolated from cells using Trizol reagent according to manufacturer's instruction, treated with Turbo DNase to remove any contaminating DNA, and purified using RNeasy mini kit. Total RNA was used to generate cDNA with the SuperScript first-strand synthesis system. A standard 10 µl reaction contained 1 µg RNA, 400 ng random hexamers, and 0.5 mM dNTPs. The mixture was incubated at 65°C for 5 min, then on ice for 1 min. 5x reverse transcriptase (RT) buffer (1x final concentration), dithiothreitol (10 mM), ribonuclease inhibitor and 50 units of SuperScript III RT were added and the reaction was incubated at 22°C for 10 min, then at 50°C for 50 min, and finally at 70°C for 15 min. Quantitative real time RT-PCR (qRT-PCR) reactions were performed in 96-well plates using the iQ SYBR Green supermix kit and consisted of incubations at 95 °C for 1.5 min followed by 40 amplification cycles of 95°C for 15 sec and 55°C for 30 sec. Primers for the qRT-PCR were purchased by Sigma and the sequences of the primers are shown in Table 1. The cycle threshold (CT) for each reaction (indicating the amount of PCR product and level of mRNA expression) was determined using the iCycler iQ™ Detection System. All samples were assayed in triplicate and the expression levels were normalized against that of endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Chromatin Immunoprecipitation (ChIP) assay. The ChIP assay was performed as previously described [16] with some modifications. For each condition five 10 cm plates were seeded with 1.5×10^6 cells and cultured overnight in normal culture medium. The following day cultures were washed with PBS and incubated overnight with serum-free and phenol-free RPMI and then incubated for 24 h with or without H_2O_2. After incubation cells were fixed with 1% formaldehyde in serum-free and phenol red-free RPMI for 10 min at room temperature, washed with PBS, and subsequently quenched with 10 ml of 0.125 M glycine in serum-free and phenol red-free RPMI for 5 min at room temperature. Cultures were then washed with PBS and cells were harvested in 1 ml PBS with freshly added protease inhibitor cocktail, per dish. Harvested cells from the 5 plates were combined, and pelleted by centrifugation. Cross-linked cell pellets were resuspended in 250 µl of lysis buffer (25 mM Tris-Cl, pH 8.1, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 3 mM EDTA and protease inhibitor cocktail) and incubated on ice for 10 min. Fragmentation of chromosomes was achieved by sonication using a Sonicator S-4000 (pulsed for 30 sec on and 1 min off 24 times at 85% and 12 times at 100% duty cycle) yielding DNA fragments with an average length 0.2-1.5 kb. Cell debris was removed by centrifugation at 20,000 x g for 10 min at 4°C. The supernatant was removed and brought to a final volume of 300 µl with lysis buffer. 5-10 µl of supernatant was reserved for input DNA. For immunoprecipitation, the remaining supernatant was equally divided between two tubes and either 7 µl of monoclonal mouse TTF-1 antibody or 7 µl of mouse IgG_1 (used as a negative control), was added, followed by incubation overnight at 4°C. Sonicated salmon sperm DNA/Protein A agarose beads were then added to the samples, incubation was continued at 4°C for 2 h, and beads were pelleted by a brief centrifugation. Pellets were washed once with 150 µl of TSE 150 mM NaCl buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1 and 150 mM NaCl), once with 150 µl of TSE 500 mM NaCl buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1 and 500 mM NaCl), once with LiCl buffer solution III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA and 10 mM Tris-Cl, pH 8.1) and twice with TE buffer at 4°C with gentle agitation. After washing, immunocomplexes were eluted off the beads by incubation at room temperature with 200 µl of 1% SDS and 0.1 M $NaHCO_3$. The eluate from the immunoprecipitation and the input sample were incubated at 65°C overnight to reverse protein-DNA cross-linking followed by DNA extraction using the Qiaquick PCR

purification kit. The DNA was then used as template for quantitative PCR analysis using primers specific to either the SP-A or SP-B TTF-1 binding sites in the respective promoter regions. Results are expressed as a percent of input DNA.

Immunoblot analysis

Sample preparation. Cells were cultured in 10 cm dishes and treated with or without H_2O_2 as described above under the ChIP assay except that plates were not pooled before analysis. After treatment, 10 cm diameter dishes were washed twice with ice-cold PBS, harvested into 1 ml PBS, and cells pelleted at 1000 x g for 5 min.

Nuclear extracts to be used for immunoblotting of TTF-1 protein were prepared as described previously [17]. In brief, the cell pellet was lysed by resuspension in 100 µl of lysis buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% Nonidet P-40, 1 mM dithiothreitol and 0.5 mM PMSF), and incubated on ice for 5 min. The sample was centrifuged at 1000 x g for 5 min and the nuclear pellet was resuspended in 100 µl of buffer B (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol, 1 mM dithiothreitol, 0.5 mM PMSF), and nuclear proteins were extracted by incubation for 10 min with occasional gentle vortexing. The sample was then centrifuged at 20,000 x g for 10 min and the supernatant was recovered and used for immunoblotting. Protein concentration in the supernatant was determined using the DC protein assay kit.

Cell pellets to be used for immunoblotting of protein kinases were resuspended in 100 µl of Cell Lysis Buffer with 0.5 mM phenylmethanesulfonylfluoride (PMSF), Complete Protease Inhibitor and PhosSTOP for 10 min on ice. After brief sonication, the lysate was cleared by centrifugation at 20,000 x g for 10 min, and supernatant was used for immunoblotting. Protein concentration was determined using the BCA protein assay kit.

