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Thioredoxin Overexpression Modulates Remodeling Factors in Stress Responses to Cigarette Smoke

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Cigarette smoke (CS) generates reactive oxygen species (ROS) to produce oxidative damage of bronchial epithelial cells. Prolonged repair responses lead to airway remodeling and irreversible airflow limitation. Thioredoxin (TRX) is a redox protein that scavenges ROS to prevent oxidative stress. The aim of this study was to investigate the mechanisms underlying TRX-mediated CS-induced stress relevant to airway remodeling. Results showed that CS stimulated ROS generation and apoptosis in normal human bronchial epithelial (BEAS-2B) cells, and interfered with gene expression of remodeling factors, such as activation of transforming growth factor (TGF)-β1, epidermal growth factor receptor (EGFR), and cyclin-dependent kinase inhibitor (p21), but repressed matrix metalloproteinases (MMP)-9. In particular, TRX-overexpressing bronchial epithelial (TRX-TD) cells reduced CS-induced apoptosis, and suppressed airway remodeling through attenuation of TGF-β1, EGFR, and p21 and upregulation of MMP-9 expression. TGF-β1 was shown to regulate MMP-9 as evidenced by suppression of MMP-9 protein induction by TGF-β1 antibody. In addition, CS produced apoptosis of BEAS-2B cells via TRX oxidation, which activated signal transduction factors, including apoptosis signal-regulating kinase (ASK) 1 and c-Jun N-terminal kinase (JNK). In contrast, TRX-TD cells exposed to CS retained reduced-form TRX, and inactivated ASK1 and JNK to attenuate apoptosis. This study indicated TRX overexpression was involved in CS-induced apoptosis and prevented airway remodeling through ASK1-JNK inactivation and MMP-9 augmentation.

In biological organisms, oxidation-reduction (redox) reactions occur dynamically during metabolism. However, imbalance of redox reaction produces oxidative stress and results in cell injury, inflammation, and tissue damage. Cigarette smoke (CS) is one of the major external sources of reactive oxygen species (ROS) relevant to human lung diseases. Because of the multiple compounds and complexes in CS (Hoffmann & Hoffmann, 1997), tobacco smoking may produce lung inflammation (Hellermann et al., 2002), chronic obstructive pulmonary diseases (Wald & Hackshaw, 1996), and even lung cancer (Hecht, 2003).

Airway epithelium provides a protective barrier against external environments. Several in vitro studies demonstrated CS extract induces oxidative stress and results in apoptosis or necrosis (Banzet et al., 1999; Carnevali et al., 2003). Damaged epithelial cells may prolong the period of epithelial repair and express remodeling mediators, such as growth factors and matrix metalloproteinases (MMP) that contribute to structural changes in bronchial epithelium (i.e., airway remodeling) (Hamilton et al., 2003). This remodeling produces irreversible airflow limitation and increased airway hyperresponsiveness (James et al., 1989). Gene expression of several growth factors including transforming growth factor (TGF)-β1, epidermal growth factor receptor (EGFR), and cyclin-dependent kinase inhibitor (p21) are increased in inflammatory responses. MMP induce digestion of basement membranes to prevent airway inflammation (Yoon et al., 2007) and tumor progression (Martin & Matrisian, 2007).

Thioredoxin (TRX), a known antioxidant with redox active sequence Cys-Gly-Pro-Cys, is ubiquitous in mammals (Hirota et al., 2002; Holmgren, 1985). TRX regulates cellular redox balance, promotes cell growth, inhibits apoptosis, and modulates inflammation (Nakamura et al., 2005; Watson et al., 2004). TRX is presumed to be essential for cell survival, as knockout mice lacking TRX do not survive (Powis & Montfort, 2001; Nonn et al., 2003). Because of the stress-inducible characteristics, TRX is expressed in response to CS, ROS, ionization radiation, and air pollutants (Hirota et al., 2002; Nakamura et al., 1997; Powis & Montfort, 2001). Genetic suppression or inhibition of TRX results in increased ROS generation and apoptosis (Hansen et al., 2006). In TRX transgenic mice there is decreased alveolar

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damage from hyperoxia-induced apoptosis (Yamada et al., 2007), and suppressed systemic inflammatory responses in response to CS (Sato et al., 2006).

Cells respond to ROS-induced apoptosis via a variety of signal transduction pathways (Tesfaigzi, 2006). The mitogen-activated protein kinase (MAPK) family regulates growth signaling in response to stresses (Wada & Penninger, 2004). Subgroups of the MAPK pathway, c-Jun N-terminal kinase (JNK) and p38 MAPK, show stress-activated characteristics and regulate cell survival or apoptosis. JNK is required for stress-induced apoptosis of normal cells. Further, apoptosis signal-regulating kinase (ASK) 1 is activated by ROS involved in apoptosis, and upregulates p38 and JNK expression (Tobiume et al., 2001). Thus, MAPK plays a crucial role in cell proliferation and apoptosis in response to oxidative stress. However, the signaling transduction resulting from CS exposure in airway epithelial cells is still not completely understood.

