## Role of thioredoxin nitration in bleomycin (BLM)-induced pulmonary fibrosis in rats

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Role of thioredoxin nitration in bleomycin (BLM)-induced pulmonary fibrosis in rats

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Abstract

Oxidant stimulation has been suggested to play an important role in the pathogenesis of idiopathic pulmonary fibrosis (IPF). Our study aimed to investigate the role and mechanisms of thioredoxin (Trx) nitration during the development of IPF. Rat IPF model was established by intratracheal instillation of bleomycin (BLM). Male Wistar rats were randomly divided into control group and BLM-treated group in which rats were intratracheally instilled with a single dose of BLM (5.0 mg/kg body weight in 1.0 ml phosphate buffered saline). At 7 or 28 days after instillation, rats were euthanized. Histopathologic and biochemical exams were performed. The activity and protein level of thioredoxin were assessed. The thioredoxin nitration level was determined using immunoprecipitation (IP) and immunoblotting (IB) techniques. Our results demonstrated that protein tyrosine nitration increased in BLM group compared with control group. Trx activity decreased in BLM group compared with control group, while its expression and nitration level increased dramatically in BLM group compared with control group. Our results indicated that Trx nitration might be involved in the pathogenesis of IPF.

Keywords: pulmonary fibrosis; thioredoxin (Trx); nitration; apoptosis
INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a fibrotic lung disease with very poor prognosis and unknown etiology. The annual incidence of IPF is rising and is estimated to be 14.0-47.2/100,000 persons in the United States of America, with the median survival time of less than 5 years (Ley and Collard 2013; Raghu et al. 2011). However, the pathogenesis of IPF is still poorly understood. Oxidative stress has been reported to play an essential role in the process of IPF by regulating apoptosis rates of alveolar epithelial cells (Strieter and Mehrad 2009; Thannickal and Horowitz 2006).

Thioredoxin, a 12-kDa protein ubiquitously expressed in all living cells, is reviewed to have various biological functions related to cell proliferation and apoptosis (Lu and Holmgren 2012). Various experiments results have demonstrated that inhibition of Trx would cause apoptosis through ASK1-P38-MAPK pathway in many diseases (Lu J and Holmgren 2012; Sato et al. 1995). It has been reported that oxidative stress stimulated cell death by activating caspase3, inducing cytochrome c release from mitochondria, inducing DNA fragmentation and activation of P38-MAPK pathway in IPF patients (Bargagli et al. 2009). All those results suggest that Trx might play a critical role in cell proliferation and cell death in pulmonary fibrosis. However, whether Trx activity is reduced in pulmonary fibrosis and whether this alteration contributes to increased alveolar epithelial cells apoptosis have never been investigated so far.

Reactive oxygen species (ROS) have long been reported to cause oxidative protein modifications and to be a major mechanism in pulmonary fibrosis (Strieter
and Mehrad 2009). However, many questions can not be exclusively explained by ROS production, and many fundamental questions are to be solved. Recent data have revealed that nitric oxide-derived reactive nitrogen species (RNS) as potential targets in several diseases acted as contributor of protein modifications (Furukawa et al. 2011; Cai and Yan 2013). Zeki Y and his colleagues revealed that aminoguanidine exerted its antifibrosis effects on bleomycin-induced lung fibrosis in rats by inhibiting nitric oxide mediated tyrosine nitration of proteins (Yildirim et al. 2004). However, which specific protein was nitrated and what role the nitrated protein played in IPF remained elusive.

Therefore, the main aims of this study were: 1) to determine the change of Trx expression and activity in BLM-induced pulmonary fibrosis; 2) to identify the mechanisms responsible for the change of Trx in pulmonary fibrosis.
MATERIALS AND METHODS

Animal model of bleomycin-induced pulmonary fibrosis

All experimental procedures were reviewed and approved by the Animal Research Committee of ZhengZhou University, ZhengZhou, China. Sixty Wistar male rats (SPF grade, 8 weeks old and weighing 200–240 g) were purchased from Vital River Laboratories (Beijing, China). Rats were maintained in a climate-controlled room and had free access to standard rat chow and water. All rats were acclimated to laboratory conditions for 7 days prior to the experiment. Male Wistar Rats were intratracheally injected with bleomycin A5 (5.0 mg/kg body weight in 1.0 ml phosphate buffered saline. Taihe Pharmaceutical, Tianjin, China) as described previously (Fang et al. 2009). The rats were euthanized using an overdose of chloral hydrate (10%) euthanized using an overdose of chloral hydrate (10%) at 7 days or 28 days after bleomycin A5 injection. Control animals received the same volume of intratracheal saline instead of bleomycin. Pulmonary fibrosis was assessed by lung hydroxyproline content as well as lung histology. Lung histology was performed as described in the following section.