Immunoblotting protocol. Nuclear extracts described as prepared above were used for immunoblotting with anti TTF-1 antibody. Lysates containing protein were boiled for 5 min in SDS sample buffer and an equal amount of protein (20-50 µg) loaded in each lane of a 10% or 12% SDS-polyacrylamide gel as indicated. After gel electrophoresis proteins were transferred to a nitrocellulose membrane. Membranes were incubated for 1h in 0.1% Tween – phosphate buffered saline (PBST) containing 5% nonfat milk powder for blocking and then incubated with primary mouse monoclonal anti-TTF-1 antibody (1:1000 dilution) in fresh blocking solution overnight at 4°C. After washing three times for 10 min in 0.1% PBST membranes were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody for 1h at room temperature. Blots were developed using the enhanced chemiluminescence Western blot detection system and quantified with a STORM 860 PhosphoImager system. Blots were then stripped using 1 M glycine, washed with PBST and immunoblotted and visualized as described above for TTF-1 except that anti-actin antibody (1:1000) was used.

To detect protein kinases, analysis was performed as described above except that membranes were incubated with 0.1% Tween – Tris buffered saline (TBST) containing 5% nonfat milk powder for blocking incubation with antibody. For phosphorylated kinases primary rabbit phosphospecific antibodies to pERK1/2, p-p38 MAPK, and pSTAT3 (Tyr705) were used. For detection of total kinases (phosphorylated and nonphosphorylated) rabbit antibodies to ERK1/2 and p38 MAPK antibodies, and mouse antibody to STAT3 was utilized. All antibodies were from Cell Signaling Technology and were used at 1:1000 dilution and immunoblotting analysis was performed as described above except for the use of in 0.1% TBST (rather than 0.1% PBST). Blots were stripped and probed with additional antibodies (actin, total ERK1/2, p38 MAPK, STAT3) as described above.

Statistical analyses

The statistical test used to determine significance was dependent upon the specific experiment and is indicated in figure legends. Statistical tests utilized included the paired t-test, and repeated-measures one-way ANOVA with either a Dunnett or Tukey post test. $p<0.05$ was considered significant.

Results

H_2O_2 Decreases Surfactant Protein A, B and ABCA3 mRNA levels, but increases the level of SP-D mRNA

To investigate the potential impact of oxidative stress on regulation of surfactant related genes, we examined the effect of H_2O_2 on expression of SP-A, SP-B, SP-D, and ABCA3 in H441 human adenocarcinoma pulmonary epithelial cells. (Since H441 cells do not express SP-C [18], the effect of H_2O_2 on SP-C expression was not examined). H_2O_2 treatment (150 µM) of

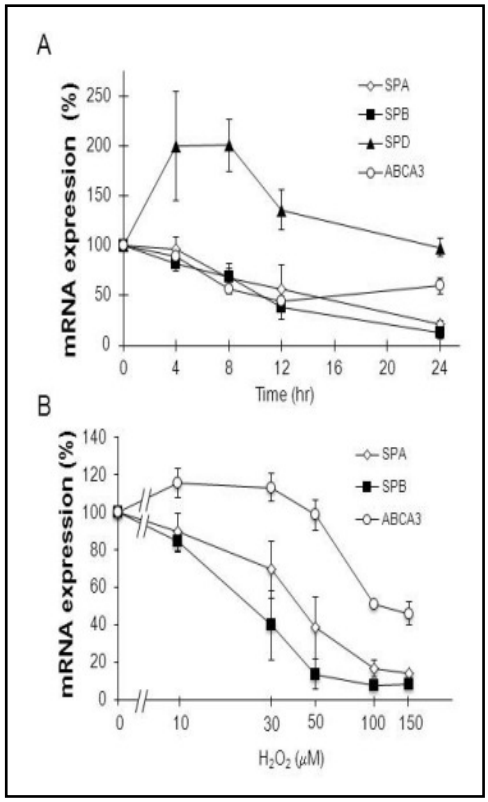

Fig. 1. Effects of H_2O_2 on SP-A, SP-B, SP-D, and ABCA3 mRNA levels in H441 cells. After the indicated treatments total RNA was isolated, and qRT-PCR was performed as described under Methods to determine mRNA level. Data are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level and expressed as a percent of untreated controls. A: H441 cells were incubated in the presence or absence of 150 μM H_2O_2 for the indicated time periods. Statistical significance was determined using a repeated measures one-way ANOVA with a Dunnett post test. For SP-A, 12 ($p<0.05$) and 24 ($p<0.01$) h values were significantly different than the 4 h value. For SP-B, 12 and 24 h values were significantly different ($p<0.01$) than the 4 h value. For SP-D, the 24 h value was significantly different ($p<0.05$) than the 4 h value. For ABCA3, 8, 12, and 24 h values were statistically different ($p<0.01$) than the 4 h value. B: H441 cells were treated with various concentrations of H2O2 for either 24 hr (SP-A and SP-B), or 12 hr (ABCA3). Statistical significance was determined using a repeated measures one-way ANOVA with a Dunnett post test. For the SP-A and SP-B results all values were significantly different than the value for 10 μM H_2O_2 with $p<0.01$ for all except SP-A at 30 μM where $p<0.05$. For ABCA3, all values at concentrations at or above 50 μM are statistically different from the 10 μM value ($p<0.05$). Data shown are the means ± S.D. of three separate experiments.

H441 cells resulted in a time-dependent reduction in SP-A and SP-B mRNA levels with the maximal effect at 24 h when SP-A and SP-B mRNA levels were decreased to ~15-20% of untreated controls (Fig. 1A). H_2O_2 also decreased ABCA3 mRNA levels, however, the maximal decrease was observed at 12 h and ABCA3 mRNA was only reduced to ~50% of untreated controls (Fig. 1A). In contrast, the SP-D mRNA level was increased to ~200% at early time points (4 and 8h) after treatment and then returned to control levels by 24 h (Fig. 1A). The inhibition of H_2O_2 was concentration dependent as demonstrated in Figure 1B. There was a decrease in SP-A, SP-B and ABCA3 mRNA levels with a maximal effect observed at 100-150 μM H_2O_2. As we were primarily interested in examining the pathways involved in the inhibition of expression of surfactant related genes, we only further examined H_2O_2 inhibition of SP-A and SP-B.