CS generates ROS leading to airway remodeling in bronchial epithelium. Once transformed, the remodeled lung is resistant to therapy (Yamauchi & Inoue, 2007); thus, it is necessary to understand the mechanisms underlying TRX mediated CS-induced apoptosis and airway remodeling. In this study, a TRX-overexpressing bronchial epithelial (TRX-TD) cell line was constructed to examine how TRX-mediated expression of remodeling factors in human bronchial epithelial cells is affected by CS-induced oxidative stress.

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METHODS

Cell Culture

Normal human bronchial epithelial cells, BEAS-2B (ATCC CRL-9609), were grown on coated tissue culture plates (a mixture of 0.01 mg/ml fibronetin, 0.03 mg/ml vitrogen 100, and 0.01mg/ml bovine serum albumin in 10 ml LHC-9 medium) in LHC-9 medium (Invitrogen, Carlsbad, CA). LHC-9 medium is a serum-free medium supplemented with 0.5 ng/ml recombinant epidermal growth factor, 500 ng/ml hydrocortisone, 0.005 mg/ml insulin, 0.035 mg/ml bovine pituitary extract, 500 n*M* ethanolamine, 500 n*M* phosphoethanolamine, 0.01 mg/ml transferrin, 6.5 ng/ml 3,3′,5-triiodothyronine, 500 ng/ml epinephrine, 0.1 ng/ml retinoic acid, and trace elements.

Plasmid Construction of TRX Gene-Tagged Flag

Human TRX cDNA was prepared by polymerase chain reaction (PCR) based on the sequence derived from the NCBI (NM_003329). The TRX was flag-tagged in a p3XFlag-CMV-14 vector (between the restriction sites BamHI and SalI) according to the manufacturer's recommended protocol (Sigma, St. Louis, MO). After sequence verification, the C-terminal of the TRX gene-attached 3flag was inserted into a tetracycline-controllable $pTRE_2$hyg vector (BD Biosciences, San Jose, CA) through restriction enzymes catalysis of ClaI and SalI (for identification of exogenous TRX expression). The plasmid $pTRE_2$hyg-TRX-3flag was then transfected into DH5α competent cells (Invitrogen, Carlsbad, CA). The presence of the plasmid DNA was verified by restriction endonuclease digestion and agarose gel electrophoresis.

TRX Overexpression in BEAS-2B Cells

BEAS-2B cells were transfected with the pTet-on regulator plasmid DNA by lipofectamine and OPTI-MEM I medium (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. After selection with 1 mg/ml G418 (Sigma, St. Louis, MO) for approximately 2 mo, the $pTRE_2$hyg-TRX-3flag plasmid DNA was transfected into the established Tet-on regulator expression BEAS-2B cells. After G418 selection, the BEAS-2B Tet-on $pTRE_2$hyg-TRX-3flag cells were developed, and overexpressed TRX (via Tet-on system) was switched on by 2 μg/ml doxycycline for 24 h. The TRX-overexpressing cells (TRX-TD; doxycycline-induced BEAS-2B Tet-on $pTRE_2$hyg-TRX-3flag cells) were examined for the expression of human flag mRNA and protein, respectively, by quantitative real time PCR and flow cytometric analysis.

Insulin Reduction Assay

Dithiothreitol (DTT; Sigma, St. Louis, MO) was used as a reductant in the insulin reduction assay as described (Wollman et al. 1988). The reaction reagents were composed of 130 μ*M* insulin (soluble at pH 2–3 and adjusted to pH 8), 100 m*M* potassium phosphate (pH 7), 2 m*M* ethylenediamine tetraacetic acid (EDTA), and 2 m*M* DTT. The protein level of constructed TRX-3flag expression in BEAS-2B cells was purified by immunoprecipitation using anti-flag antibody (Ab) and protein G-agarose (Sigma, St. Louis, MO). The competitive assay of TRX reduction capability was detected with DTT at 650 nm, kinetic 1-min intervals for 2 h. TRX protein from *Escherichia coli* (Sigma, St. Louis, MO) was used as a positive control.

Intracelluar TRX-Flag Immunostaining

The expression of exogenous transfected TRX was monitored by intracellular flag immunostaining. TRX-TD cells were fixed with commercial fixation solution (eBioscience, San Diego, CA), vortexed, incubated in the dark at room temperature for 20 min, and permeated for staining (permeabilization buffer, eBioscience, San Diego, CA). Optimal concentration of monoclonal M2 anti-flag-FITC Ab (Sigma, St. Louis, MO) was added to cells and determined by flow cytometry (CyFlow, Partect). The data were analyzed using WinMDI 2.8 software.

