Nitrative inactivation of thioredoxin-1 loses its protective effect in bleomycin-induced pulmonary fibrosis

Lei Wang (wanglei138@aliyun.com)
The First Affiliated Hospital of Zhengzhou University

Wan-yang Xu
The First Affiliated Hospital of Zhengzhou University

Yi-wen Tang
The First Affiliated Hospital of Zhengzhou University

Feng Hu
The First Affiliated Hospital of Zhengzhou University

Research Article

Keywords: thioredoxin, nitration, apoptosis, MAPK, pulmonary fibrosis

Posted Date: April 18th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1552277/v1

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Abstract

Background

Pulmonary fibrosis is common in the development of inflammatory lung diseases with no effective clinical drug treatment currently. As an essential redox enzyme, thioredoxin (Trx) has been reported to be involved in pulmonary fibrosis, but the mechanism is to be revealed.

Methods

Bleomycin-indued pulmonary fibrosis mouse model was established, Trx activity and nitrated Trx were examined. p38-MAPK apoptosis pathway was determined in lung tissues. Additionally, before BLM administration, C57/BL6 mice were treated with aminoguanidine (AG, a peroxynitrite scavenger), recombinant human Trx-1 (rhTrx-1), or SIN-1 (a peroxynitrite donor) nitrated Trx-1 (N-Trx-1).

Results

In bleomycin (BLM)-induced pulmonary fibrosis model in C57/BL6 mice, we observed that nitrated Trx increased, while its activity decreased, with the increase of lung cells apoptosis rate by p38-MAPK pathway. We demonstrated that AG or rhTrx-1, but not N-Trx-1 significantly reduced pulmonary fibrosis.

Conclusion

Blockade of Trx-1 nitration, or supplementation of exogenous rhTrx-1, might represent novel therapies to attenuate pulmonary fibrosis in idiopathic pulmonary fibrosis patients.

Background

Pulmonary fibrosis can be the results of lung injury resulting from radiation, severe infection, chemotherapy, environmental exposure or many unknown reasons, as idiopathic pulmonary fibrosis (IPF) [1]. As a fetal pulmonary fibrosis, the annual incidence of IPF is rising and is estimated to be over 150 000 patients annually in the USA, and more than 5 million worldwide, resulting in a median survival time less than 5 years [2, 3]. However, as its pathogenesis is not fully revealed, effective therapeutic interventions are still needed [4].

Oxidative stress has been revealed to be involved in the pathological process of IPF by regulating the apoptosis rate of alveolar epithelial cells (AECs) through Mitogen-activated protein kinases (MAPK) pathway [5]. However, many fundamental questions cannot be explained by ROS overproduction alone. As another resource of redox stimulations, nitric oxide-derived reactive (RNS) has been reported to be another contributor of cell injury by protein modifications [6, 7].
Trx1 is a 12 k Da redox protein with important antioxidative and cell signaling functions, which has been revealed to take part in the pathogenesis of several diseases, such as ischemia, stroke and lung diseases [8, 9, 10]. Some researchers investigated that Trx suppressed BLM-induced pulmonary fibrosis progression in mice [11, 12]. Tao and her colleagues have revealed that in the myocardial ischemia models of the rats, Trx's activity decreased after being nitrated by RNS, resulting in myocardial apoptosis by ASK1-p38MAPK pathway [13]. In our previous research, we have demonstrated the role of Trx's nitrination modification in BLM-induced pulmonary fibrosis in rats [14]. But our previous results have not provided direct cause-effect relations among Trx nitration, apoptosis and pulmonary fibrosis. Therefore, in the BLM-induced pulmonary fibrosis model, we investigated the direct evidence to support our hypothesis that increased nitration inactivation of Trx-1 is causatively related to BLM-induced pulmonary fibrosis in mice by regulating p38-MAPK apoptosis pathway.