To examine whether H_2O_2 affected cell viability the effect of 150 μM H_2O_2 on number of viable cells after 4, 8, 12 and 24 h of treatment was determined as described under Methods. Cell viability did not decrease significantly with H_2O_2 treatment over the time examined (data not shown) indicating that there was no significant cytotoxicity with 150 μM H_2O_2 treatment. In addition, neither total RNA yield nor GAPDH mRNA levels were significantly altered at the various concentrations of H_2O_2 used at any of the time points (data not shown).

Antioxidants block H_2O_2-mediated effects on SP-A, SP-B, and ABCA3 mRNA

Previous work examining the effects H_2O_2 on various cell types has used the antioxidant N-acetyl cysteine or catalase to help determine whether the effects of H_2O_2 are related to its oxidative effects [19, 20]. To determine whether the decreased expression of SP-A, SP-B and ABCA3 was specific to the oxidative effects of H_2O_2, we pretreated H441 cells with the antioxidants N-acetyl cysteine (NAC, 10 mM) or catalase (100 U/ml). These agents either completely prevented or significantly attenuated the H_2O_2-mediated decrease in SP-A, SP-B, and ABCA3 mRNA expression (Fig. 2A-C). These results suggest that the observed effect of H_2O_2 on SP-A, SP-B, and ABCA3 gene expression is the result of the oxidative effects of H_2O_2.

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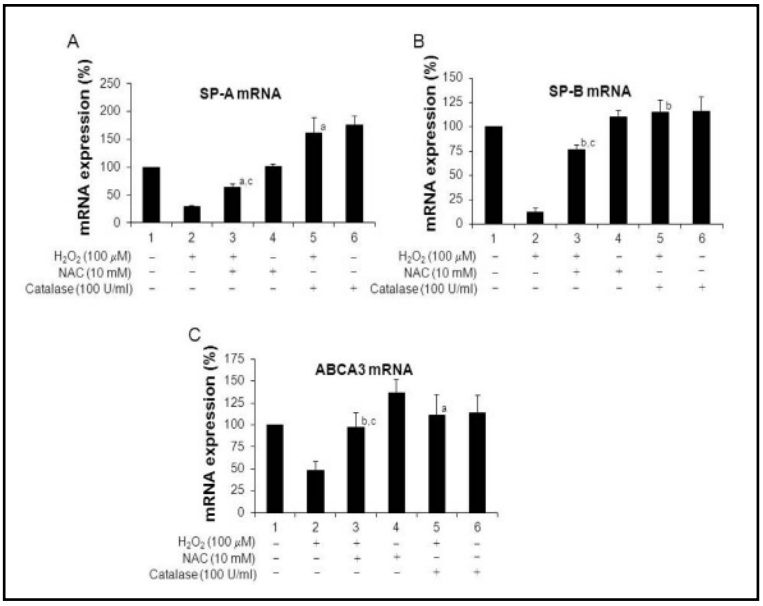

Fig. 2. Effect of antioxidants on the H_2O_2-induced decreased in SP-A, SP-B, and ABCA3 mRNA levels. H441 cells were treated with or without N-acetyl-L-cysteine (NAC) or catalase for 1 h before incubation for 12 (ABCA3) or 24 (SP-A, SP-B) hours with or without H_2O_2. RNA was isolated, and qRT-PCR was performed as described under Methods to determine mRNA level. Data are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level and expressed as a percent of untreated controls. Data shown are the means ± S.D. of three separate experiments. Statistical significance was determined using a repeated measures one-way ANOVA with a Tukey post test. a, $p<0.01$, b, $p<0.001$, compared to H_2O_2only; c, $p<0.01$ compared to NAC only.

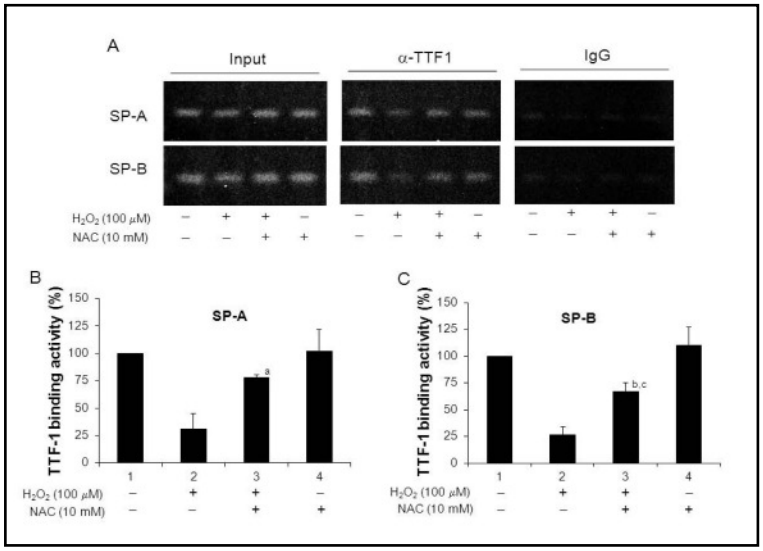

Fig. 3. Effect of H_2O_2 on TTF-1 binding to SP-A and SP-B promoter regions. H441 cells were treated with or without N-acetyl-L-cysteine (NAC) for 1 h before incubation for 24 hours with or without H_2O_2. ChIP analysis was performed as described under Methods using anti-TTF-1 antibody or normal IgG as a negative control. Input DNA (soluble chromatin before immunoprecipitation) was used as a positive control. A: Ethidium bromide staining of one representative experiment showing amplified products specific for SP-A (top) and SP-B (bottom) promoter regions after ChIP analysis. B and C: The amount of TTF-1 binding to SP-A and SP-B promoter regions was quantitated by qPCR of precipitated DNA. Results are expressed as a percent of the binding observed in untreated cells. Data shown are the means ± S.D. of three separate experiments. Statistical significance was determined using a repeated measures one-way ANOVA with a Tukey post test. a, $p<0.05$, b, $p<0.01$, compared to H_2O_2 only; c, $p<0.01$ compared to NAC only.

