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Smoking, aging, and expression of proteins related to the FOXO3 signaling pathway in lung tissues

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ABSTRACT. We investigated the effects of smoking and aging on proteins involved in the forkhead box O3 (FOXO3) signaling pathways in the lungs. Sixteen senescence-accelerated mouse-resistant 1 (SAMR1) and senescence-accelerated mouse-prone 8 (SAMP8) mice at 3 months of age were divided into a normally aged, smoke-exposed group (4 SAMR1 mice), a normally aged, air-exposed group (4 SAMR1 mice), an aging-accelerated, smoke-exposed group (4 SAMP8 mice), and an aging-accelerated, air-exposed group (4 SAMP8 mice). Expression of genes and proteins related to the FOXO3 signaling pathways in each group was examined by western blot analysis and immunohistochemistry. FOXO3a expression was significantly increased in the normally aged, air-exposed group compared with the aging-accelerated, air-exposed group. FOXO3a expression was significantly reduced in the aging-accelerated, smoke-exposed group compared with the aging-accelerated, air-exposed group. Sirtuin 1, manganese superoxide dismutase, and phosphatidylinositol 3-kinase (PI3K)/Akt expression de-

creased significantly in the smoke-exposed groups compared with the air-exposed groups and in the aging-accelerated groups compared with the normally aged groups. Signal transduction pathways mediated by the transcription factor FOXO3a (such as the PI3K/Akt pathway) may be involved in the accelerated aging of lung tissues in chronic obstructive pulmonary disease. Smoking inactivates the transcription factor FOXO3, thus accelerating lung tissue aging during chronic obstructive pulmonary disease.

Key words: Aging; FOXO3; PI3K/Akt; Signaling pathway; Smoking

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a common disease with a high incidence rate that is significantly associated with disability and mortality (Liu et al., 2014; Erdem, 2014). In developing countries such as China, the high incidence of COPD is a major public health concern (Zhou and Chen, 2013; Zhang et al., 2013). COPD is correlated with various factors, including infection, genetics, and environmental exposure (particularly cigarette smoke). Epidemiological investigations have shown that COPD is significantly more prevalent in the elderly and in smokers than in the general population (Yang et al., 2013; Liu et al., 2014). In adults aged 40 years or more, COPD incidence increases significantly with increasing age, and age itself is a risk factor for COPD, independently of the number of cigarettes smoked per year (Burney et al., 2013; Daldoul et al., 2013). In lung tissues, peripheral blood mononuclear cells and alveolar macrophages from patients with COPD exhibit decreased expression of a variety of longevity- and aging-related molecules, including sirtuin 1 (SIRT1) and sirtuin 6, which belong to the class III histone deacetylase family, histone deacetylase 2, which belong to the class I histone deacetylases, senescence marker protein 30, and klotho (Yao et al., 2013; Hwang et al., 2014). These proteins are thought to exert anti-aging effects, suggesting that longevity- and aging-related signaling pathways are related to pathophysiological changes in COPD.

Forkhead box class O (FOXO) transcription factors constitute a subfamily within the highly conserved forkhead box (FOX) family (Moriishi et al., 2014; Estevez et al., 2014). In mammals, the FOXO subfamily includes at least 4 members, FOXO1, FOXO3a, FOXO4, and FOXO6 (Monsalve and Olmos, 2011). FOXO proteins are transcription factors that regulate the expression of numerous target genes related to cell cycle progression, apoptosis, longevity and aging, stress response, and metabolism. FOXO proteins are thought to be related to various aging-related signal transduction pathways, including the Akt (Wimmer et al., 2014), c-Jun N-terminal kinase (van den Berg et al., 2013), and IκB kinase pathways (Salminen and Kaarniranta, 2010), which play key roles in the regulation of aging and longevity. However, the contribution of the FOXO subfamily proteins to age-related changes in lung tissue and to senile emphysema remains unclear.

Pulmonary symptoms (such as pulmonary emphysema) and systemic symptoms (including increased facial wrinkles, cardiovascular diseases, osteoporosis, and increased risk of cancer) of COPD are very similar to the clinical manifestations of aging. Therefore, we hypothesized that the energy metabolism and aging-related signal transduction pathways mediated by the transcription factor FOXO3a are also involved in accelerated lung aging in COPD patients.

MATERIAL AND METHODS

Experimental animals and grouping

Sixteen 3-month-old senescence-accelerated mouse-resistant 1 (SAMR1) and senescence-accelerated mouse-prone 8 (SAMP8) mice were divided into the following groups: a normally aged, smoke-exposed group, which included 4 SAMR1 mice; a normally aged, air-exposed group, which included 4 SAMR1 mice; an aging-accelerated, smoke-exposed group, which included 4 SAMP8 mice; and an aging-accelerated, air-exposed group, which contained 4 SAMP8 mice. As previously described (Hu et al., 2013; Vlahos and Bozinovski, 2014), an animal model of COPD was established in the aging-accelerated mouse model, so that aging-accelerated mice were exposed to cigarette smoke for 8 weeks in a computer-controlled cigarette smoking system.