Material And Methods

Chemicals and Reagents

Bleomycin A5 (BLMA5) (J1716055) was purchased from Aladdin (Shanghai, China). Recombinant Trx (MB1039) was purchased from Meilunbio (Dalian, China). Aminoguanidine (A111133), a peroxynitrite scavenger was purchased from Aladdin (Shanghai, China). Hydroxyproline assay kit was provided by Wanleibio (Shenyang, China). Antibody against Trx (ab26320) was purchased from Abcam (Cambridge, United Kingdom). Nitrotyrosine antibody (06-284) was purchased from Millipore (Danvers, MA, United States). Antibody against ASK1 (SC-390275) was purchased from Santa Cruz Biotechnology (United States). Antibodies against p38, iNOS and second antibodies were all obtained from Wanleibio (Shenyang, China). p38 activity kit (9790) was purchased from Cell Signal Technology Inc. (Danvers, MA, United States). Classic IP Kit (26146) was purchased from Thermo (NewYork, United States). SIN-1 was purchased from Cayman Chemical (Michigan, United States). MnTBAP chloride (CAS:55266-18-7) was purchased from Santa Cruz (United States). Tunnel (WL029a) kit and caspase3 (WLA047) kit were provided by Wanleibio (Shenyang, China).

Animal model of BLM-induced pulmonary fibrosis

All experimental procedures were reviewed and approved by The Animal Research Committee of Zheng Zhou University, Zheng Zhou, China. C57/BL6 mice (SPF grade, 8 weeks old and weight 20-25g) were purchased from Vital River Laboratories (Beijing, China). Mice housed in a room with controlled temperature (22±2°C) and humanity (65±5%) had free access to standard mice chow and water. All mice were acclimated to laboratory conditions for 7 days prior to the experiment. The mice were administered BLMA5 by intracheal injection (5.0 mg/kg body mass in phosphate-buffered saline) as described previously [14]. At 7 or 28 days after administration of BLMA5, the mice were euthanized using an overdose of chloral hydrate (10%). Before BLM administration, mice were randomized to receive either vehicle (0.9%NaCl), aminoguanidine (AG, a peroxynitrite scavenger, 40 mg/kg, continuously for 14 days), recombinant human Trx-1 (40ug/20g, continuously every 2 day for 14 days), or nitrated Trx-1 (please see
details, continuously every 2 day for 14 days) by intraperitoneal injection. Pulmonary fibrosis was assessed from the lung histology. Lung histology was performed as described [15].

**Histopathological examination of lung**

Part of the upper lungs were inflated with 1 ml of 10% paraformaldehyde / PBS solution and embedded in paraffin. Sections (5µm thick) were treated for sample preparation. The lung tissues were stained with hematoxylin-eosin (H&E) and Masson's trichrome, then evaluated under a light microscopy conducted by experienced pathologists. The lung fibrosis score, based on the severity and extent of lung fibrosis present in the peribronchial and interstitial tissues was assessed by a pulmonary pathologist blinded to the experimental protocol. Lungs were assigned a severity score from 0 to 4 (0=absence of fibrosis, 1=1% to 25%, 2= 26% to 50%, 3=51% to 75%, 4=76% to 100% fibrosis) [16].

**Determination of pulmonary cells apoptosis**

Pulmonary cells apoptosis was determined by terminal deoxynucleotidyltransferase-mediated dUTP nick and labeling (TUNEL) staining and caspase-3 activity assay. Sections were stained as the manufacturer's protocol. Briefly, the sections were deparaffinized and treated by ethylenediaminetetraacetic acid solution while being heated in a microwave. The sections were then reacted in a solution of terminal deoxynucleotidyl transferase enzyme mixed with biotinylated nucleotide. Horseradish peroxidase-labeled streptavidin was bound to these biotinylated nucleotides, which were detected using the peroxidase substrate, hydrogen peroxide, and diaminobenzidine (DAB). The index of apoptosis (number of TUNEL positive nuclei / total number of nuclei×100) was automatically calculated for further analysis. The caspase-3 activity was determined as the instruction provided by the manufacturer.