H_2O_2 reduces TTF-1 binding to SP-A and SP-B promoter regions

Thyroid transcription factor 1 (TTF-1/NKX2.1, hereafter referred to as TTF-1), a member of Nkx family of homeodomain transcription factors, is involved in transcriptional regulation of a number of genes and is essential for the transcriptional regulation of SP-A, SP-B and ABCA3 [21-23]. To determine whether H_2O_2 alters TTF-1 DNA binding to the promoter regions of SP-A and SP-B, we performed chromatin immunoprecipitation (ChIP) assays using anti-TTF-1 antibody. As shown in Figure 3, H_2O_2 (100 μM) decreased TTF-1 binding to the SP-A and SP-B promoter regions by ~75%. In addition, the H_2O_2 induced decreased in TTF-1 binding was attenuated by pretreatment with the antioxidant NAC. These results strongly suggest that the H_2O_2 induced decrease in SP-A and SP-B gene expression is due, at least in

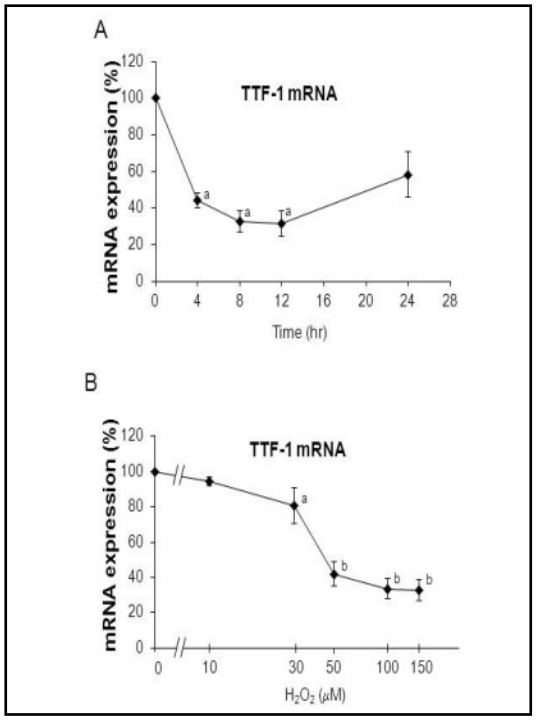

Fig. 4. Effect of H_2O_2 on TTF-1 gene expression in H441 cells. After the indicated treatments total RNA was isolated, and qRT-PCR was performed as described under Methods to determine TTF-1 mRNA level. Data are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level and expressed as a percent of untreated controls. A: H441 cells were incubated in the presence or absence of 150 μM H_2O_2 for the indicated time periods. Significance was determined using a paired t test. a, $p<0.05$ versus untreated controls. B: H441 cells were treated with various concentrations of H_2O_2 for 8 hr. Significance was determined using a repeated measures one-way ANOVA with a Dunnett post test. a, $p<0.05$, b, $p<0.01$ compared to the 10 μM value. Data shown are the means ± S.D. of three separate experiments.

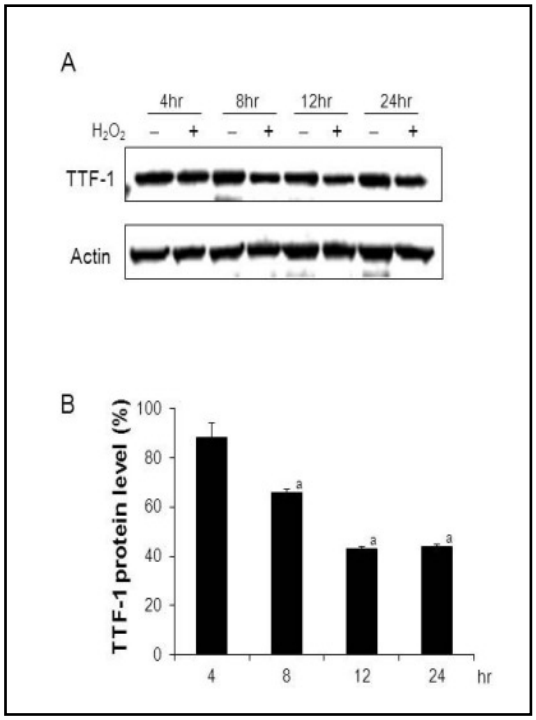

Fig. 5. Effect of H_2O_2 on TTF-1 protein expression. H441 cells were treated with or without H_2O_2 for 4-24 hr, and nuclear extracts were prepared. Equal amount of protein lysates (~30 μg) were analyzed by immunoblotting as described under Methods to detect TTF-1 protein. A: Results from one representative immunoblot. B: Protein bands were quantified using a STORM 860 phosphorImager and normalized to actin. Results are expressed as a percent of the 4 hour untreated control. Data shown are the means ± S.D. of three separate experiments. Significance was determined using a repeated measures one-way ANOVA with a Dunnett post test. a, $p<0.01$, compared to treatment with H2O2 for 4 h.

part, to reduced TTF-1 binding to the SP-A and SP-B promoters.