Preparation of histopathological sections of the lung tissues

Tissue samples were collected from the medial and outer one-third of the lung along the sagittal plane. Tissue samples were fixed, paraffin-embedded, and sliced into 4-μm sections. The tissue sections were de-waxed in xylene and rehydrated in a graded series of ethanol and water. The sections were stained with hematoxylin for 5 min, rinsed with tap water, differentiated in hydrochloric acid-ethanol for 30 s (a few dips in acid alcohol), immersed in tap water for 15 min, and incubated with eosin solution for 2 min. After staining, the sections were conventionally dehydrated, cleared, and mounted.

Immunohistochemistry

Expression of FOXO3a was examined using the streptavidin-biotin-peroxidase immunohistochemical method. The anti-FOXO3a antibody kit, the streptavidin-biotin-peroxidase immunohistochemical staining kit, and the 3,3'-diaminobenzidine chromogenic kit were purchased from Fuzhou Maixin Biotechnology Development Co., Ltd. (Fuzhou, China). The experimental procedure was as follows. 1) Paraffin-embedded tissue sections were de-waxed with xylene, rehydrated in a graded ethanol series, and washed 3 times with phosphate buffered saline (PBS) for 3 min each. 2) Antigen retrieval was achieved by immersing the sections in boiling ethylenediaminetetraacetic acid in a pressure cooker for 2.5 min. The sections were cooled to room temperature and washed 3 times with PBS for 3 min each. 3) The tissue sections were incubated in 3% hydrogen peroxide solution for 10 min at room temperature to block endogenous peroxidase activity. The sections were then washed 3 times with distilled water for 3 min each, followed by 2-3-min washes in PBS. 4) The sections were blocked with normal serum for 15 min at room temperature. 5) After blocking, the serum was removed. Rabbit anti-human ribonucleotide reductase subunit M1 polyclonal antibody was gently added drop-wise over the sections, and the sections were incubated in a refrigerator at 4°C overnight. 6) After washing 3 times with PBS for 5 min each, the sections were incubated with biotinylated secondary antibody for 15 min at room temperature and then washed 3 times with PBS for 3 min each. 7) A working solution of horseradish peroxidase conjugated-streptavidin was added drop-wise to the tissue sections, and the sections were incubated for 15 min at room temperature. The sections were then washed 3 times with PBS for 5 min each. 8) 3,3'-Di-

aminobenzidine chromogenic reagent was added drop-wise, and the staining intensity was monitored under a microscope to determine the duration of the chromogenic reaction. The chromogenic reaction was stopped by washing the sections in tap water. The sections were counterstained with hematoxylin for 5 min, washed with tap water, differentiated in hydrochloric acid-ethanol, and washed again with tap water for 15 min to restore the blue color. 9) The sections were dehydrated using a graded ethanol series, cleared with xylene, and mounted with neutral gum.

Western blotting

Total protein was extracted from the lung tissues, and the protein concentration was determined using the bicinchoninic acid protein assay kit. As previously described (Yue et al., 2013; Huang et al., 2013), 100 μg protein was diluted 1:4 (v/v) in 5X sodium dodecyl sulfate sample buffer and denatured for 5 min in a boiling water bath at 99°C. The denatured protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with purified anti-FOXO-3 primary antibody (Abcam, Cambridge, UK) diluted in 5% non-fat milk overnight at 4°C with gentle shaking. The membrane was washed with Tris-buffered saline-Tween 20 solution and incubated with a horseradish peroxidase-conjugated secondary antibody in 5% non-fat milk (working concentration 1:5000) for 2 h at room temperature. The membrane was washed again, and the target proteins were visualized using chemiluminescence reagents and autoradiography. The autoradiographic film was digitized, and the relative expression level of the target protein was calculated as the grey-scale value of the target protein band divided by the grey-scale value of the internal reference protein.

RESULTS

Immunohistochemical analyses showed that in both the normally aged groups and the aging-accelerated groups, FOXO3a expression in mice exposed to cigarette smoke was significantly decreased (Figure 1). Thus, smoking inhibited the release of FOXO3a in the lung tissues.