Histopathological examination of lung

Part of the left upper lung was harvested and fixed in 4% paraformaldehyde-PBS, embedded in paraffin and cut in 5um-thick sections following standard procedures for sample preparation. The lung tissues were stained with H&E staining and Masson staining. The light microscope (Olympus D72, Japan) was used to examine H&E-stained slides with ×100 magnifications as well as Masson-stained
slides with ×100 magnifications. The fibrosis severity was scored in the Szapiel manner under a light microscope (Tascilar et al. 2007). Percentage tissue showing fibrotic and (or) reparative changes in the interstitium and alveolar walls was graded from 0 to 4 (0 = absence of fibrosis, and 1 = 1% to 25%, 2 = 26% to 50%, 3 = 51% to 75%, and 4 = 76% to 100% fibrosis) (Leme et al. 2002). Scores in BLM-induced group were then averaged and compared with controls. The image analysis software of Masson-stained slides was Image-Pro Plus Version 6.0.

Hydroxyproline assay

To assess the degree of collagen deposition, hydroxyproline content of the left upper lung was determined by the colorimetric method as previously described (Russo et al. 2009). On 7 and 28 days after bleomycin administration, rat left upper lungs were harvested for determination of hydroxyproline content following the manufacturer’s instructions. The total lung hydroxyproline content was expressed as milligrams per gram lung tissue.

Quantification of tissue nitrotyrosine content

Paraformaldehyde-fixed lung tissues were cut into semi-thin sections of 4 to 5µm thick and stained with a primary antibody against nitro-tyrosine (Minipore, USA). Immunostaining was done with Vectastain ABC kit (Vector Laboratories), and slides were analyzed by light microscopy. Quantification of lung tissue nitrotyrosine content was performed by Millipore nitro-tyrosine assay kit according to the manufacturer's instruction. Results were presented as micrograms per milligram (µg/mg) protein.

Western blotting assays for Trx expression
Lung tissues were cut into small pieces and homogenized in lysis buffer (50 mM/L Tris·HCl, pH 7.4; 150 mM NaCl; 1% sodium deoxycholate; 0.1% SDS) on ice. The protein concentration was determined by BCA using the protein assay kit (applygen, China). 60 ug protein was loaded and separated by electrophoresis on sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to 0.22 um polyvinylidene difluoride (PVDF) membranes (Dingguo, China). Membranes were blocked using 5% non-fat milk in TRIS-buffered saline with Tween-20 (TBST) for 1 h and then were incubated with Trx antibody (1:1000 dilutions, Cell Signaling Technology, USA) overnight at 4 °C. After washing with TBST, protein bands were detected with secondary antibody conjugated with horseradish peroxidase (1:6000 dilutions, Cell Signaling, USA). The membrane was washed 3 times in TBST with each time of 10 min. The blot was developed with a super-signal chemiluminescent detection kit (Pierce) and visualized with a Kodak Image Station 400. The blot density was analyzed with Kodak 1D software (version 3.6).

Determination of thioredoxin enzyme activity

Trx activity was determined via the insulin disulfide reduction assay (Holmgren et al. 1995). In brief, 40 mg of tissue homogenate were pre-incubated at 37 °C for 15 min with 2 mL activation buffer (100 mM HEPES, 2 mM EDTA, 1 mg/mL BSA, and 2 mM DTT) for Trx reduction. Samples were then mixed with 20 µL reaction buffer (100 mM HEPES, 2.0 mM EDTA, 0.2 mM NADPH, and 140 mM insulin). The reaction was initiated by addition of mammalian Trx reductase (1 mL, 15 mU, Sigma) or water to controls, and samples were incubated for 30 min at 37 °C. The reaction
was terminated by adding 125 µL of stopping solution (0.2 m Tris–CL, 10M guanidine–HCl, and 1.7 mM 3-carboxy-4-nitrophenyl disulfide, DTNB) followed by absorption measurement at 412nm.

Detection of Trx nitration

Trx nitration detection was performed as previously described (Vadseth et al, 2004). Pulmonary tissues were homogenized with lysis buffer. Endogenous Trx-1 was immunoprecipitated with a monoclonal anti-murine Trx-1 antibody (Santa, USA). Trx-1 nitration was detected with a monoclonal antibody (Upstate, Charlottesville, VA, USA) against nitro-tyrosine. The blot was developed with Supersignal-Western reagent (Pierce) and visualized with a Kodak Image Station 400. The blot density was analysed with Kodak 1D software (version 3.6).