H_2O_2 reduces TTF-1 mRNA and protein levels

To determine whether the reduction in TTF-1 binding activity caused by H_2O_2 was due to decreased expression of TTF-1, the effect of H_2O_2 on TTF-1 expression was examined. H_2O_2 treatment of H441 cells resulted in a time- and concentration-dependent decrease in TTF-1 mRNA levels with the maximal reduction observed by 8 h (~70%); by 24 h TTF-1 mRNA levels begin to increase towards untreated controls (Fig. 4A). As shown in Fig. 4B, The observed concentration dependence is similar to that observed for effects on SP-A, SP-B and ABCA3 mRNA levels with 100-150 μM showing a maximal effect. Pretreatment with the antioxidants NAC or catalase completely prevented this reduction (data not shown). Immunoblotting using anti TTF-1 antibody was performed to determine whether H_2O_2 also decreased TTF-1 protein levels. As shown in Figure 5, H_2O_2 decreased TTF-1 protein levels over time with a maximal decrease of ~60% at 12h, suggesting that reduced TTF-1 protein level contributed to the H_2O_2-mediated reduction in SP-A, SP-B and ABCA3 mRNA levels.

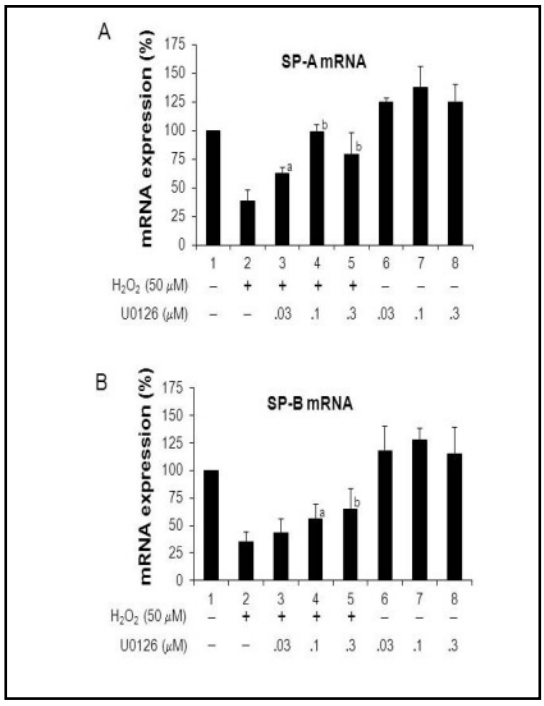

Fig. 6. Effect of U0126 on the H_2O_2-induced decrease in SP-A and SP-B gene expression. H441 cells were treated for 1 hour with various concentrations of U0126 and then incubated for an additional 24 hours in the absence or presence of H_2O_2. Total RNA was then isolated, and qRT-PCR was performed as described under Methods to quantitate either SP-A (A) or SP-B (B) mRNA. Data are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level and expressed as a percent of untreated control. Data shown are the means ± S.D. of three separate experiments. Significance was determined using a repeated measures one-way ANOVA with a Dunnett post test. a, $p<0.05$, b, $p<0.01$ compared to H_2O_2 only.

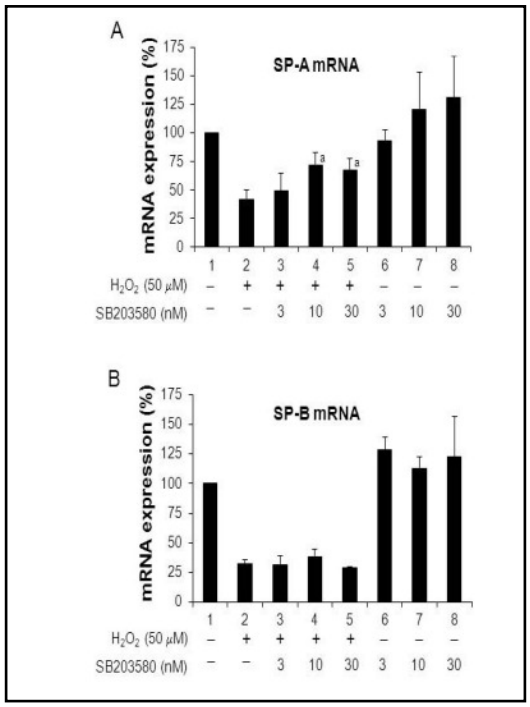

Fig. 7. Effect of SB203580 on H_2O_2-induced decrease in SP-A and SP-B gene expression. H441 cells were treated for 1 hour with various concentrations of SB203580 and then incubated for an additional 24 hours in the absence or presence of H_2O_2. Total RNA was then isolated, and qRT-PCR was performed as described under Methods to quantitate either SP-A (A) or SP-B (B) mRNA. Data are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level and expressed as a percent of untreated control. Data shown are the means ± S.D. of three separate experiments. Significance was determined using a repeated measures one-way ANOVA with a Dunnett post test. a, $p<0.01$ compared to H_2O_2 only.

