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FOXO3 Deficiency Leads to Increased Susceptibility to Cigarette Smoke-Induced Inflammation, Airspace Enlargement, and Chronic Obstructive Pulmonary Disease

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Forkhead box class O 3a (FOXO3) is a member of the FoxO transcription factor subfamily, which regulates the expression of target genes not only through DNA binding as a transcription factor, but also through protein–protein interaction. Although FOXO3 is a well-known transcription factor involved in diverse biological processes, the role of FOXO3 in cigarette smoke (CS)-induced lung inflammation and injury has not been studied. It is, therefore, hypothesized that deficiency of FoxO3 leads to increased susceptibility to CS-induced lung inflammatory response and airspace enlargement. In this article, we show that the levels of FOXO3 are significantly decreased in lungs of smokers and patients with chronic obstructive pulmonary disease, as well as in lungs of mice exposed to CS. Genetic ablation of FoxO3 led to pulmonary emphysema and exaggerated inflammatory response in lungs of mice exposed to CS. We further showed that CS induced the translocation of FoxO3 into the nucleus where FoxO3 interacted with NF-κB and disrupted NF-κB DNA-binding ability, leading to inhibition of its activity. Targeted disruption of FoxO3 also resulted in downregulation of antioxidant genes in mouse lungs in response to CS exposure. These results suggest that FoxO3 plays a pivotal role in regulation of lung inflammatory response and antioxidant genes, and deficiency of FoxO3 results in development of chronic obstructive pulmonary disease/emphysema. The Journal of Immunology, 2011, 187: 987–998.

Cigarette smoke (CS) is the major risk factor for the development of chronic obstructive pulmonary disease (COPD)/emphysema via inducing an abnormal and persistent inflammatory response with an increase of inflammatory cells, such as neutrophils, macrophages, dendritic cells, and T cells in the lungs (1–3). COPD is a highly prevalent and debilitating lung disease, but the molecular and cellular mechanisms responsible for its pathogenesis are not clear. This is due to lack of understanding of the specific cellular and molecular pathways triggered by CS/oxidants in the lungs. It is currently thought that increased lung oxidative stress, abnormal immune-inflammatory response, and cellular senescence play an important role in development of COPD/emphysema (1, 4–6). It has also been proposed that abnormal inflammatory responses are associated with increased cell senescence incited by CS in the development of COPD/emphysema (6, 7).

Forkhead box class O 3a (FoxO3) is a member of the FoxO subfamily, identified as transcription factors with forkhead DNA-binding domains, which regulate the expression of several genes involved in diverse biological processes, such as apoptosis, cell cycle progression, vascular remodeling, development, senescence, oxidative stress resistance, innate immune homeostasis, and inflammation (8–17). FoxO3 controls T cell response and regulates neutrophil function during inflammation (18, 19). Although CS induces pulmonary inflammation and modulates innate immune response (20), the role of FoxO3 in CS-mediated inflammation is not known.

FoxO3 functions as a transcription factor through its direct DNA binding and interaction with other proteins (21–24). In addition, the function of FoxO3 is modulated by posttranslational modifications, such as phosphorylation and acetylation. Translocation into nucleus and degradation of FoxO3 in the proteasome are regulated by its phosphorylation via divergent cell signaling (11, 25, 26). Furthermore, acetylation of FoxO3 by CREB-binding protein can alter its DNA-binding and transcription activity, which can be restored by sirtuin 1 (SIRT1)-mediated deacetylation (27–31). We and others have shown that SIRT1 level/activity is decreased in response to CS exposure in vitro in macrophages and epithelial cells, as well as in vivo in lungs of patients with COPD (32–35). Therefore, CS-mediated SIRT1 reduction will result in acetylation of FoxO3 and alter its transactivation ability, thereby regulating antioxidant gene transcription. Furthermore, because FoxO3 regulates RelA/p65, a subunit of NF-κB (25, 36), it is possible that FoxO3 deficiency in lungs can lead to exaggerated lung inflammatory response.
We hypothesized that FoxO3 plays an important role in regulating CS-induced lung inflammation and airspace enlargement (emphysema) through the modification of NF-kB activity and antioxidant genes in mouse lungs, as well as in patients with COPD. We, therefore, determined the abundance and localization of FoxO3 in lungs of smokers and patients with COPD, and investigated whether deficiency of FoxO3 would lead to increased susceptibility to CS-induced lung inflammation and emphysema in mouse lungs. We further studied the potential mechanisms of FoxO3-mediated protection against CS-induced lung inflammatory response by determining the ability of FoxO3 to interact with RelA/p65 and dampen NF-kB activation.

Materials and Methods

Human lung tissues and spumum cells

Lung tissue specimens were from 37 subjects and patients: 10 lifelong nonsmokers; 10 current smokers with normal lung function; and 17 patients with COPD (11 former and 6 current smokers; 10 patients had been prescribed inhaled and/or low-dose oral corticosteroids) undergoing resection for suspected lung tumor (either malignant or nonmalignant local carcinoma or hamartoma) or lung transplantation from the Department of Medicine and Pathology, Helsinki University Hospital, as described in our previous study (33). None of the patients had suffered from acute exacerbation for 2 mo. Tumor-free peripheral lung tissues were immediately stored at −80°C for immunohistological analysis and/or embedded in paraffin for immunohistochemistry. Spumus samples were obtained from all subjects and patients as previously described (37). The use of the tissues and spumus was approved by the ethics committee of the Helsinki University Hospital, Helsinki, Finland. All subjects and patients provided informed consent. The clinical characteristics of the subjects and patients used were described in detail previously (33, 37).

Animals

Heterozygous FoxO3 (FoxO3+/−) mice (FVB/129S6 Fdxo3tm1.1Rdp) were obtained from the Mutant Mouse Regional Resource Centers, the University of California at Davis (Davis, CA; stock no. 016132-UCD). The generation and genotyping of mice was performed as previously described (38). Wild-type (FoxO3−/−), on FVB/129S6 mixed background) and knockout (FoxO3−/−) littermates were housed in the Inhalation Core Facility at the University of Rochester before being exposed to air or CS. All experimental protocols were approved by the University Committee on Animal Research at the University of Rochester.