**Quantification of tissue nitrotyrosine content**

Paraformaldehyde-fixed lung tissues were cut into semi-thin sections 4 to 5 µm thick and stained with a primary antibody against nitrotyrosine. Immunostaining was determined as described in our previous study [14]. Quantification of lung tissue nitrotyrosine content was performed by ELISA assay. Results are presented as micrograms per milligram(µg/mg) protein.

**Western blotting analysis**

Lung tissues were cut into small pieces and homogenized in lysis buffer on ice. The protein concentration was determined according to a protein assay kit, where 40 µg protein was loaded and separated by electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and transferred to 0.22 µm polyvinylidene difluoride (PVDF) membranes. Membranes were blocked using 5% nonfat milk in TRIS-buffered saline with Tween-20 (TBST) for 1 h and then were incubated with Trx antibody or iNOS antibody overnight. After washing with TBST, protein bands were detected with secondary antibody conjugated with horseradish peroxidase. The membranes were washed 3 times in TBST for 10 min each. 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 Vision Works LS Acquisition and Analysis software.

**Determination of Trx enzyme activity**

Trx activity was determined by the insulin disulfide reduction assay [17]. In brief, 40 mg of tissue homogenates were pre-incubated at 37°C for 15 min with 2 mL activation buffer (100 mmol/L HEPES, 2 mmol/L EDTA, 1 mg/mL bovine serum albumin, and 2 mmol/L dithiothreitol) for Trx reduction. Samples were then mixed with 20 uL reaction buffer (100 mmol/L HEPES, 2.0 mmol/L EDTA, 0.2 mmol/L NADPH, and 140 mmol/L insulin), the reaction was initiated by the addition of mammalian Trx reductase (1 mL, 15 mU, Sigma) or water to the controls, and the samples were incubated for 30 min at 37°C. Finally, the reaction was stopped by adding 125uL of stopping solution (0.2 mmol/L Tris-HCl, 10 mmol/L guanidine-HCl, and 1.7 mmol/L3-carboxy-4-nitrophenyl disulfide; DTNB) followed by absorption measurement at 412 nm.

**Immunoprecipitation and immunoblotting**

Trx-1 nitration and Trx-1/ASK1 interaction detection were performed as described [18]. In brief, Trx-1 in homogenized lung tissues was immunoprecipitated with a monoclonal antibody against Trx-1. Trx-1 nitration and Trx-1/ASK1 interaction were examined, respectively, by immunoblotting using a primary antibody against nitrotyrosine or against ASK1 after sample separation. After incubation with horseradish peroxidase conjugated secondary antibody, the blot was developed with an ECL-Plus chemiluminescence reagent kit and visualized with UVP Bio-Imaging Systems. Blot densities were analyzed with Vision Works LS Acquisition and Analysis software.

**p38 MAPK activity assay**

p38 MAPK activity assay was performed by p38 MAPK assay kit according to the manufacturer's instructions.

**In vitro nitration of Trx-1**

Human Trx-1 was subjected to in vitro nitration with a provided procedure described by Guo et al [19]. In brief, purified human Trx-1(dissolved in 0.1 µM phosphate buffer, pH 7.4, final concentration of 50 µM) was incubated at 37°C for 30 min with SIN-1 (final concentration of 100 µM). Unreacted SIN-1 was removed by ultrafiltration over membranes with a 3-kDa cutoff.

**Statistical analysis**

All values in the text and figures were presented as mean±SD of independent experiments. All data (except immunoblotting density) were subjected to two-way ANOVA followed by Bonferroni correction for post hoc t test. Immunoblotting densities were 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 mice

As C57/BL6 mice are more susceptible to BLM induced fibrosis [20], C57/BL6 mice were selected in the present study. After BLM exposure, the mice weight dropped significantly [data not shown]. In the lungs of control group, both the HE staining and Masson staining results showed that intact and clear alveoli, normal interstitium, few inflammatory cells were observed in the lungs at 7 d or 28 d. However, BLM administration caused progressive lung damage, demonstrating as destruction of lung alveoli, inflammatory cells infiltration and thickening of lung interstitium (Fig. 1).