Western Blotting

Cells were lysed with protein extract buffer containing 0.6 *M* KCl, 1% Triton X-100, 0.02 *M* Tris-HCl (pH 7), 1 m*M* phenylmethylsulfonyl fluoride, and 50 μg/ml aprotinin (all from

Sigma, St. Louis, MO) and centrifuged at 8000 × g (3 min, 4°C). Protein samples in the supernatant were immediately transferred and the concentration was measured using a DC protein assay (Bio-Rad, Hercules, CA) according to Bradford (1976). For Western analysis of TRX redox status, the lysates were incubated with alkylated 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (Molecular Probes, Eugene, OR) followed by nonreducing Western blot to distinguish between reduced (alkylated) and oxidized (nonalkylated) proteins (Chen et al., 2006). Protein in the samples were then electrophoresed over a 12.5% sodium dodecyl sulfate polyacrylamide gel, and subsequently transferred to a nitrocellulose membrane (Millipore, Billerica, MA). The membrane-bound proteins were then immunostained with anti-human TRX (Abcam, Cambridge, MA), flag (Sigma, St. Louis, MO), MMP-9 (R & D Systems, Inc., Minneapolis, MN), and phospho-ASK1, phospho-JNK, or β-tubulin (Cell Signaling, Danvers, MA) Ab, followed by treatment with secondary anti-IgG horse radish peroxidase (HRP) Ab (Chemicon, Billerica, MA). Any tagged proteins were then detected using a chemiluminescence reagent (Perkin Elmer, Boston) and photographed in a G:Box ChemiXT 16 system (Syngene, Frederick, MD).

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Cigarette Smoke Extraction and Exposure

CS extract was prepared as described by Hoshino et al. (2001). Mainstream smoke of 7 research-use cigarettes (University of Kentucky 2R4F; 10 mg tar and 0.8 mg nicotine per cigarette) was withdrawn steadily via a peristaltic pump at a rate of 50 rpm and bubbling through a 27.5-cm height vessel containing 10 ml LHC-9 medium. CS extract consisted of multiple compounds with maximal absorbance at 305 nm wavelength; the concentration of 100% CS extract has a 0.5 absorbance at 305 nm. In order to quantify and reproduce the daily preparation of CS extract, the absorbance at 305 nm was measured for CS content. The CS extract freshly obtained was then filtered through a 0.22-μm filter, and diluted to the desired percentage prior to cell exposure.

Cell Viability Assay

LIVE/DEAD assay (Molecular Probes, Invitrogen, Carlsbad, CA) was carried out as per manufacturer protocol. In brief, CS-exposed cultures were washed once with 200 μl/well of phosphate-buffered saline (PBS) (for 96 well plates), and 100 μl phosphate-buffered saline (PBS) was added back to the cells. Then 100 μl of 4 μ*M* calcein and 2 μ*M* ethidium homodimer-1 mixture was added to cells in dark. After 30 min of incubation at 37°C, cells were detected with fluorescence of calcein and ethidium homodimer-1 at excitation/emission wavelengths 485/538 nm and 538/620 nm separated into live or dead cells.

Measurement of Intracellular ROS Generation

Cells were incubated with 5 μ*M* CM-H_2DCFDA (5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester; Molecular Probes, Invitrogen Corporation, Carlsbad, CA) for 30 min. CM-H_2DCFDA is a cell-permeant and nonfluorescent compound when reduced, and emits fluorescence when oxidized by ROS (hydrogen peroxide and hydroxyl radical). The adherent cells were trypsinized, washed with PBS three times, and then resuspended in 1 ml PBS. The fluorescent signal reflecting ROS was determined by flow cytometry. Data analysis was performed using WinMDI 2.8 software.

Apoptosis Determination

The level of apoptosis was determined using an Annexin V-FITC apoptosis detection kit (R & D Systems, Inc., Minneapolis, MN). The trypsinized cells were washed twice with cold PBS and resuspended in 18 binding buffer. One hundred microliters of suspension of 10^5 cells was treated 5 μl Annexin V. The staining cells were incubated at 4°C in the dark for 15 min and 400 μl binding buffer was added before FACS analysis. The fluorescent signal of flow cytometry was performed by WinMDI 2.8 software. The percent apoptosis was referred to sham groups within 95% confidence intervals of intensity.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA were extracted using RNA Trizol (Invitrogen, Carlsbad, CA) reagent to determine the gene expression at mRNA level by quantitative real-time PCR. cDNA was synthesized from total RNA by reverse transcription with oligo-dT primer and RNase-free water at 65°C for 5 min (1 cycle) to anneal primer with mRNA. After that, the product was reverse-transcribed to cDNA with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and dNTP at 37°C for 90 min and 70°C for 15 min (1 cycle).

Quantitative Real-Time PCR

Real-time PCR was performed to quantify the message expression pattern of RT-PCR-synthesized cDNA products. One hundred nanograms cDNA was analyzed using 2 × power SYBR Green PCR master mix, forward and reverse primers, and RNase-free water by an ABI 7300 sequence detection system (Applied Biosystems, Inc., Foster City, CA). PCR primers were: TRX sense 5′-GGA CGC TGC GGG TGA TA-3′ and anti-sense 5′-GAG AGG GAA TGA AAG AAA GGC TT-3′; TGF-β1 sense 5′-ACA ATT CCT GGC GAT ACC TCA-3′ and anti-sense 5′-GGC GAA AGC CCT CAA TTT C-3′; EGFR sense 5′-GGA GAG GAG AAC TGC CAG AAA CT-3′ and anti-sense 5′-GCA GCC TGC AGC ACA CTG-3′; p21 sense 5′-GGG ACA GCA GAG GAA GAC CAT-3′ and anti-sense 5′-GGA GTG GTA GAA ATC TGT CAT GCT-3′; MMP-9 sense 5′-ACC ACC TCG AAC TTT GAC AGC-3′′ and anti-sense 5′-TCT AAG CCC AGC GCG TG-3′; β-actin sense 5′-TTG TTA CAG GAA GTC CCT TGC C-3′ and anti-sense 5′-ATG CTA TCA CCT CCC CTG TGT G-3′. The reaction