Generation of chimeras by bone marrow transplantation

Bone marrow transplantation (BMT) chimeras were generated by radioablation of recipient bone marrow followed by the reconstitution with donor bone marrow cells as previously described (39). In brief, the bone marrow was extracted from a minimum of three donors of the appropriate mouse strain by flushing femurs and tibias into HBSS with 1% FCS, dispersing through a 21-gauge needle, and pooling. Erythrocytes were removed by hypotonic lysis. The cells were counted, resuspended in media at 5 × 10^7 cells/ml, and delivered to the recipient mice by tail vein injection (1 × 10^7 cells/mouse). After BMT, animals were allowed to reconstitute for 8 wk under the microisolator conditions. The mortality rate by a failure of the engraftment posttransplantation was only 31.4% (16 dead mice/51 BMT trial mice), suggesting that the survived mice (68.6%) posttransplantation of bone marrow cells were donated by BMT (donor to recipient: WT to WT, WT to KO, KO to WT, and KO to KO) were exposed to air or CS for 3 d. Chimerism was confirmed by FoxO3 mRNA expression in peripheral blood cells and lung tissue using quantitative real-time PCR (Supplemental Table 1).

Quantification of FoxO3 expression in peripheral blood and lung tissues

Blood was collected by cardiac puncture from the air-exposed BMT chimeric mice. To perform cardiac puncture, mice were anesthetized by sodium pentobarbital (50 mg/kg, i.p.); then we accessed the right ventricle with a 23-gauge needle, and 400–500 μl blood was aspirated into a 3-ml syringe. Blood was immediately discharged into a 2-ml microtube tube precooled with 1.3 ml RNA later Tissue Collection RNA Stabilization Solution (Ambion, Austin, TX), mixed by inversion, and stored at −20°C. Mouse RiboPure-Blood RNA Isolation kit (Ambion, Austin, TX) was used for extraction of RNA. In brief, the samples were centrifuged and the RNA later solution removed before disruption of the blood pellet in a guanidinium-based lysis solution, followed by organic extraction and purification of total RNA fraction. RNA yields were determined by UV absorbance using a Nano Drop instrument (ND-1000 Spectrophotometer; NanoDrop Technologies). To validate the expression of FoxO3 in blood cells and lung tissues, we performed a quantitative real-time PCR by Bio-Rad iCycler real-time system using the SYBR Green PCR Master mix from SABiosciences (Fredrick, MD). Specific primers against FoxO3 (product no. PPM03393E) and 18S rRNA (PPM57735E) were purchased from SABiosciences. Expression of FoxO3 was normalized to 18s rRNA levels. RNA relative abundance was quantified by the comparative 2−ΔΔCt methods.

CS exposure

Mice were exposed to CS for 3 d and 8 wk using the Baumgartner-Jaeger CSM2082i cigarette smoking machine (CH Technologies, Westwood, NJ) and for 4 mo using the Teague TE-10 smoking machine (Teague Enterprises, Davis, CA) in the Inhalation Core Facility at the University of Rochester. For 3 d and 8 wk of CS exposure, mice were placed in individual compartments of a wire cage, which was placed inside a closed plastic box connected to the smoke source. The smoke was generated from 2RF research cigarettes containing 11.7 mg total particulate matter (TPM), 9.7 mg tar, and 0.76 mg nicotine per cigarette (University of Kentucky, Lexington, KY). Mice received two 1-h exposures per day, 1 h apart, according to the Federal Trade Commission protocol (1 puff/min of 2-s duration and 35-ml volume) for 3 d to 8 wk (40–42). Mainstream CS was diluted with filtered air and directed into the exposure chamber. The smoke exposure (TPM per cubic meter of air) was monitored in real time with a MicroDust Proaerosol monitor (Casella CEL, Bedford, U.K.) and verified daily by gravimetric sampling. The smoke concentration was set at a nominal value of ∼300 mg/m³ TPM by adjusting the number of cigarettes used to produce smoke and the flow rate of the dilution air. Control groups were exposed to filtered air in an identical manner for the same duration of time. Carbon monoxide concentration in the chamber was 290–300 parts per million. For 4 mo of CS exposure, mice received a continuous 5-h exposure per day, 5 d a week for 4 mo. Each lighted cigarette was puffed for 2 s and once every minute for a total of 6 puffs with the flow rate of 1.05 l/min, standard puff of 35 cm, the smoke machine was adjusted to produce a mixture of sidestream smoke (89%) and mainstream smoke (11%) by smoldering five cigarettes at one time, and the smoke chamber atmosphere was monitored for total suspended particulates (90 mg/m³) and carbon monoxide (350 ppm) (40, 43).

Mean linear intercept analyses

Mouse lungs (which had not been lavaged) were inflated by 1% low-melting agarose at a pressure of 25 cm H₂O and then fixed with 4% neutral buffered formalin. Tissues were embedded in paraffin and sectioned (4 μm). Lung sections were deparaffinized and rehydrated by passing through a series of xylene and graded alcohol, and were stained with H&E. Alveolar size was estimated from the mean linear intercept (Lm) of the airspace, which is a measure of airspace enlargement/emphysema (40, 42). Lm was calculated for each sample based on 10 random fields observed at a magnification of ×200 using a cross line.

Measurements of lung mechanical properties

Lung mechanical properties of mice were determined using Scireq Flexivent apparatus (Montreal, QC, Canada). Quasi-static compliance, lung resistance, and tissue elastance were measured in mice anesthetized by sodium pentobarbital (50 mg/kg i.p.) and paralyzed by pancuronium (0.5 mg/kg i.p.). A tracheotomy was performed, and an 18-gauge cannula was inserted 3 mm into an anterior nick in the exposed trachea and connected to a computer-controlled rodent ventilator. Initially, the mice were ventilated with room air (150 breaths/min) at a volume of 10 ml/kg body mass. After 3 min of ventilation, measurement of lung mechanical properties was initiated by a computer-generated program to measure quasi-static compliance, lung resistance, and tissue elastance (40, 44–46). These measurements were repeated three times for each animal.