Statistical analysis

All values in the text and figures were presented as mean ± SD. All data were subjected to two-way ANOVA followed by Bonferroni correction for post hoc t test. Immunoblotting density was analyzed with the Kruskal–Wallis test followed by Dunn post hoc test. Probabilities of 0.05 or less were considered to be statistically significant.

RESULTS

BLM induced pulmonary fibrosis in rats

BLM exposure resulted in significant drop of rat weight. The HE staining results showed that intact and clear alveoli, normal interstitium, few inflammatory cells were observed in the lungs of control group at each time point. However, BLM
administration caused progressive lung injury, demonstrating as destruction of lung alveoli, inflammatory cells infiltration and thickening of lung interstitium with time extension (Fig. 1A). The Masson’s staining results also showed that intact alveoli, normal interstitium in the lungs of control group at each time point, BLM administration caused gradually exacerbated lung alveoli destruction, interstitium thickening, and fibroblasts diffusion in rat lungs. Semiquantified histological scoring supported the above data, thus confirming BLM induced pulmonary fibrosis in rats (**P<0.01 vs control group, Table 1 and Fig.1B).

Hydroxyproline content increased in the pulmonary fibrosis in rats

Hydroxyproline content in lung tissues were measured as a surrogate of lung collagen deposition. At day 7 after fibrosis induction with bleomycin, the lung hydroxyproline content was 70% higher than that of controls. At 28 day after fibrosis induction with bleomycin, the lung hydroxyproline content was 113 % higher than that of controls (Fig.2). These results indicated that BLM induced pulmonary fibrosis in rats successfully.

The expression and activity of thioredoxin in BLM-induced pulmonary fibrosis

Thioredoxin has been reported to be involved in the progress of idiopathic pulmonary fibrosis. In the lung tissues of usual interstitial pneumonia (UIP) patients, Tiitto L has reported that the expression of thioredoxin increased significantly compared with controls (Titto et al. 2003). However, the change of thioredoxin activity during the development of pulmonary fibrosis has not been reported previously. We observed the expression of thioredoxin increased at 7 day and 21 day
in BLM-induced pulmonary fibrosis model in rats (Fig 3A). However, with the increase of Trx protein level, pulmonary Trx activity was reduced both at 7 day and 21 day after BLM administration (Fig 3B). The results indicated that reduced Trx activity in pulmonary fibrosis might be caused by other factors, such as post-translational modifications rather than reduced protein expression.

The expression of nitrotyrosine in BLM-induced pulmonary fibrosis in rats.

Several studies have demonstrated that thioredoxin was susceptible to nitrative modification, which resulted in inhibition of its activity in cardiovascular diseases (Tao et al. 2004; Zhang et al. 2007). To determine whether reduced thioredoxin activity in fibrotic lungs was caused by increased nitrative stress, we examined nitrative stress in pulmonary fibrosis. Nitrotyrosine content in the fibrosis lung was a footprint of in vivo ONOO\(^{-}\) formation and an index of nitrative stress. So we evaluated nitrotyrosine content by immunohistochemistry and enzyme-linked immunosorbent assay (ELISA). In control group, there was no obvious nitrotyrosine in the lungs. In the BLM-induced fibrotic lungs, the expression of nitrotyrosine increased dramatically (Fig 4A). Quantitative analysis confirmed BLM-induced nitrotyrosine production in rats (Fig 4B). The results indicated that nitrative stress might play an important role in BLM-induced pulmonary fibrosis.

The content of thioredoxin nitration in BLM-induced pulmonary fibrosis

Through proteomic techniques, many investigators have demonstrated that a variety of proteins were nitrated in diseased tissues (Haendeler 2006). To determine whether thioredoxin was an essential target of nitrative stress, we examined nitrated
thioredoxin level using Co-IP and Western blotting methods. As illustrated in Figure 5, at 7 d and 28 d nitrated Trx was not obviously detected in control rats, while Trx nitration increased significantly in BLM–treated rats.

**DISCUSSION**

In this study, we have made several observations. Firstly, in BLM-induced pulmonary fibrosis model we discovered that tyrosine nitration increased in early and late phase of fibrosis, which is consistent with previous study (Yildirim et al. 2004). Secondly, we observed that the activity of thioredoxin decreased in lung tissues of BLM-treated rats. Thirdly, we found that thioredoxin was a target of nitrative modification in BLM-induced pulmonary fibrosis, which meant that thioredoxin nitration might play an important role in BLM-induced pulmonary fibrosis.