MAPK family members, ERK and p38 MAPK are involved in the H_2O_2-mediated decrease in SP-A and SP-B mRNA levels

Since ROS have been reported to activate MAPKs [10], we investigated whether MAPK signaling was associated with H_2O_2-induced inhibition of SP-A and SP-B gene expression using specific inhibitors of different MAPK family members. U0126 is a specific inhibitor of MEK1/2 which phosphorylates and activates the ERK1/2 family of MAPKs [24]. Treatment with U0126 (0.03-0.3 μM) blocked the H_2O_2-mediated reduction in SP-A gene expression in a concentration-dependent manner (Fig. 6A) and partially blocked the H_2O_2-mediated reduction in SP-B expression (Fig. 6B). SB203580, an inhibitor of p38 MAPKs [25] (3-30 nM), partially blocked the H_2O_2-mediated reduction in SP-A mRNA (Fig. 7A), but had no effect on the H_2O_2-mediated reduction in SP-B mRNA (Fig. 7B). These concentrations of SB203580 were used based on previous published work demonstrating inhibition p38 MAPK in H441 cells [26]. SP600125, a specific inhibitor of the JNK family of MAPKs, had no effect on the H_2O_2-mediated reduction in SP-A or SP-B mRNA (data not shown). Since the ERK1/2 inhibitor blocked the H_2O_2-induced decrease in both SP-A and SP-B gene expression the effect of U0126 on the H_2O_2 induced decrease in TTF-1 expression was examined. Interestingly U0126 partially blocked the effect of H_2O_2 on TTF-1 mRNA levels (data not shown).

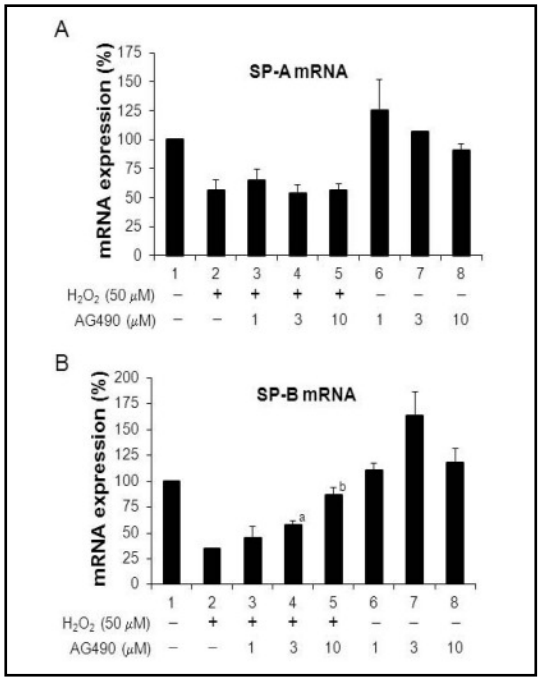

Fig. 8. Effect of AG490 on H_2O_2-induced decrease in SP-A and SP-B gene expression. H441 cells were treated for 1 hour with various concentrations of AG490 and then incubated for an additional 24 hours in the absence or presence of H_2O_2. Total RNA was then isolated, and qRT-PCR was performed as described under Methods to quantitate either SP-A (A) or SP-B (B) mRNA. Data are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level and expressed as a percent of untreated control. Data shown are the means ± S.D. of three separate experiments. Significance was determined using a repeated measures one-way ANOVA with a Dunnett post test. a, $p<0.05$, b, $p<0.01$ compared to H_2O_2 only.

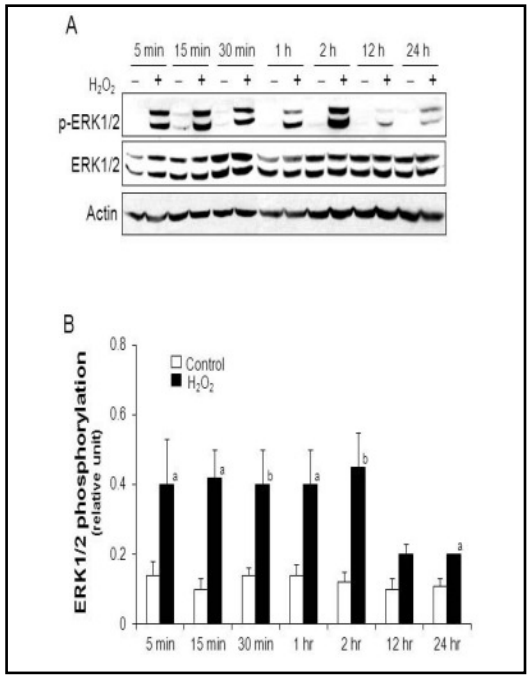

Fig. 9. Effect of H_2O_2 on the phosphorylation of ERK1/2. H441 cells were incubated in the presence or absence of 100 μM H_2O_2 for the indicated times. Cells lysates were prepared and used for immunoblotting with antibody specific for either phosphorylated ERK1/2 (p-ERK1/2), total ERK1/2 or actin as described under Methods. A: Shown is one representative immunoblot of demonstrating H_2O_2 mediated phosphorylation of ERK1/2. B: Protein bands were quantified using a STORM 860 phosphorImager and normalized to actin. Data shown are the means ± S.D. of three separate experiments. Significance was determined using a paired t test. a, $p<0.05$, b, $p<0.01$ versus untreated controls.

The JAK-STAT pathway is associated with H_2O_2-mediated inhibition of SP-B but not SP-A gene expression

Previous studies have demonstrated that the JAK2-STAT3 signaling pathway is involved in H_2O_2 signaling [11, 12, 27]. To determine whether this signaling pathway plays a role in the observed H_2O_2-mediated decrease in SP-A and SP-B mRNA levels, H441 cells were treated with AG490 a well-known, specific, inhibitor of JAK2/3 [28] in the presence or absence of H_2O_2. As shown in Figure 8A, the H_2O_2-mediated decrease in SP-A mRNA levels was not affected by AG490. In contrast, the H_2O_2-mediated decrease in SP-B mRNA was blocked by AG490 in concentration-dependent manner with 10 μM AG490 almost completely preventing the effect of H_2O_2 (Fig. 8B) suggesting that the JAK-STAT pathway is involved in H_2O_2-mediated reduction in SP-B, but not SP-A mRNA.