Exercise performance tests

Exercise performance of air- and CS-exposed mice at 4 mo was assessed using a motorized rodent treadmill (Columbus Instruments, Columbus, OH). In brief, mice were placed on a motorized rodent treadmill with an electric grid at the rear to adapt and run on a rodent treadmill 2 d before conducting an exercise performance test. Familiarization runs were 10 min in duration with a treadmill incline of 10°. Treadmill speed on the first day was 10 m/min and then 12 m/min on the second day. For the performance test, mice were placed on the treadmill and allowed to adapt to the sur-
roundings for 3–5 min before starting. The treadmill was started at a speed of 8.5 m/min with a 0° incline. After 9 min, the speed and incline were raised to 10 m/min and 5°, respectively, for the second stage of the test. Speed was then increased by 2.5 m/min every 3 min to a maximum of 40 m/min, and the incline progressively increased 5° every 9 min to a maximum of 15°. Exercise was continued until exhaustion, defined as an inability to maintain running speed despite repeated contact with the electric grid. Each mouse was immediately removed from the treadmill when exhaustion had been determined and was returned to its home cage. Running time and running distance were measured for each mouse at the end of exercise performance test (47).

**Immunohistochemistry**

Lung sections were deparaffinized in xylene, rehydrated through graded alcohols to PBS, and then treated with 3% H2O2 for 10 min to quench endogenous peroxidase activity. To identify the localization of FOXO3 in human lung sections, we used Picture-Double Staining Kit (Invitrogen, Camarillo, CA) according to the manufacturer’s instructions. Immunohistochemistry for FOXO3 was performed using a rabbit polyclonal anti-FoxO3 Ab (Abnova, Taipei, Taiwan). Omitting the primary Ab served as negative control, which had no tissue staining. The assessment of immunostaining intensity was performed semiquantitatively (10 random microscopic fields per lung section in 3 different sections) and in a blinded fashion as described previously (33, 48). Macrophages in mouse lung sections were identified using a rat anti-mouse Mac-3 Ab (BD Pharmingen, Franklin Lakes, NJ), Vectastain ABC kit (rat IgG), and diaminobenzidine endogenous peroxidase activity. To identify the localization of FOXO3 in human lung sections, we used Picture-Double Staining Kit (Invitrogen, Carlsbad, CA), following the manufacturer’s instructions. The number of Mac-3+ cells in lung sections (six random microscopic fields per lung section in three different sections) was counted manually at 200 magnification and averaged (42).

**Bronchoalveolar lavage**

Mice were anesthetized at 24 h after the last exposure by an i.p. injection of pentobarbital sodium (100 mg/kg; Abbott Laboratories, Abbott Park, IL) followed by exsanguination. The lungs were lavaged three times with 0.7 ml saline via a cannula inserted into the trachea. The aliquots were combined and centrifuged, and the bronchoalveolar lavage (BAL) inflammatory cell pellet was resuspended in saline. The cells were stained with trypan blue (Invitrogen, Carlsbad, CA), and the total cell number was counted using a hemocytometer. Cytospin slides (Thermo Shandon, Pittsburgh, PA) were prepared using 50,000 cells/slide, and differential cell counts (∼500 cells/slide) were performed on cytospin-prepared slides stained with Diff-Quik (Dade Behring, Newark, DE).

**Proinflammatory mediator analysis**

The levels of proinflammatory mediators, such as cytokine chemottractant protein-1 (MCP-1), keratinocyte-derived chemokine (KC), and IFN-γ-inducible protein 10 (IP-10) in lung homogenates were measured by ELISA using corresponding duo-Ab kits (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions. The results were expressed in the samples as pg/mg protein.

**Assay of NF-κB DNA-binding activity**

NF-κB DNA-binding activity in nuclear extracts was measured using the Trans-AM RelA/p65 transcription factor assay kit (Active Motif, Carlsbad, CA), following the manufacturer’s instructions. In brief, 2.5 μg nuclear extracts was incubated in wells precoated with NF-κB consensus oligonucleotide. Plates were then washed before addition of anti-RelA/p65 Ab. An HRP Ab was used for signal detection and quantification. The absorbance was determined on a spectrophotometer (Microplate reader model 680; Bio-Rad) at 450 nm.

**IKK2 inhibitor administration**

IκB kinase 2 (IKKK) inhibitor IMD-0354 was purchased from Tocris (Ellisville, MO). Vehicle (0.5% carboxymethylcellulose) or IMD-0354 (30 mg/kg) was administered to mice by i.p. injection for 3 d daily at 2 h before CS exposure.

**Immunoblotting**

Cyttoplasmic and nuclear proteins (20 μg) from mouse lungs were separated on a 6.5–12% NaDodSO4–polyacrylamide gel by electrophoresis (SDS-PAGE). Separated proteins were transferred onto nitrocellulose membranes (Amersham, Arlington Heights, IL), and blocked for 1 h at room temperature with 5% BSA (Sigma-Aldrich). The membranes were then probed with a specific primary Ab (1:1000 dilution in 5% BSA in PBS containing 0.1% Tween 20) of acetylated lysine, GAPDH (Cell Signaling Technology, Beverly, MA), FoxO3, manganese superoxide dismutase (MnSOD; Upstate, Temecula, CA), catalase, β-actin (Sigma), and CuZnSOD (Assay Designs, Ann Arbor, MI) at 4°C overnight. After three washing steps (10 min each), the levels of protein were detected by probing with secondary anti-rabbit or anti-mouse Ab (1:10,000 dilution in 5% BSA) linked to HRP for 1 h, and bound complexes were detected using the ECL method (Perkin Elmer, Waltham, MA). Equivalent loading of the gel was determined by quantitation of protein, as well as by reprobing the membranes for β-actin or GAPDH. The ImageJ densitometry software (Version 1.41; National Institutes of Health, Bethesda, MD) was used for gel band quantitative densitometric analysis.

**Cell culture**

The human bronchial epithelial cell line H292 was purchased from the American Type Tissue Culture Collection (Manassas, VA), H292 cells were grown in 100-mm dishes (4 × 106 cells), containing RPMI 1640 supplemented with 10% FBS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 100 μg/ml penicillin, and 100 U/ml streptomycin in humidified atmosphere under 7.5% CO2 at 37°C.