mixture was incubated 50°C for 2 min, 95°C for 10 min, and 95°C for 15 s, 52°C for 30 s, 72°C for 45 s for 40 cycles, with extension 95°C for 15 s and 60°C for 1 min. The relative level of mRNA expression is a ratio of optical density of the experimental groups to that of β-actin. Independent triplicate experiments were performed.

Statistical Analysis

Results were described as mean ± standard deviation of at least three separate experiments. All statistical analysis was conducted by the statistical package SPSS13.0. Statistically significant differences among groups were determined using Student's *t*-test and one-way analysis of variance (ANOVA). A two-tailed p value <.05 was considered statistically significant.

RESULTS

Constructed TRX Plasmid Expression in BEAS-2B Cells

The reducing activity of TRX protein was determined by insulin reduction assay (Figure 1A). BEAS-2B cells were transfected the constructed TRX-3flag gene which expressed its protein and redox capability. In order to determine whether exogenous TRX transfection displayed in BEAS-2B cells, analysis of intracellular TRX-flag protein was conducted. Expression of TRX-flag protein in TRX-TD cells was significantly higher than BEAS-2B cells (intensity: 6 ± 0.6 and 15.4 ± 1.9) (Figure 1B). The transgenic TRX-flag protein was detectable in TRX-TD cells but not BEAS-2B cells (Figure 1C). TRX-TD cells expressed both endogenous and exogenous TRX protein (Figure 1D). These results indicated that TRX-TD cells contained both endogenous and exogenous TRX, which led to overexpression of TRX.

Cell Viability in CS Exposure

CS served as the source of ROS in this study. BEAS-2B cells and TRX-TD cells were treated with different concentrations from 0 to 100% CS for 24 h and then assessed for cell viability. The results illustrated a concentration-response relationship between CS exposure and cell viability (Figure 2). TRX-TD cells had a higher survival ratio than BEAS-2B cells following

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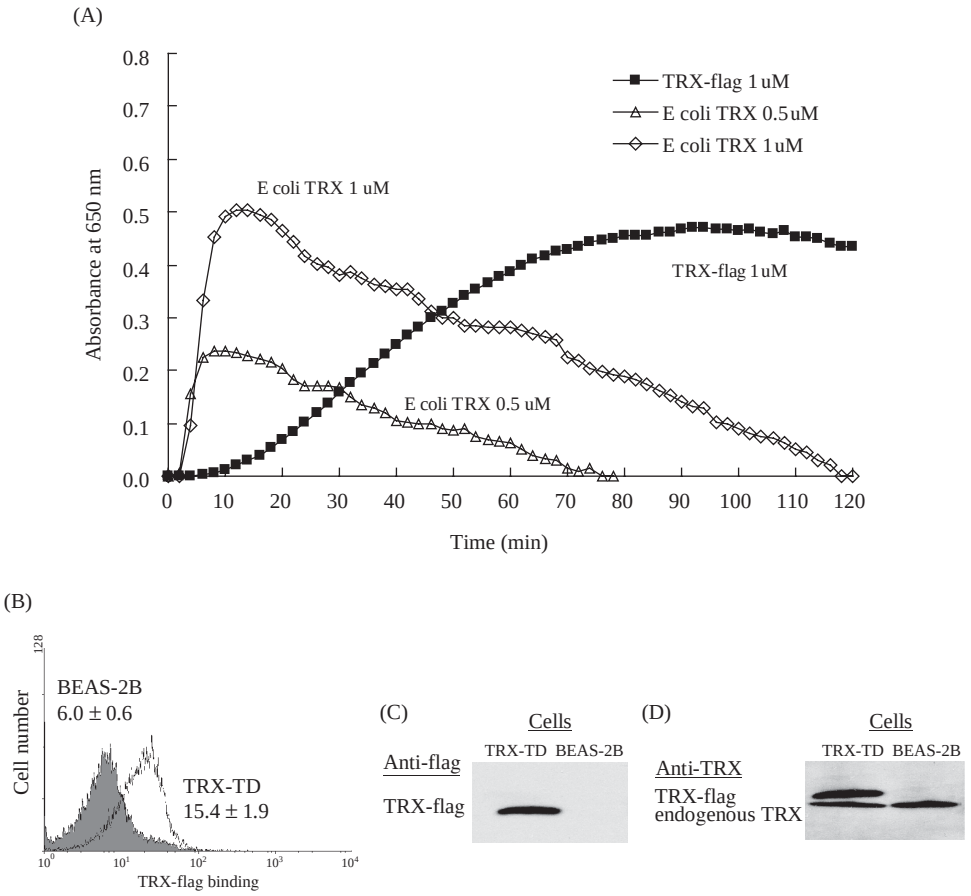

FIG. 1. Expression of TRX and TRX-flag protein in BEAS-2B and TRX-TD cells. (A) Reducing activity of TRX protein. (B) Cytometric analysis of intracellular TRX-flag protein. Data presented as mean ± standard deviation of the intensity of fluorescence signal. Western blotting with (C) exogenous TRX-flag protein and (D) endogenous TRX protein.