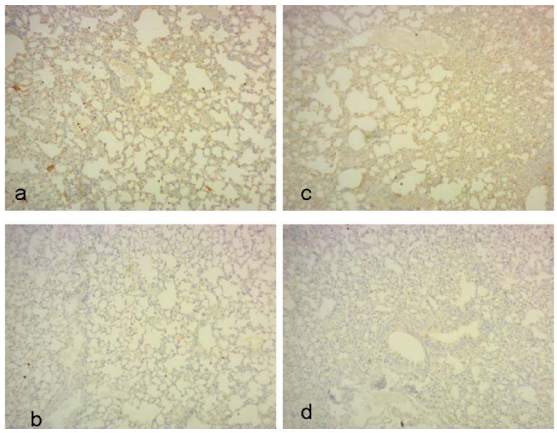

Figure 1. Immunohistochemical analyses. **a.** Normally aged, air-exposed group; **b**. normally aged, smoke-exposed group; **c.** aging-accelerated, air-exposed group; **d.** aging-accelerated, smoke-exposed group.

Western blot analysis showed that FOXO3a expression was significantly increased in the aging-accelerated, air-exposed group compared with the normally aged, air-exposed group. In addition, FOXO3a expression in the aging-accelerated, smoke-exposed group was significantly lower than in the aging-accelerated, air-exposed group (Figure 2).

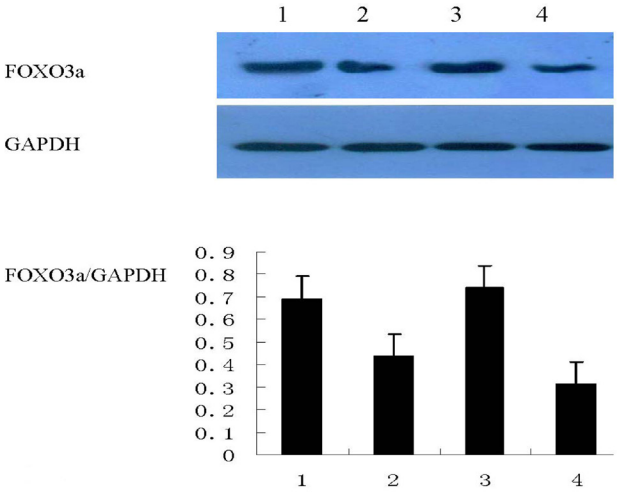

Figure 2. Western blot analyses of FOXO3a. *Lane 1* = normally aged, air-exposed group; *lane 2* = normally aged, smoke-exposed group; *lane 3* = aging-accelerated, air-exposed group; *lane 4* = aging-accelerated, smoke-exposed group.

SIRT1 expression was also significantly lower in the smoke-exposed groups compared with the air-exposed groups and in the aging-accelerated groups compared with the normally aged groups (Figure 3). Similarly, manganese superoxide dismutase (MnSOD) expression was significantly reduced in the smoke-exposed groups compared with the air-exposed groups and in the aging-accelerated groups compared with the normally aged groups (Figure 4).

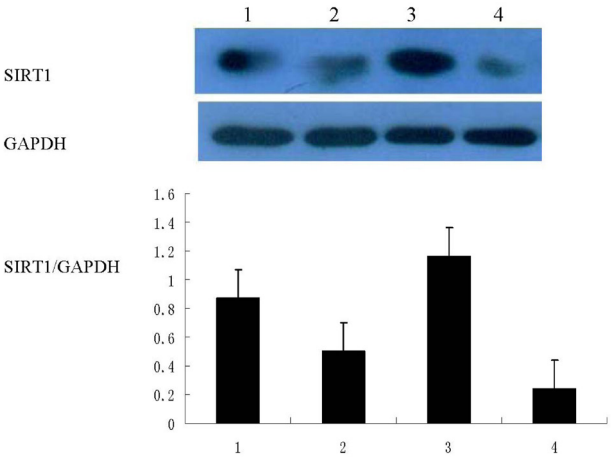

Figure 3. Western blot analyses of SIRT1. *Lane 1* = normally aged, air-exposed group; *lane 2* = normally aged, smoke-exposed group; *lane 3* = aging-accelerated, air-exposed group; *lane 4* = aging-accelerated, smoke-exposed group.