**Preparation of CS extract**

We used the same cigarettes that were used in exposure of mice to CS. CS extract (CSE) was prepared by bubbling smoke from one cigarette into 10 ml serum-free RPMI 1640 medium at a rate of 1 cigarette/min, as described previously (49–51). The pH of the CSE was adjusted to 7.4, and it was sterile-filtered through a 0.45-μm filter (25-mm Acrodisc; Pall Corporation, Ann Arbor, MI). CSE preparation was standardized by measuring the absorbance (OD: 1.00 ± 0.05) at a wavelength of 320 nm. The pattern of absorbance (spectrogram) observed at 320 nm showed little variation between different preparations of CSE. CSE was freshly prepared for each experiment and diluted with culture media supplemented with 10% FBS immediately before use. Control medium was prepared by bubbling air through 10 ml serum-free RPMI 1640 medium, adjusting pH to 7.4, and sterile filtering as described earlier.

**Immunocytochemistry**

The cells treated with or without indicated concentration of CSE for 24 h or H2O2 for 1 h were fixed in acetone/methanol (1:1, v/v) at −20°C for 20 min. The cells were incubated with 10% normal goat serum and then stained with FoxO3 Ab (1:500; Upstate, Temecula, CA) followed by 1-h incubation with Alexa 594-labeled secondary Ab (1:1000; Molecular Probes, Eugene, OR). Anti-fade DAPI Fluoromount-G (Southern Biotech, Birmingham, AL) was used for nuclei staining and mounting. Images were captured using a fluorescent microscope (BX51; Olympus Optical, Tokyo, Japan).

**Immunoprecipitation**

Immunoprecipitation was performed with 2 μg Abs in 300 μl nuclear extracts or 1 mg whole-tissue lysates. Abs were incubated with protein conjugated at 4°C on a rocker. Protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added to each sample at 4°C on a rocker for 1 h. The samples were then centrifuged at 13,000 × g at 4°C for 5 min. The supernatant was discarded, and the beads were washed five times and then subjected to immunoblot as described earlier.

**RT-PCR**

Total RNA was isolated from nonlavaged lung tissue specimens (stored in RNAlater; Ambion, Austin, TX) using RNAeasy kit (Qiagen, Valencia, CA). RT-PCR was performed using oligo(dT) primers and superscript reverse transcriptase (Invitrogen Life Sciences), following the manufacturer’s instructions. The PCR conditions and the primer pairs were as described: MnSOD and CuZnSOD, 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s during 30 cycles; catalase, 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s during 28 cycles; GAPDH, 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s during 30 cycles; and actin, 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s during 32 cycles with a final extension for 10 min at 72°C in a PTC-200 DNA thermal cycler (MJ Research, Waltham, MA). The primer pairs were as follows (forward and reverse, respectively): MnSOD, 5′-AGCGTGGCTGTAACACTCA-3′ and 5′-AGCATGGCTGTCAGAGTTC-3′; CuZnSOD, 5′-ATCCACTCTGGACGAGAAG-3′ and 5′-TTCACCTTCGGCCAAATG-3′; catalase, 5′-AACTCCATACCATGTCGACA-3′ and 5′-CGCTGTTGTAGGAAGGCCGTC-3′; GAPDH, 5′-ACGACCCCTTCTGAGCAGG-3′ and 5′-CCTCCTGCTGTAGAATA-3′. Amplified products were resolved by 1.5% agarose gel electrophoresis, stained with SYBR Safe dye (Invitrogen), visualized and scanned by a white/UV transilluminator, and quantified by densitometry.

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Statistical analysis

Data were presented as mean ± SEM. Statistical analysis of significance was calculated using one-way ANOVA followed by Tukey’s post hoc test for multigroup comparisons using StatView software. A p value <0.05 was considered significant.

Results

FOXO3 is downregulated in lungs of patients with COPD and in lungs of mice exposed to CS

To determine the possible involvement of FOXO3 in pathogenesis of COPD, the abundance of FOXO3 was determined in peripheral lung samples from nonsmokers, smokers, and patients with COPD by immunohistochemistry and immunoblot analysis. Immunohistochemical staining of fixed peripheral lung tissues showed that FOXO3 was predominantly localized in airways/alveolar epithelium in nonsmokers, which was decreased both in lungs of smokers and patients with COPD (Fig. 1A, 1B). The levels of FOXO3 were significantly decreased in peripheral lung tissues of smokers and were further reduced in patients with COPD as compared with nonsmokers as shown by immunoblotting (Fig. 1C, 1D). In sputum cells, which mainly consist of macrophages and neutrophils, the levels of FOXO3 were also significantly reduced in smokers and patients with COPD (Fig. 1E, 1F). A similar result for FOXO3 staining was also confirmed in tissue macrophages by immunohistochemistry staining in serial lung sections of patients with COPD (93%) and smokers (84%) versus nonsmokers (11%; data not shown). By immunoprecipitation assay, we found that acetylation of FOXO3 was increased in lungs of smokers (Fig. 1G, 1H).

The levels of FOXO3 were significantly lower in lungs of CS-exposed WT mice than that of air-exposed WT mice (Fig. 2A, 2B). The reduction in levels of FOXO3 was more pronounced in CS-exposed WT mice after 4 mo of CS exposure compared with those at 3 d of CS exposure. Furthermore, CS caused acetylation of FOXO3 both at 3 d and 4 mo of CS exposures in mouse lungs (Fig. 2C, 2D). These results suggest that FOXO3 is downregulated and acetylated in response to CS.

FoxO3 deficiency results in airspace enlargement/emphysema and alters lung mechanical properties in mice in response to 4 mo of CS exposure

To investigate the potential effect of FoxO3 on CS-induced air-space enlargement/emphysema, FoxO3−/− and WT littermates were exposed to CS for 3 d, 8 wk, and 4 mo. We then determined the susceptibility of FoxO3−/− mice for CS-induced airspace enlargement/emphysema by lung histopathological and functional measurements, and exercise performance test. There was no significant difference in lung histopathology by H&E staining between air- or CS-exposed WT and FoxO3−/− mouse lungs at 3 d (Fig. 3A) and 8 wk (Fig. 3B) of CS exposures, which was assessed by determining the Lm (Fig. 3D, 3E). However, 4 mo of CS exposure led to significantly increased airspace enlargement as measured by Lm in the lungs of CS-exposed FoxO3−/− mice as compared with CS-exposed WT mice (Fig. 3C, 3F). CS exposure at 4 mo did not cause airspace enlargement in WT mice. Similarly, the lung resistance and elastance were significantly decreased in FoxO3−/− mice exposed to CS for 4 mo, whereas the static lung compliance was not altered.