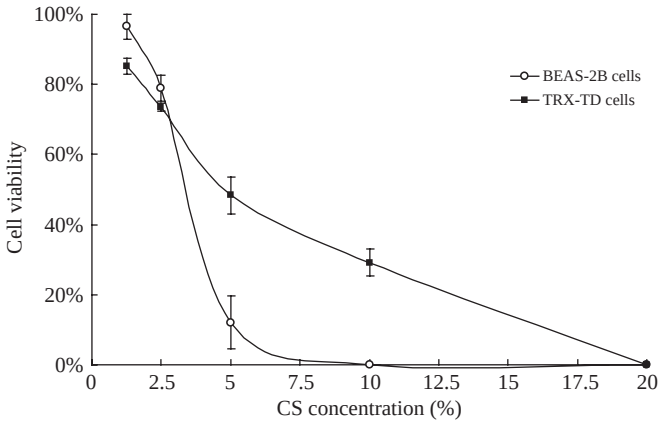

FIG. 2. Concentration response of cell viability in BEAS-2B and TRX-TD cells after 24-h CS exposure.

exposure to 5% CS (48.4 ± 4.3% and 12 ± 6.8%). TRX-TD cells were more resistant to CS exposure than BEAS-2B cells. The survival ratio of cells exposed 2.5% CS for 24 h was approximately 80%, and thus the concentration of 2.5% CS for 24-h exposure was used for further experiments. At this concentration, cells displayed manifestations of oxidative stress but survived.

TRX Overexpression Reduced ROS Generation and Apoptosis Following CS Exposure

Treatment of BEAS-2B cells with a potent oxidant 500 μ*M* H_2O_2 for 2 h was used as positive control of intracellular ROS generation (Figure 3A). A 24-h exposure to 2.5% CS was used to generate ROS in BEAS-2B cells. In contrast, TRX-TD cells over expressing TRX reduced ROS generation. CS-exposed BEAS-2B cells showed 1.43-fold increased mRNA expression in response to TRX referred compared to nonexposed BEAS-2B cells (Figure 3B). TRX-TD cells expressed TRX 4.3-fold higher than BEAS-2B cells. CS exposure markedly induced 124.13-fold expression of TRX transcript in TRX-TD cells. This indicated that CS induced both endogenous and exogenous TRX expression. After CS exposure, TRX-TD cells (7%) had a decreased level of apoptosis compared with BEAS-2B cells (11%) (Figure 3C). TRX-TD cells overexpressing TRX suppressed CS-induced apoptosis.

TRX Overexpression Mediated Inference of Remodeling Factors

CS induced gene expression of TGF-β1, EGFR, and p21 in BEAS-2B cells compared to nonexposed BEAS-2B cells (6.89, 3.11, and 1.65-fold increase, respectively) (Figure 4A). In contrast, CS suppressed the expression of MMP-9 (0.84-fold decrease). Further, following CS exposure TRX-TD cells displayed lower expression of TGF-β1 and EGFR than BEAS-2B cells (respectively, fold changes 1.27 vs. 6.89 and 1.59 vs. 3.11), and repressed