Thiordoxin system, including Trx, thioredoxin reductase (TR), NADPH, was a very important antioxidative system, and had been reported to have many effects, such as regulating cellular reduction/oxidation (redox) status and cell proliferation/cell survival process (Lu and Holmgren 2012). Trx system has also been reported to regulate pathologic process of several tumors (Powis and Kirkpatrick 2007). Besides its essential role in regulating cancer proliferation, Trx system was also involved in cardiovascular diseases, heart failure, stroke, inflammation, metabolic syndrome and other diseases (Mahmood et al. 2013). However, thioredoxin’s function in pulmonary diseases have not been revealed thoroughly. It was reported that in asthma and COPD diseases, the expression of thioredoxin increased dramatically, which meant that induction of thioredoxin in COPD or asthma
might represent an attempt to protect the lung from injuries (Zhang et al. 2008; Ito et al. 2011). Hoshino T et al have proved that thioredoxin had anti-fibrosis effects in BLM-induced fibrosis in rats (Hoshino et al. 2003); Gon Y found that in lungs of BLM-treated mice, the expression of thioredoxin was strongly induced in bronchial epithelial cells (Gon et al. 2001). Tiitto L discovered that compared with control patients, in the lungs of IPF patients the expression of thioredoxin increased (Titto et al. 2003). Our results confirmed the expression of thioredoxin was increased in BLM-induced pulmonary fibrosis in rats [Fig 3A]. All these results proved that thioredoxin might play a key role in the process of pulmonary fibrosis. However, it has not been revealed how thioredoxin’s activity was affected in IPF.

Recent studies have demonstrated that besides being regulated by protein level, Trx activity regulated by post-translational modifications might also play important roles in many human diseases by influencing cellular signaling. Three forms of post-translational modifications of Trx have been investigated thoroughly, which included oxidation, glutathionylation and s-nitrosylation (Haendeler 2006). Thioredoxin exerted several functions via cysteines 32 and 35. When oxidized at cysteines 32 and 35, thioredoxin couldn’t bind its target protein, resulting in Trx inactivation (Watson et al. 2003). Michelet L reported Trx activity decreased when glutathionylazed at cysteine 73 under oxidative stress (Michelet et al. 2005). Recently s-nitrosylation of thioredoxin at cysteine 73 has been thoroughly studied by Tao L et al., which showed that nitrosylation of thioredoxin contributed to the cardioprotective and anti-apoptotic functions of thioredoxin in cells and mice (Tao et al. 2004).
Besides three previously reported posttranslational modifications in Trx, Tao L and Zhang HX discovered the fourth modification: nitration. Their researches showed that nitration of thioredoxin at the tyrosine residue could cause its inactivation in ischemia-reperfusion model of rats (Tao et al. 2004; Zhang et al. 2007). In our study, we firstly demonstrated that in IPF model the expression level of thioredoxin increased, however its activity decreased [Fig 3B]. We proposed that thioredoxin’s posttranslational modifications played an important role during the process of IPF. We also observed that the nitrotyrosine content increased in the lungs of IPF model [Fig 4]. At last, using Co-IP and Western blotting methods, we discovered that nitrated thioredoxin increased in the lungs of IPF model [Fig 5]. Our results demonstrated for the first time that Trx nitration increased in the IPF model in rats.

Apoptosis takes part in many pathophysiologic processes through highly conserved signaling pathways. MAPK pathway is involved in apoptosis pathway under many conditions. After exposure to oxidative stress and environmental stimuli, MAPKS are activated to influence apoptosis, proliferation and differentiation (Nishida and Otsu 2006). Three distinct MAPKS pathways have been reported in mammalian cells: extracellular signal regulated kinase (ERK), c-Jun amino-terminal kinase (JNK)/stress-activated protein kinase and P38 MAPK. As an upstream activator of JNK and P38 MAPK, ASK1 activity is inhibited by several cellular factors, such as Trx, glutaredoxin, and phosphoserine-binding protein under physiologic conditions( Raman et al. 2007). The interaction between thioredoxin and ASK1 is determined by the redox state of Trx (Li et al. 2013). In the process of IPF,
apoptosis has been reviewed thoroughly (Thannickal and Horowitz 2006). It was reported that increased apoptosis of alveolar epithelial cells (ACEs) was observed in areas of IPF by P38 MAPK pathway (Barbas-Filho et al. 2001). However, in IPF patients, myofibroblasts were observed to resist apoptosis (Plataki et al. 2005). In IPF model of rats, we observed that thioredoxin activity decreased, and the nitrated thioredoxin increased using Co-IP and Western blotting methods. We presumed that thioredoxin was a key target of nitration modification, and nitrated Trx could promote apoptosis through activating ASK1-P38 MAPK pathway, which at last resulted in caspase3 activation, cytochrome c release from mitochondria and DNA fragmentation. Our results indicated that thioredoxin nitration could be a therapeutic target in the therapy of IPF. As thioredoxin was essential in regulating redox state, we didn’t exclude that ROS overload was involved in the process of IPF through modifying thioredoxin with nitration.