FIGURE 1. FOXO3 is downregulated in lungs of smokers and patients with COPD. A, Abundance and localization of FOXO3 in lung alveolar/airway epithelial cells of nonsmokers, smokers, and patients with COPD, as shown by immunohistochemical staining. Red represents the presence of FOXO3 (arrow), which was decreased in lungs of smokers and patients with COPD. Original magnification × 200. B, Immunostaining scores for FOXO3 per cell type in alveolar and airway regions of the lung. The assessment of immunostaining intensity was performed semiquantitatively and in a blinded fashion. Immunoblot analysis of FOXO3 in whole-tissue lysates extracted from the lung tissue (C) and sputum cells (E) of nonsmokers, smokers, and patients with COPD. After densitometric analysis, the values of FOXO3 in lung tissue (D) and sputum cells (F) were normalized against β-actin (loading control). The relative levels of FOXO3 were significantly decreased in lung tissues of smokers and patients with COPD compared with nonsmokers. G, Whole-tissue lysates extracted from the lung tissues of nonsmokers, smokers, and patients with COPD were immunoprecipitated with anti-FoxO3 Ab, and immunoprecipitates were subjected to immunoblot and probed with anti-acetylated lysine Ab. H, Relative intensity of acetylated lysine/FoxO3 represents the increased acetylation of FOXO3 in lungs of smokers. Data are shown as mean ± SEM (n = 3–4 per group). *p < 0.05, **p < 0.01, ***p < 0.001, significant compared with nonsmokers; #p < 0.05, significant compared with smokers. Alv, alveoli; Aw, airway; (−) control, negative control that was stained without primary Ab; E, epithelial cells.
compliance was significantly increased in these mice as compared with WT mice exposed to 4 mo of CS (Fig. 4A). A similar trend in lung mechanical properties was observed in FoxO3−/− mice exposed to 6 mo of CS (data not shown). In addition, FoxO3−/− mice exposed to CS for 4 mo showed a significant reduction in exercise performance (i.e., running time and distance) compared with CS-exposed WT mice (Fig. 4B). WT mice exposed to CS for 4 mo also showed a slight reduction in exercise performance. The exercise performance in the CS-exposed FoxO3−/− mice was persistently decreased at 6 mo of CS exposure (data not shown). There was no statistically significant difference in body weight or mortality between air-exposed WT and FoxO3−/− mice up to 6 mo of CS exposure (data not shown). These data are consistent with a previous report that showed no significant differences in body weight and mortality up to 48 wk of age in FoxO3−/− mice (38). Taken together, these data suggest that FoxO3 deficiency leads to an increased susceptibility to chronic CS-induced airspace enlargement/emphysema.

**FIGURE 2.** FoxO3 is downregulated in lungs of mice in response to CS. A. Immunoblot analysis of FoxO3 in whole-tissue lysates extracted from lungs of mouse exposed to CS for 3 d and 4 mo. B. After densitometric analysis, the values of FoxO3 were normalized against β-actin (a loading control). C. Nuclear fraction from lungs of mouse exposed to CS for 3 d and 4 mo were immunoprecipitated with anti-FoxO3 Ab, and immunoprecipitates were subjected to immunoblot analysis and probed with anti–acetyl-lysine Ab. IgG was used as an isotype control. D. The relative density of acetylated FoxO3 showed increased acetylation of FoxO3 in CS-exposed mouse lungs at 3 d and 4 mo. A representative blot is shown. Data are shown as mean ± SEM (n = 3–4 per group). **p < 0.001, significant compared with air-exposed WT mice.

**FIGURE 3.** Increased airspace enlargement in lungs of FoxO3−/− mice exposed to CS for 4 mo. H&E-stained lung sections from WT and FoxO3−/− mice exposed to air or CS for 3 d (A), 8 wk (B), and 4 mo (C) are shown. Arrows indicate airspace enlargement. A–C, Original magnification ×200. Lm was analyzed in H&E-stained slides (D, 3 d; E, 8 wk; F, 4 mo). Lung sections from FoxO3−/− mice exposed to CS for 4 mo show an increased airspace enlargement when compared with CS-exposed WT mice. Data are shown as mean ± SEM (n = 3 per group). **p < 0.001, significant compared with corresponding air-exposed mice; **p < 0.001, significant compared with CS-exposed WT mice.
FoxO3-deficient mice show an increased susceptibility to lung inflammation in response to CS exposure

CS induces the lung inflammatory response, which is associated with development of emphysema in the mouse (1, 49, 53). To determine the susceptibility of FoxO3−/− mice to CS-induced lung inflammation, the inflammatory cell influx into the BAL fluid and lung tissue was assessed using the Diff-Quik and immunohistochemical stainings, respectively. There was no significant change in total cell numbers in BAL fluids after 3 d and 8 wk of CS exposures in any group (Fig. 5A, 5B), but CS exposure for 4 mo resulted in a significant increase in total cell numbers in BAL fluid of WT mice, which was further augmented in FoxO3−/− mice (Fig. 5C). In both 3 d and 8 wk of CS exposures, increased neutrophil influx in BAL fluid was observed (Fig. 5D, 5E), whereas no change in neutrophil influx in BAL fluid was seen in WT mice exposed to CS for 4 mo (Fig. 5F). Nevertheless, CS-exposed FoxO3−/− mice showed a significant increase in neutrophil influx in BAL fluid as compared with WT mice exposed to CS for 3 d, 8 wk, and 4 mo (Fig. 5D–F). Similarly, CS exposures for 3 d (Fig. 6A, 6D), 8 wk (Fig. 6B, 6E), and 4 mo (Fig. 6C, 6F) increased the macrophage infiltration in lung interstitium of WT mice, which was further augmented in corresponding CS-exposed FoxO3−/− mice. The increased inflammatory cell recruitment in lungs of CS-exposed FoxO3−/− mice was persistently increased at
6 mo of CS exposure (data not shown). In addition, the deficiency in FoxO3 intensified the release of proinflammatory mediators, such as MCP-1, KC, and IP-10, triggered by CS exposure for 3 d (Fig. 7A), 8 wk (Fig. 7B), and 4 mo (Fig. 7C). These data suggest that FoxO3-deficient mice show increased susceptibility to lung inflammatory response by CS exposure, which may...
contribute to enhanced airspace enlargement/emphysema in these mice.