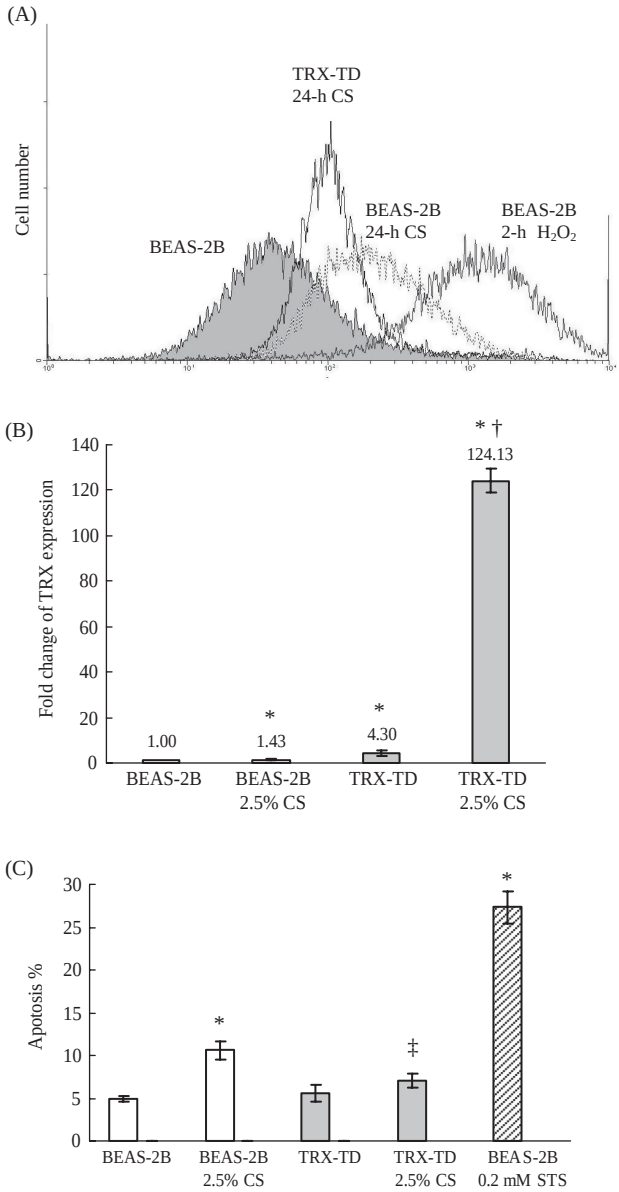

FIG. 3. Intracellular ROS generation, TRX expression, and apoptosis following exposure to 2.5% CS for 24 h. (A) ROS generation in BEAS-2B and TRX-TD cells. BEAS-2B cells treated with 500 μ*M* H_2O_2 for 2 h as a positive control. (B) Quantification of TRX transcript by real-time PCR. (C) Determination of apoptosis with Annexin-V-FITC staining and FACS detector. STS: staurosporine produces mitochondria damage as positive control of apoptosis. Asterisk indicates significant at $p < .05$ compared with BEAS-2B cells; $^{\dagger}p < .05$, compared with TRX-TD cells; $^{\ddagger}p < .05$, compared with CS-exposed BEAS-2B cells.

p21 transcript (0.93 vs. 1.65). Otherwise, CS exposure augmented MMP-9 expression in TRX-TD cells (3.99-fold increase).

Following exposure to CS, MMP-9 protein was diminished in BEAS-2B cells but activated in TRX-TD cells (Figure 4B). To investigate whether TGF-β1 regulated MMP-9, TGF-β1 Ab was added to neutralize TGF-β1 protein secreted into culture

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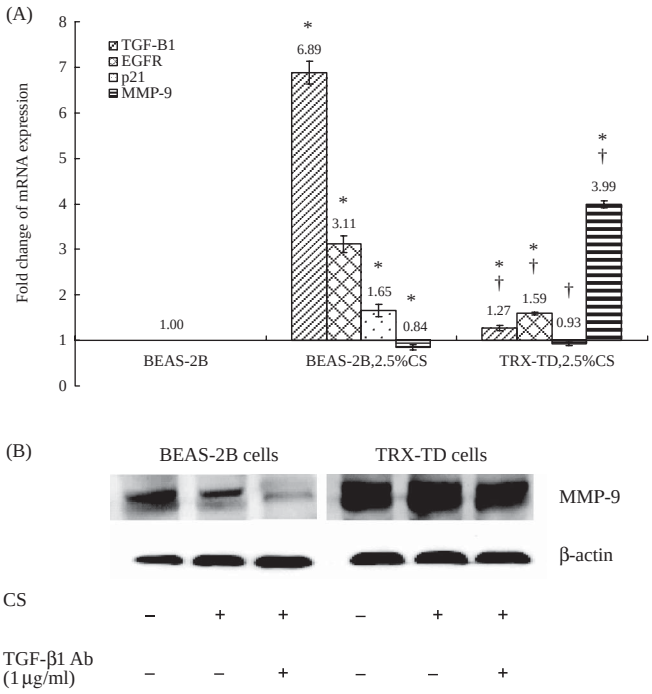

FIG. 4. Gene expression of remodeling factors in response to 24-h CS exposure. (A) Analysis of mRNA expression of TGF-β1, EGFR, p21, and MMP-9 by real-time PCR. (B) Determination of MMP-9 protein using Western blotting. TGF-β1 Ab was used to neutralize TGF-β1 protein secreted into culture medium. Significant differences indicated by asterisk for $p < .05$, compared with BEAS-2B cells; $^{\dagger}p < .05$, compared with CS-exposed BEAS-2B cells.

medium. Interestingly, induction of MMP-9 protein was suppressed by adding TGF-β1 Ab.

CS-Induced TRX and MAPK Expression

CS induced TRX expression and oxidization (Figure 5). With respect to the activation of transfected TRX-flag, TRX-TD cells contained higher amounts of reduced TRX than BEAS-2B cells following CS exposure. CS-exposed BEAS-2B cells displayed phosphorylation of ASK1 and JNK. In TRX-TD cells, phospho-ASK1 and phospho-JNK were activated following CS exposure, and subsequently TRX overexpression led to inactivation of phosphorylation.