However, there were still several limitations in our research. Firstly, our results have not provided direct relations among thioredoxin nitration, apoptosis and fibrosis. Secondly, we mainly focused on BLM-induced pulmonary fibrosis in rats, the pathology of which was different from IPF patients. In the following studies, we will pretreat rats with EKU 134 (an ONOO- scavenger) when making pulmonary fibrosis model with BLM administration, to confirm the function of ASK1-P38 MAPK pathway in fibrosis. Furthermore, we will exam the thioredoxin nitration in IPF patients.

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References


Table 1. Semiquantified histological analysis of lung alveolitis and pulmonary fibrosis on day 7 and 28 after bleomycin administration

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<th>Day 28</th>
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<tr>
<td></td>
<td>Alveolitis</td>
<td>Fibrosis</td>
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<tr>
<td>Control</td>
<td>1.00±0.24</td>
<td>1.00±0.48</td>
</tr>
<tr>
<td>BLM</td>
<td>3.70±0.92**</td>
<td>2.15±0.98**</td>
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Data are means ± SD, n = 6 in each group. ** P < 0.01 vs. control group.
H&E(A) and Masson staining (B) for pathological changes in rat lung caused by BLM. BLM (5.0mg/kg weight in 1.0 ml phosphate buffered saline, intratracheal) was given once to rats. Sections of pulmonary tissue were prepared on 7, 28 days after BLM administration, and subjected to histopathological and microscopic examination with ×100 magnifications.

Hydroxyproline content increased in the pulmonary fibrosis in rat. Hydroxyproline levels, a marker of pulmonary fibrosis, were measured in harvested lungs 7d or 28d after BLM administration. Data are means ± SD, n = 6 in each group. *P< 0.05 vs. control; #P< 0.05 vs. BLM of 7d.

Effect of BLM on the expression and activity of thioredoxin in rats. BLM (5.0mg/kg weight in 1.0 ml phosphate buffered saline, intratracheal) was given once to rats. Sections of pulmonary tissue were prepared on 7, 28 days after BLM administration, the expression (3A) and activity of thioredoxin (3B) were examined. Lane 1=control; lane 2=BLM (7 d); lane 3=control (28 d); lane 4= BL M (28 d) (n=6). Data are means ± SD, n = 6 in each group. *P< 0.05 vs. control; **P< 0.01 vs. control (28 d); #P< 0.05 vs. BLM of 7d.

Nitrotyrosine content in pulmonary determined by immunohistochemistry and ELISA assay. BLM (5.0mg/kg weight in 1.0 ml phosphate buffered saline, intratracheal) was given once to rats. Sections of pulmonary tissue were prepared on 7, 28 days after BLM administration, the nitrotyrosine content was examined by immunohistochemistry (4A) and ELISA method (4B). Data are means ± SD, n = 6 in each group. *P< 0.05 vs. control (7 d); **P< 0.01 vs. control (28 d); #P< 0.05 vs. BLM of 7d.

Trx-1 nitration determined by immunoprecipitation (IP) method. BLM (5.0mg/kg
weight in 1.0 ml phosphate buffered saline, intratracheal) was given once to rats. Sections of pulmonary tissue were prepared on 7, 28 days after BLM administration. A, Trx-1 nitration determined by immunoprecipitation (IP). Representative immunoblot (IB) graphs are shown. Lane 1=control; lane 2=BLM (7 d); lane 3=control (28 d); lane 4= BLM (28 d). B, Summary of density analysis results from 6 rats /group. *$P<0.05$ vs. control (7 d); **$P<0.01$ vs. control (28 d); #$P<0.05$ vs. BLM of 7d.
Figure 1.
677x254mm (300 x 300 DPI)
Figure 2.
258x214mm (300 x 300 DPI)
Figure 3.
508x338mm (300 x 300 DPI)
Figure 4.
677x338mm (300 x 300 DPI)
Figure 5.
762x254mm (300 x 300 DPI)