Both hematopoietic and nonhematopoietic cells are responsible for enhanced lung inflammatory response in FoxO3-deficient mice

To identify the cells responsible for the altered response to CS exposure in FoxO3−/− mice, BMT chimeras were generated by radioablation, and the reconstituted mice (donors to recipients; WT to WT, WT to KO, KO to WT, and KO to KO) were exposed to air or CS for 3 d at 8 wk after BMT. All CS-exposed chimeras showed increased susceptibility to CS-induced lung inflammation compared with air-exposed chimeras, and the effect was augmented in CS-exposed KO to KO (FoxO3 deficient in both hematopoietic and structural cells) chimeras (Table I), WT to KO (FoxO3 deficient in only lung structural cells) and KO to WT (FoxO3 deficient in only hematopoietic cells) chimeras showed increased inflammatory response to CS exposure as compared with WT to WT (FoxO3 expressed in both hematopoietic and structural cells) chimeras, but it was lower than KO to KO chimeras, suggesting that both the structural and hematopoietic cells are responsible for the altered responses seen by CS exposure in FoxO3−/− mice.

Deficiency of FoxO3 leads to augmented NF-κB activation because of loss of its interaction with RelA/p65 in lungs of mice in response to CS

Given that NF-κB plays a crucial role in expression of proinflammatory genes in response to diverse stimuli (40, 42), we speculated that FoxO3−/− mice might show augmented NF-κB activity in response to CS. To test this contention, we performed the Trans-AM RelA/p65 DNA-binding assay and found that 3 d of CS-exposed FoxO3−/− mice showed a 1.4-fold increase in RelA/p65 DNA-binding activity as compared with CS-exposed WT mice (Fig. 8A). However, there was no significant difference in RelA/p65 DNA-binding activity between air-exposed WT and FoxO3−/− mice. The total and acetylated levels of RelA/p65 at Lys310 residue in lung nuclear fractions were evaluated, but no detectable difference was seen between CS-exposed WT and FoxO3−/− mice exposed to CS for 3 d (Supplemental Fig. 1A, 1B).

To examine the possibility that FoxO3 might inhibit RelA/p65 DNA-binding activity through their interaction in the nucleus, we determined whether FoxO3 and RelA/p65 could form a complex in the nucleus. By immunoprecipitation assay, it was found that CS exposure increased the interaction of FoxO3 with RelA/p65 (Fig. 8B), which was further confirmed by reciprocal immunoprecipitation (Fig. 8C). Consistent with these results, this interaction was also observed in the nuclear fractions of CSE-treated human bronchial epithelial cells (Fig. 8D). Furthermore, we found CS-induced nuclear translocation of FoxO3 in vivo in mouse lungs and in vitro in lung epithelial cells (Supplemental Fig. 2A–C). Taken together, these results indicate that FoxO3 is translocated into the nucleus where it interacts with RelA/p65 in response to CS, resulting in inhibition of NF-κB DNA-binding activity.

To further validate the role of increased NF-κB activity in enhanced lung inflammation of FoxO3−/− mice, both WT and FoxO3−/− mice were administered with an IKK2 inhibitor, IMD-0534 (Tocris, Ellissippi, MO), as previously described (40). The administration of IKK2 inhibitor resulted in attenuation of CS-mediated proinflammatory cytokine release in WT and FoxO3−/− mice (Supplemental Fig. 3). Interestingly, there was a 14% reduction in KC levels of WT mouse lungs in response to CS, but a marked reduction (34%) was seen in FoxO3−/− mice (p < 0.05). These data suggest that the CS-induced heightened lung inflammation in FoxO3−/− mice is, at least in part, mediated by increased NF-κB activity.

Antioxidant genes are regulated by FoxO3

FoxO3 plays a role in detoxification of cellular reactive oxygen species (ROS) levels through the regulation of antioxidant gene transcription in response to oxidative stress (22). We hypothesized that deficiency of FoxO3 leads to transcriptional suppression of antioxidant genes, resulting in reduction in detoxification of ROS in response to CS. We determined the mRNA and protein expression levels of MnSOD and catalase, which are well-known target stress genes that are transcriptionally regulated by FoxO3. After 3 d of CS exposure, both mRNA expression and protein abundance of MnSOD and catalase were increased in CS-exposed WT mice as compared with air-exposed WT mice, whereas CS-exposed FoxO3−/− mice showed a significant reduction in mRNA and protein levels of MnSOD and catalase as compared with CS-exposed WT mice (Fig. 9A, 9B). At 4 mo of CS exposure, the upregulated levels of MnSOD and catalase were reduced in CS-exposed WT mice as compared with 3 d of CS exposure for WT mice. There were no significant changes in MnSOD and catalase mRNA levels between WT and FoxO3−/− mice at 4 mo of CS exposure (Fig. 9A, 9B). Expression levels of CuZnSOD, which was used for nontarget control, showed no difference between WT and FoxO3−/− mice even though the level was increased in response to CS exposure. These data suggest that the absence of FoxO3 reduces the expression of antioxidant genes in response to CS, resulting in imbalance between oxidants and antioxidants in the lungs.