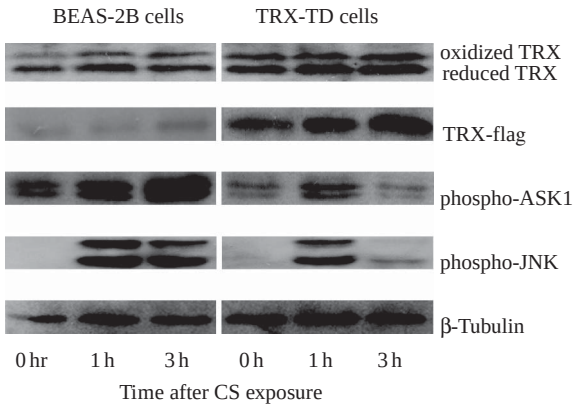

FIG. 5. Western blot analysis of redox TRX, TRX-flag, and phosphorylation of ASK1 and JNK in BEAS-2B and TRX-TD cells after CS exposure.

DISCUSSION

CS is known to generate ROS in human lung epithelial cells (Ryter et al., 2007; Yao et al., 2007). This study investigated intracellular ROS generation and apoptosis in human bronchial epithelial cells following CS exposure. TRX possesses stress-inducible characteristics to prevent cell injury and apoptosis following exposure to CS (Sato et al., 2006) and diesel exhaust particles (Kaimul Ahsan et al., 2005). Because of redox capability, TRX suppresses oxidative stress and inflammatory responses (Watson et al., 2004). Consistent with this study, TRX was upregulated in CS-exposed BEAS-2B cells and TRX-TD cells, enabling reduction of ROS generation and apoptosis. Data suggested that TRX overexpression protected airway cells against oxidative stress induced by CS.

TGF-β plays a crucial rule in the signal network of cell growth and differentiation. TGF-β inhibits cyclin D-dependent kinase 4 through induction of p21 pathway required for inhibition of cell growth (Massague et al., 2000). TGF-β1 induces cell apoptosis which precedes inflammatory responses (Blobe et al., 2000). Yamasaki et al. (2008) showed that TGF-β1 transgenic mice manifest marked bronchoalveolar and tissue inflammation, fibrosis, and pulmonary alveolar remodeling. Murillo et al. (2005) demonstrated that TGF-β1 activates EGFR for anti-apoptosis and cell survival. Abnormal activation of EGFR suppressed apoptosis, enabling tumor progression (Liu et al., 2007), and this was found in lung cancer (Sharma et al., 2007). EGFR plays a direct role in pro-inflammatory responses by inducing interleukin (IL)-8 release from bronchial epithelial cells, which contribute to and prolong neutrophilic inflammation and tissue injury (Hamilton et al., 2003). Fedorov et al. (2005) demonstrated that the thickness of the lamina reticularis is significantly correlated with epithelial EGFR. EGFR impaired apoptosis and induced cell proliferation through increased p21 expression (Sheng et al., 2006).

TGF-β1 stimulates the expression of MMP-9 for cell migration (Seomun et al., 2008). Airway epithelial wound repair enhances the activation of MMP-9 (Bove et al., 2007). MMP-9 induces digestion of basement membrane to prevent airway from remodeling. Yoon et al. (2007) found that MMP-9 exerted a protective capability during ozone-induced lung neutrophilic inflammation and hyperpermeability. The quantitative PCR results in the current study indicated that CS induced TGF-β1, EGFR, and p21 expression in BEAS-2B cells but diminished MMP-9 expression. Evidence indicated that CS exposure activated TGF-β1 and EGFR expression to initiate cellular responses, such as apoptosis and cell proliferation.

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TGF-β1 mediates p21 to arrest cell cycle and inhibit apoptosis (Gartel & Tyner, 2002). In particular, p21 blocks cell apoptosis by interacting with pro-apoptotic molecules such as ASK1, procaspase-3 and caspase-8. However, the results here suggested that induction of p21 was not sufficient for anti-apoptosis, and thus resulted in activated ASK1 phosphorylation. The regulation of TGF-β1 and p21 failed to protect airway cells from effects of CS, and thus led to cell apoptosis. Furthermore, expression of MMP-9 in BEAS-2B cells was suppressed by CS exposure, which would result in the progression of airway remodeling.

TRX is involved in cell growth and differentiation (Arner & Holmgren, 2000), and regulation of tumor suppressor p53 to transactivate p21 (Ueno et al., 1999). p21 is an essential factor of cell cycle progression, DNA repair, and apoptosis (Harper et al., 1993; Xiong et al., 1993). Data of this study showed that TGF-β1 activated EGFR to upregulate p21 for damage repair in CS-exposed BEAS-2B cells. Otherwise, TRX-TD cells reduced the transcript level of TGF-β1 to prevent CS-induced apoptosis, and inhibited irregular cell proliferation and repair by downregulating EGFR and p21 expression. Expression of TGF-β1, EGFR, and p21 was preserved at steady state after CS exposure, which indicated TRX overexpression was involved in homeostatic mechanisms adequate to suppress CS-induced stress. This finding suggested that TRX might reduce gene expression of TGF-β1, EGFR, and p21 to eliminate the influence of any irregular cell proliferation of extracellular matrices in airway epithelial cells. In contrast, TRX overexpression enhanced MMP-9 transcription in TRX-TD cells. Upregulation of MMP-9 is thought to modulate airway remodeling (Ohbayashi & Shimokata, 2005), and likely to exert a protective role for bronchial epithelial cells in response to CS exposure. In addition, activation of MMP-9 protein was suppressed by treatment with an anti-TGF-β1 Ab, which suggested that the presence of TGF-β1 appeared to neutralize CS-induced mediators in the medium, and attenuated the generation of MMP-9 protein.