H_2O_2 stimulates phosphorylation of ERK1/2, p38 MAPK and STAT3

To determine whether H_2O_2 activates the signaling pathways suggested by the kinase inhibitors studies, we examined the effects of H_2O_2 on the phosphorylation of ERK1/2, p38 MAPK and STAT3. Protein lysates from H_2O_2-treated H441 cells were analyzed by immunoblotting using antibodies specific for phosphorylated forms of the indicated kinases. H_2O_2 treatment resulted in a rapid increase in ERK1/2 and p38 MAPK phosphorylation. The increase in phosphorylated ERK1/2 (pERK1/2) was evident at 5 min, sustained for at least 2 hr,

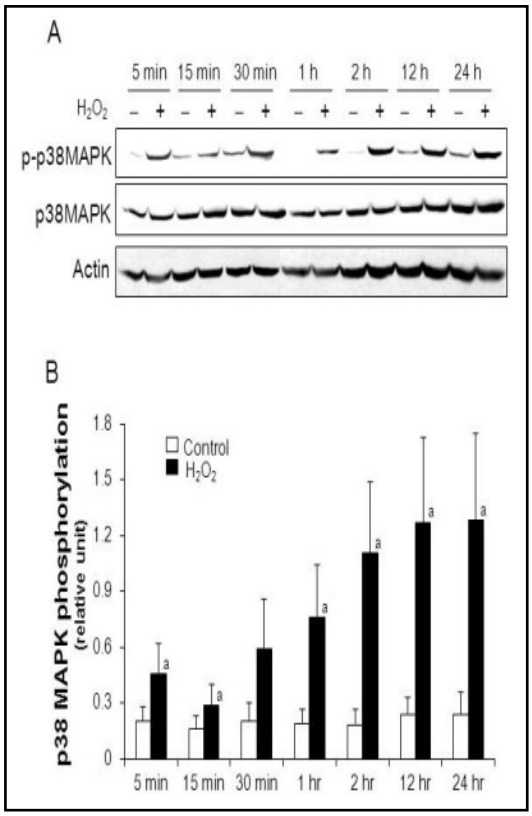

Fig. 10. Effect of H_2O_2 on the phosphorylation of p38 MAPK. H441 cells were incubated in the presence or absence of 100 μM H_2O_2 for the indicated times. Cell lysates were prepared as described under Methods and used for immunoblotting with antibody specific for either phosphorylated p38 MAPK (p-p38 MAPK), total p38 MAPK, or actin. A: Shown is one representative immunoblot demonstrating H_2O_2 mediated phosphorylation of p38 MAPK. B: Protein bands were quantified using a STORM 860 phosphorImager and normalized to actin. Data shown are the means ± S.D. of three separate experiments. Significance was determined using a paired t test. a, $p<0.05$ versus untreated controls.

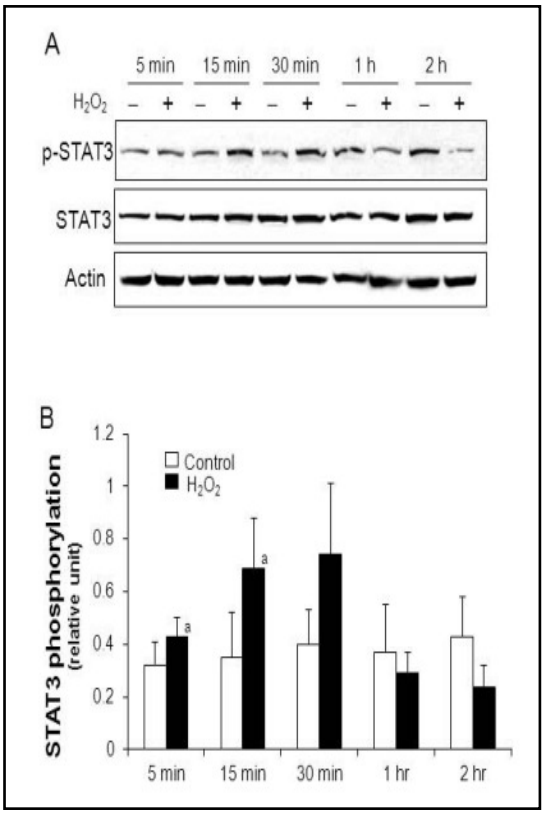

Fig. 11. Effect of H_2O_2 on the phosphorylation of STAT. H441 cells were incubated in the presence or absence of 100 μM H_2O_2 for the indicated times. Cell lysates were prepared as described under Methods and used for immunoblotting with antibody specific for either STAT phosphorylated on Tyr 705 (p-STAT), total STAT, or actin. A: Shown is one representative immunoblot demonstrating H_2O_2 mediated phosphorylation of STAT. B: Protein bands were quantified using a STORM 860 phosphorImager and normalized to actin. Data shown are the means ± S.D. of three separate experiments. Significance was determined using a paired t test. a, $p<0.05$ versus untreated controls.

and returned to almost basal levels by 12 h (Fig. 9). H_2O_2 treatment also rapidly stimulated p38 MAPK phosphorylation (p-p38 MAPK), however phosphorylation of p38 MAPK was sustained for at least 24 hr (Fig. 10). We also examined the phosphorylation of the Tyr705 site of STAT3 (STAT3-Tyr705) (Fig. 11), using antibodies specific to this site (α-pSTAT3 [Y705]). The Tyr705 site demonstrated a modest, but statistically significant increase in phosphorylation at 5 min and 15 min, however phosphorylation was decreased to basal levels at 1 hr (Fig. 11).