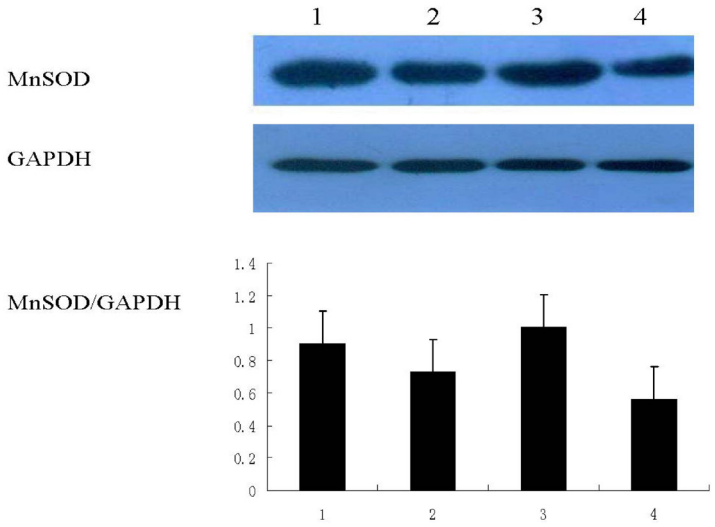

Figure 4. Western blot analyses of MnSOD. *Lane 1* = normally aged, air-exposed group; *lane 2* = normally aged, smoke-exposed group; *lane 3* = aging-accelerated, air-exposed group; *lane 4* = aging-accelerated, smoke-exposed group.

The expression of phosphatidylinositol 3-kinase (PI3K)/Akt decreased significantly in the smoke-exposed groups compared with the air-exposed groups and in the aging-accelerated groups compared with the normally aged groups (Figure 5).

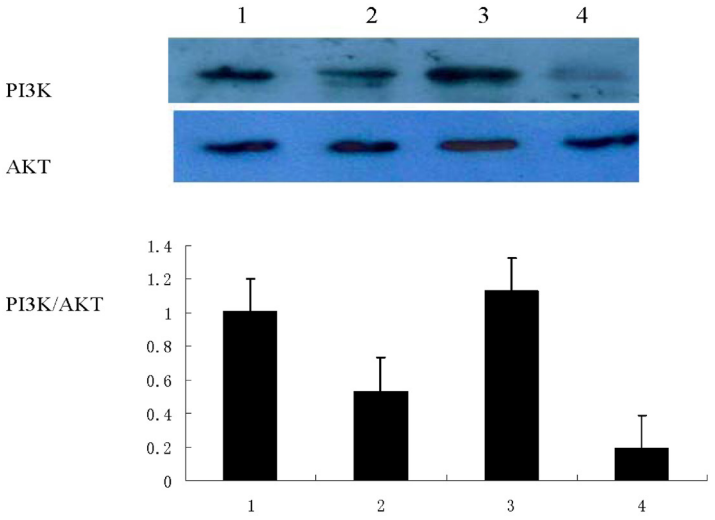

Figure 5. Western blot analyses of PI3K/AKT. *Lane 1* = normally aged, air-exposed group; *lane 2* = normally aged, smoke-exposed group; *lane 3* = aging-accelerated, air-exposed group; *lane 4* = aging-accelerated, smoke-exposed group.

DISCUSSION

We found that signal transduction pathways mediated by the transcription factor FOXO3a (such as the PI3K/Akt pathway) were involved in accelerated lung aging in COPD. In addition, smoking inactivated the transcription factor FOXO3a, accelerating lung aging in COPD.

Previous studies on COPD have primarily focused on inflammation, oxidative stress, and the protease/anti-protease imbalance, which cannot explain the similarities between the clinical manifestations of COPD and aging (Yao et al., 2013). FOXO signaling pathways are important for regulating metabolism and life span in mammals. We investigated the molecular mechanisms of lung aging in COPD to determine the pathogenesis of this chronic age-related disease.

We found that FOXO3a expression increased significantly in the normally aged air-exposed group compared with the aging-accelerated, air-exposed group. In addition, FOXO3a expression was significantly reduced in the aging-accelerated, smoke-exposed group compared with the aging-accelerated, air-exposed group. The expression of SIRT1, MnSOD, and PI3K/Akt was significantly decreased in the smoke-exposed groups compared with air-exposed groups and in aging-accelerated groups compared with normally aged groups. FOXO transcription factors regulate the expression of various target genes involved in cell cycle progression, apoptosis, longevity/aging, stress response, and metabolism, which are closely related to human aging. In COPD patients, smoking inactivates the transcription factor FOXO3, accelerating the aging of lung tissues.

Furthermore, the expression of SIRT1 and MnSOD was significantly decreased in both the smoke-exposed groups and the aging-accelerated groups, indicating that smoking and aging play important roles in the pathogenesis of COPD. In lung tissues from COPD patients, decreases in SIRT1 and MnSOD protein levels result in the acetylation and inactivation of FOXO3a, which eventually accelerates senescence and apoptosis in lung cells. Therefore, reduced SIRT1 and MnSOD protein levels may be an important mechanism of COPD pathogenesis.

In summary, signal transduction pathways mediated by the FOXO3a transcription factor accelerate lung aging in COPD patients. Smoking also accelerates lung aging.

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