TRX regulates the ROS-induced MAPK pathway (Hsieh & Papaconstantinou, 2006). When TRX is oxidized, ASK1 becomes active, and cells approach apoptosis by upregulation of downstream JNK and p38 expression (Yoshida et al., 2003). JNK is important for induction of apoptosis. Inhibition of JNK signal expression would abrogate apoptosis from oxidative injury. Lee et al. (2002) found that TRX mediates JNK expression. MAPK was induced by CS following acute exposure (Yoneda et al., 2003). In present study, CS exposure oxidized TRX to activate ASK1 and JNK in BEAS-2B cells, and resulted in apoptosis. Otherwise, TRX-TD cells normally express TRX and ASK1 at steady state. When exposed to CS, TRX-TD cells were found to overexpress TRX and attenuate phosphorylation of ASK1 and JNK. This indicated a fraction of TRX was oxidized early, and the reduced form was retained to inactivate ASK1 phosphorylation and suppress apoptosis. Therefore, TRX overexpression protected airway cells from ASK1-activated apoptosis through inhibition of JNK pathway.

MAPK signaling is related to EGFR expression. EGF induces MAPK signaling and proliferation in pulmonary epithelial cells (Semlali et al., 2008). Furthermore, MMP-9 is MAPK dependent and regulated by TGF-β1 and EGFR (Uttamsingh et al., 2007). TRX ameliorates CS-induced lung inflammation and emphysema (Sato et al., 2008). Based on previous studies and our findings, Figure 6 presents an illustration of how TRX mediates remodeling factors following CS exposure. In general, CS-stimulated ROS generation induces expression of TGF-β1 and EGFR to activate p21 and ASK1-JNK signaling for cell repair and apoptosis. Damaged epithelial cells may prolong the period of epithelial repair and contribute to airway remodeling. When TRX overexpression occurs following CS exposure, p21 and ASK1-JNK signaling are repressed, and MMP-9 is upregulated for inhibition of apoptosis and remodeling. TRX overexpression exerts the capacity to protect human airway epithelial cells against oxidative stress from CS.

Oxidative stress generated by CS induced MAPK phosphorylation and suppressed MMP-9 expression, leading to apoptosis

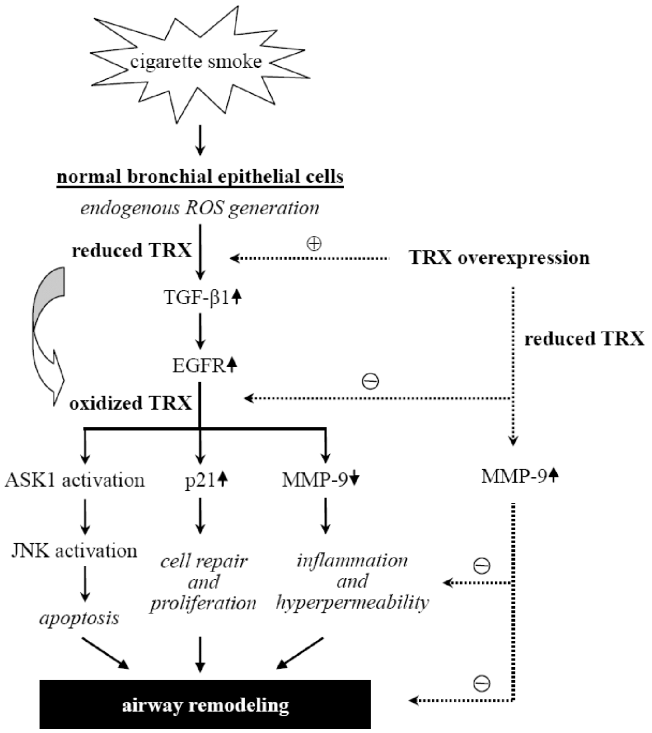

FIG. 6. An illustration of TRX mediating stress response to CS. CS induces airway epithelial cells to generate ROS, and upregulates the expression of TGF-β1 and EGFR to activate p21 and ASK1-JNK signaling for cell repair and apoptosis. Sloughed epithelial cells are involved in airway remodeling due to irregular cell proliferation and prolonged repair duration. During TRX overexpression, TRX is partially oxidized to modulate CS-induced oxidative stress, and the reduced form of TRX is retained to repress p21 and ASK1-JNK signaling and augment MMP-9 expression for diminished inflammation and hyperpermeability. Data suggest TRX modulates CS-induced stress to retard apoptosis and progression of airway remodeling in bronchial epithelial cells.

and progression of airway remodeling. TRX is a ubiquitous protein playing a role in signal transduction. This study demonstrated that transfected TRX produced redox effects and transcription/translation responses in human airway cells. TRX overexpression prevented airway cells from CS-induced apoptosis and remodeling through ASK1-JNK inactivation and MMP-9 augmentation.

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