Discussion

This study is unique as it explores the signaling pathways involved in the ROS-mediated inhibition of SP-A and SP-B gene expression. The studies demonstrate that H_2O_2 treatment decreases mRNA levels of SP-A, SP-B, and ABCA3 in the human lung epithelial cell line H441, a human adenocarcinoma cell line used frequently to study the regulation of surfactant pro-

duction. H_2O_2 treatment also decreases binding of TTF-1, a transcription factor integrally involved in the transcriptional regulation of SP-A, SP-B, and ABCA3, to SP-A and SP-B promoter regions. In addition, mRNA and protein levels of TTF-1 are decreased by H_2O_2 with a time course similar to that observed for effects on SP-A, SP-B and ABCA3. These data suggest that decreased expression of SP-A and SP-B after H_2O_2 treatment is mediated, at least in part, by reduced TTF-1 levels. The H_2O_2 induced decrease in binding of TTF-1 to the SP-A and SP-B promoters may also be due to oxidation of TTF-1 as previous studies have demonstrated that oxidation of TTF-1 reduces DNA binding affinity [29, 30]. Whether H_2O_2 also affects the level or DNA binding activity of other transcription factors involved in regulation of surfactant related genes remains to be investigated.

ROS activate multiple signaling pathways including mitogen-activated protein kinase (MAPK) [10] and Janus kinase (JAK)/signal transducers and activators of transcription (STAT) [11, 12] pathways. In addition, some of these pathways have also been implicated in inhibition of surfactant gene expression by other agents [31, 32]. We used inhibitors of specific signaling pathways to determine whether any of the pathways might be involved in the effects of H_2O_2 on gene expression of SP-A and SP-B. Our results indicate that several of these pathways are involved with the effect of H_2O_2 on gene expression of surfactant related proteins. The ERK1/2 pathway appears to be involved in the H_2O_2-mediated inhibition of SP-A, SP-B, and TTF-1 gene expression. However the p38 MAPK pathway appears to be involved in the effect of H_2O_2 on SP-A, but not SP-B, gene expression. Conversely the JAK/STAT pathway appears to be involved in the H_2O_2 mediated decrease in SP-B, but not SP-A, mRNA. Our data indicates that all three of these pathways are activated by H_2O_2 treatment in H441 cells. Together these data indicate that these multiple pathways are differentially involved in the effect of H_2O_2 on SP-A and SP-B gene expression.

As discussed above, the ERK1/2 and p38 MAPK pathways both appear to be involved in the H_2O_2 effect on SP-A mRNA levels. Previous studies have implicated MAPK family members in inhibition of SP-A expression elicited by other agents. TNF-α and TPA, have been shown to inhibit SP-A gene expression in human lung epithelial cells via p38 MAPK and ERK MAPK signaling pathways, respectively [31, 32]. The effect of H_2O_2 on SP-B appears to involve both the ERK1/2 and the JAK/STAT pathways. That there are overlapping and different pathways involved in the H_2O_2 mediated inhibition of SP-A and SP-B is not surprising as there is also overlap and differences in the factors that regulate transcription of these genes. In addition it is possible that some of the effects of H_2O_2 may be mediated by changes to the stability of the mRNA, which could also vary between SP-A and SP-B, and was not examined in this study.

There are several limitations to this study. First, H441 cells are a human pulmonary adenocarcinoma cell line; although this cell line has been used extensively to study surfactant synthesis, the effect of H_2O_2 on normal human pulmonary epithelial cells remains to be determined. Second, while we demonstrated the effect of H_2O_2 on surfactant protein mRNA levels, we did not show the protein levels. Indeed, SP-A protein levels were also reduced (data not shown) but protein levels of SP-B are very low in these cells despite detectable mRNA levels, and we could not evaluate the effect of H_2O_2 on SP-B protein levels. Finally, although we demonstrate H_2O_2 decreases TTF-1 message and protein levels we did not evaluate the effect of H_2O_2 on TTF-1 binding affinity, although data published by others suggest that binding affinity is also reduced by oxidation [29, 30]. Further studies will help better elucidate these mechanisms of ROS inhibition of surfactant production.

It is still unclear how the activation of the indicated signaling pathways affects the transcription of SP-A and SP-B. The ERK1/2 pathway could affect the levels of SP-A and SP-B by changing the level or activity of required transcription factors, including TTF-1. Activation of the ERK1/2 pathway has been reported to inhibit TTF-1 transcriptional activity and stimulate TTF-1 phosphorylation [33]. In addition, Missero et al. demonstrated that ERK1/2 directly phosphorylates TTF-1 *in vitro*, however it remains unclear whether phosphorylation of TTF-1 by ERK1/2 affects its localization or function. The phosphorylation of SP-1, another transcription factor involved in transcriptional regulation of SP-A, SP-B and TTF-1, is associated

with TNF-α inhibition of TTF-1 expression [34]. In addition, a number of kinases, including ERK1/2 phosphorylate SP-1, although the effect of phosphorylation of SP-1 on promoter activity appears dependent on the promoter context and cell type [35, 36]. It is unclear how the JAK/STAT pathway is involved in decreasing SP-B expression, however, as the JAK/STAT pathway has also been reported to be involved in the glucocorticoid induced potentiation of IL-6 stimulated SP-B gene expression [37], it is likely that the specific context in which the pathway is activated is important for determining its overall effect on SP-B expression. Future studies will be required to investigate the regulation of other transcription factors involved in surfactant protein expression.

In conclusion, H_2O_2 inhibits SP-A, SP-B and ABCA3 gene expression, and stimulates SP-D gene expression, in lung epithelial cells. Furthermore, changes in SP-A and SP-B gene expression induced by H_2O_2 are likely to be due, at least in part, to a decrease in the level of TTF-1 mRNA and protein in H_2O_2 treated cells. A number of signaling pathways appear to be involved in the effect of H_2O_2 on SP-A and/or SP-B expression including the ERK1/2, the p38 MAPK, and the JAK/STAT signaling pathways. Together these results provide a more detailed understanding of the cellular mechanisms by which oxidative stress may affect SP-A and SP-B gene expression.

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