Discussion

FoxO3 has been shown to regulate expression of several genes involved in inflammation, oxidative stress resistance, and cellular senescence, which are intertwined in the pathogenesis of COPD. However, the role of FOXO3 in lung inflammation and pathogenesis of COPD/emphysema is not known. We found that the abundance of FOXO3 was decreased in lungs of smokers, and even more prominently reduced in patients with COPD compared with nonsmokers. FoxO3 was also reduced in mouse lungs in response to CS exposure, which is a major risk factor in development of COPD. Furthermore, the deficiency in FoxO3 increased the susceptibility to CS-induced emphysema, which was reflected by airspace enlargement, impaired lung function, and decreased

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>WT to WT</th>
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<tr>
<td>KC</td>
<td>70.8 ± 4.01</td>
<td>71.3 ± 0.82</td>
<td>65.4 ± 4.86</td>
<td>87.7 ± 5.88</td>
<td>103.2 ± 4.91**</td>
<td>129.6 ± 2.16***††</td>
<td>129.2 ± 10.78***††</td>
<td>165.6 ± 11.93***†††</td>
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<tr>
<td>MCP-1</td>
<td>9.86 ± 0.52</td>
<td>10.5 ± 0.58</td>
<td>8.7 ± 1.75</td>
<td>9.2 ± 0.66</td>
<td>14.2 ± 1.24*</td>
<td>17.4 ± 1.77**</td>
<td>20.1 ± 1.68***†</td>
<td>25.9 ± 4.07***†††</td>
</tr>
</tbody>
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n = 3, mean ± SE.

*p < 0.05, **p < 0.01, ***p < 0.001, significant compared with corresponding air-exposed controls; †p < 0.05, ††p < 0.01, †††p < 0.001, significant compared with CS-exposed WT to WT chimeras.
exercise performance. All these findings suggest CS-induced re-
donduction of FOXO3 is a key contributing factor in development of COPD/emphysema. The mechanism underlying CS-induced re-
donduction of FoxO3 is unclear, but it may be associated with its posttranslational modifications, such as acetylation and phos-
phorylation. This is corroborated by the findings that increased acetylation of FOXO3 occurred in lungs of smokers and mice ex-
posed to CS.

Sustained inflammation is a characteristic feature in pathogen-
thesis of COPD/emphysema, and FoxO3 is known to be involved in
augmented inflammatory responses (18, 54). Therefore, we de-
etermined whether susceptibility of FoxO3−/− mice to development of emphysema is associated with increased lung inflammatory response. CS exposure increased inflammatory cell infiltration into the lungs accompanied with proinflammatory cytokine release, including MCP-1, KC, and IP-10 in WT mice, which was more pronounced in lungs of FoxO3−/− mice. This finding is consistent with a previous study showing that an enhanced inflammatory re-
sponse occurred in intestinal tissue of FoxO3−/− mice challenged with dextran sulfate sodium as compared with WT mice (55).

FIGURE 8. Deficiency of FoxO3 leads to augmented NF-κB–DNA-binding activity because of loss of its interaction with RelA/p65 in lungs of mice exposed to CS. A, NF-κB–DNA binding was measured by the Trans-AM transcription factor RelA/p65 ELISA kit in nuclear proteins from CS-exposed mouse lungs for 3 d. Data are shown as mean ± SEM (n = 3–4 per group). B, Nuclear fraction from mouse lungs exposed to CS for 3 d were immu-
noprecipitated (IP) with anti-RelA/p65 Abs. C, Reciprocal immunoprecipitation of nuclear fraction from mouse lungs exposed to CS for 3 d was performed with anti-FoxO3 Abs, followed by immunoblot analysis. D, H292 epithelial cells were treated with CSE (1%), and nuclear fractions from cell lysates were used for immunoprecipitation with anti-RelA/p65 Ab. IgG was used as an isotype control. Immunoprecipitates were subjected to immunoblot analysis and probed with anti-FoxO3 or anti-RelA/p65 Ab. *p < 0.05, **p < 0.01, significant compared with corresponding air-exposed mice; p < 0.05, significant compared with CS-exposed WT mice.

FIGURE 9. Antioxidant genes are regulated by FoxO3. The levels of MnSOD and catalase were measured by RT-PCR (A) and immunoblot (B) at the indicated time points of CS exposures. CuZnSOD was used as a nonspecific target protein of FoxO3, and GAPDH was used for loading controls. After densitometric analysis, the values of MnSOD and catalase were normalized against GAPDH. Gel images shown are representative of at least three separate experiments. Data are shown as mean ± SEM (n = 3–4 per group). *p < 0.05, **p < 0.01, ***p < 0.001, significant compared with corresponding air-exposed mice; ****p < 0.001, significant compared with CS-exposed WT mice.
Thus, increased inflammatory response may contribute to susceptibility in development of emphysema in FoxO3−/− mice.

FoxO3 was predominantly localized in both lung airway/alveolar epithelium and macrophages in nonsmokers, and was significantly decreased in all locations in lungs of smokers and patients with COPD. These findings raise a question as to which specific cell type is responsible for CS-induced pathological changes (e.g., inflammation and cellular senescence) in lungs of FoxO3−/− mice. We, therefore, generated the BMT chimeric mice to distinguish the role of FoxO3 in lung epithelial cells (radioresistant non-hematopoietic-derived structural cells) and inflammatory cells (radioresistant hematopoietic-derived cells) in CS-induced lung inflammation. It was found that CS-induced release of proinflammatory cytokines was increased in mice deficient for FoxO3 in hematopoietic or nonhematopoietic cells as compared with the mice expressing FoxO3 in these cells. Deficiency in FoxO3 in both the cells further increased the release of proinflammatory mediators in response to CS exposure, suggesting that both structural cells (mainly epithelial cells) and hematopoietic cells (mainly macrophages and neutrophils) are responsible for the altered responses to CS exposure seen in FoxO3−/− mice. Although it has been reported that simultaneous conditional deletion of FoxO1, FoxO3, and FoxO4 results in long-term defect of hematopoietic stem cell (56), Miyamoto et al. (57) have demonstrated that young adult FoxO3−/− mice (8–12 wk old) show normal proliferation and differentiation of hematopoietic progenitors, which is supported by normal morphology and cell numbers in lymphoid and myeloid cells in peripheral blood and bone marrow. Consistent with the latter observation, we did not find any differences in differential cell counts from myeloid cell-derived neutrophils and macrophages in air-exposed FoxO3−/− and BMT chimeric mice. A recent study has also shown that the loss of FoxO3 alone does not alter intrinsic phenotype and activation of T cells (18). Although the lymphoid cell population in BMT chimeric mice was not investigated, CD8+ T cell product, IP-10 levels were similar among the air-exposed chimeric mice (data not shown), possibly suggesting that lymphoid-lineage cells of hematopoiesis are not impaired in FoxO3−/− mice. As evidenced by a significant increase of IP-10 levels (a specific chemotactant for activated T cells), shown in chronic CS exposure, BMT chimeric mice exposed to chronic CS may broaden the understanding of the role of FoxO3 in regulation of immune response by CS exposure and in pathogenesis of COPD.

It has been shown that CS induces lung inflammation, which is accompanied by NF-κB activation in experimental mouse model, and IKK, which is implicated in activation of NF-κB, regulates FoxO3 activity (25, 40, 42, 58). Therefore, we postulated that increased susceptibility to CS-induced inflammation in FoxO3−/− mice was due to exaggerated increase in NF-κB activity. Interestingly, CS exposure led to increased DNA binding activity of NF-κB (RelA/p65) in WT mice, which was augmented in FoxO3−/− mice. These data suggest that NF-κB activity is inhibited by FoxO3, which is consistent with a previous report that FoxO3 can function as NF-κB antagonist and inhibit its activation (36). In addition, we demonstrated the FoxO3–RelA/p65 interaction in response to CS in lungs in vivo and in epithelial cells in vitro. It may be possible that FoxO3–RelA/p65 interaction specifically affects RelA/p65 activity by blocking its DNA binding in response to CS. Recent data showed that the N-terminal region of FoxO4 interacts with the Rel-homology domain of NF-κB, which was confirmed by a series of deletion mutants of FoxO4 and NF-κB (59). Because forkhead DNA-binding domain was included in the N-terminal region of FoxO4, as well as FoxO3, FoxO3 may bind directly with NF-κB through its forkhead do-

main. However, further studies are required to identify the interactive domain between FoxO3 and NF-κB, and their involvement in control of inflammatory processes.

It has been shown that RelA/p65 translocated into the nucleus in response to oxidative stress and CS, we therefore speculated that CS also induced nuclear translocation of FoxO3 to interact with RelA/p65. Brunet et al. (30) reported that FoxO3 was localized in cytoplasm when growth factors were present and was translocated into the nucleus in response to oxidative stress. Consistent with this observation, CS/oxidative stress promoted FoxO3 translocation into the nucleus in vivo in lung cells and in vitro in bronchial epithelial cells. Although the phosphorylation of FoxO3 causes its proteolysis via the ubiquitin–proteasome pathway in the cytoplasm (25), a recent study reported that oxidative stress-induced dephosphorylation of FoxO3 promoted nuclear translocation of FoxO3 without affecting the total FoxO3 levels (60). Similarly, CSE also induced dephosphorylation of FoxO3 in a time-dependent fashion in bronchial epithelial cells (data not shown), which was accompanied with nuclear translocation of FoxO3. The mechanism underlying CS-induced dephosphorylation and translocation of FoxO3 into the nucleus is not known. However, it may be possible that CS-induced dephosphorylation of FoxO3 is closely related with its translocation into the nucleus.

Oxidative stress caused by imbalance between oxidants and antioxidants is involved in pathogenesis of chronic pulmonary diseases (61). It has been reported that FoxO3 plays a role in regulation of cellular ROS levels through modulation of transcription of antioxidant genes (22, 62, 63). In this study, we showed that the mRNA and protein expression of MnSOD and catalase, which are well-known specific target genes of FoxO3, were increased in CS-exposed WT mice, whereas the levels were significantly reduced in CS-exposed FoxO3−/− mice at 3 d. Despite reduction of FoxO3 in lungs of CS-exposed WT mice, the expression of its target MnSOD and catalase was increased in CS-exposed WT mice. This discrepancy may be caused by increased acetylation of FoxO3 against acute lung inflammation. We showed that acetylation of FOXO3 was increased in lungs of smokers, as well as in lungs of mice exposed to CS, suggesting alteration in transactivation of FoxO3 for target genes, such as MnSOD and catalase. CS-induced oxidant stress may potentially modify FoxO3 via CREB-binding protein-mediated acetylation, which is shown to be the case for FoxO4 (64) and SIRT1-mediated deacetylation (30). We have recently shown that the levels of SIRT1 are reduced in lungs of smokers and patients with COPD, as well as in mouse lungs (33, 35), which can result in acetylation of FoxO3. Transcriptional activity of FoxO3 is modified mainly by posttranslational modifications and association with many different cofactors (65). Although many studies have described that posttranslational modifications, such as phosphorylation and acetylation, of FoxO3 lead to the repression of its transcriptional activity, some reports have suggested that acetylation of FoxO3 increases the target gene transcription (30, 66). Therefore, it is possible that CS-induced acetylation of FoxO3 enhances the transcription of antioxidants genes to counteract oxidative stress. In 4 mo of CS exposure, however, the expression of antioxidant genes was reduced both in CS-exposed WT and FoxO3−/− mice because of the loss of FoxO3 levels per se. Altogether, our data suggest that acetylation of FoxO3 may enhance transcription of antioxidant genes, but further study is required to assess the role of acetylation and deacetylation of FoxO3 in regulation of MnSOD and catalase under the condition of oxidative stress.

In conclusion, we show a novel role of FoxO3 in regulation of lung inflammation and in pathogenesis of COPD/emphysema. The abundance of FOXO3 was decreased in lungs of patients with
COPD. Furthermore, genetic ablation of FoxO3 in mice led to increased susceptibility to airspace enlargement/emphysma in response to CS, in association with exaggerated inflammatory response and decreased antioxidant genes in the lungs. We further provide a novel mechanistic link between FoxO3 and NF-κB both in vivo and in vitro, and suggest that FoxO3 acts as a fine-tuner that modulates CS-induced lung inflammatory response and COPD/emphysma. In addition, it may be possible that increased cellular senescence and premature lung aging, which are important events in the pathogenesis of COPD, are regulated by FoxO3 via SIRT1 deacetylation. Therefore, further studies are required to expand our understanding of the role of FoxO3 in the pathogenesis of COPD/emphysma, particularly with respect to regulation of antioxidant defense mechanisms and stress-induced premature cellular senescence in the lungs.

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Disclosures

The authors have no financial conflicts of interest.

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FoxO3 REGULATES LUNG INFLAMMATION AND EMPHYSEMA