The apoptosis rate of pulmonary cells increased in the BLM-induced pulmonary fibrosis in mice

Apoptosis of AECs has been revealed to play a critical role in IPF [21, 22]. To investigate whether BLM-induced pulmonary fibrosis was associated with increased pulmonary cells apoptosis rate, the TUNEL straining and caspase-3 activity were examined. Compared with the control group, the rate of TUNEL-positive nuclei or caspase-3 activity in BLM group increased significantly, which revealed that apoptosis might be involved in BLM-induced pulmonary fibrosis (Fig. 2).

BLM-induced protein nitration in mice

Nitrotyrosine has been revealed as a footprint of protein nitration [23, 24]. The enzyme-linked immunosorbent assay (ELISA) kit was used to examine nitrotyrosine content. BLM group manifested nitration formation, while absent in control groups. As iNOS is a key enzyme to induce NO production, its expression was determined in the research. The expression of iNOS was induced in the lungs of BLM group, which means that the increase of nitrotyrosine content might be the result of iNOS overexpression (Fig. 3).

The activity and nitrated Trx in BLM-induced pulmonary fibrosis in mice

Trx is a key regulator of oxidative stress [25], therefore, we examined Trx’s activity. Compared with the control group, in the BLM-induced pulmonary fibrosis group, Trx’s activity decreased while its expression increased. As Trx is susceptible to nitratative modification, resulting in irreversible inhibition, the expression of nitrated Trx was determined. Compared with control group, the expression of nitrated Trx increased dramatically in BLM group (Fig. 4). The results are in accordance with our previous results [14].

The inhibition of Trx-1/ASK1 interaction and increased p38MAPK activity was involved in BLM-induced pulmonary fibrosis in C57/BL6 mice

Previous researches have revealed that Trx exerts its antiapoptotic effect by binding to ASK1 (causing inhibition of the downstream proapoptotic kinases) [26]. As the fibrosis rate of pulmonary cells is more obvious at day 28 after BLM administration, therefore, 28 d was chosen in the following study. We investigated the signaling pathway by which nitration of Trx increased the BLM-induced pulmonary
fibrosis. Trx was physically associated with ASK1 in control group, and p38MAPK activity was inhibited. In BLM group, the association between Trx and ASK1 was decreased, with the increase of p38 MAPK activity. The results suggested that increased activation of the p38-MAPK signaling pathway may contribute to the increased ACE apoptosis in BLM-induced pulmonary fibrosis in mice (Fig.5).

**Blockade of Trx-1 nitrative inactivation, or Trx-1 supplement, is protective of BLM-induced pulmonary fibrosis**

To further obtain more direct evidence to support the hypothesis that increased nitrative Trx is causatively related to increased BLM-induced pulmonary fibrosis in mice, aminoguanidine (AG), or rhTrx was administered before BLM administration. As shown in Figs.6 and 7, treatment with AG reduced Trx nitration, preserved Trx activity, restored TRX1/ASK1 interaction, and inhibited p38 MAPK activity in BLM-induced pulmonary fibrosis model, suggesting that AG could prevent the nitration of Trx, resulting in inhibiting p38MAPK pathway in BLM-induced pulmonary fibrosis model. In addition, treatment with AG or Trx significantly decreased the fibrosis rate, attenuated lung cells apoptosis rate, evidenced by decreasing TUNNEL staining and caspase3 activity. Finally, in vitro incubation of Trx with a peroxynitrite donor SIN-1(100µM) for 30 min inhibited Trx activity (135±15 µmol/min/mg Trx-1 vs. 762±53 µmol/min/mg Trx-1 in vehicle-incubated Trx-1, $P<0.001$). Administration with N-Trx lost Trx’s protecting effect in BLM-induced pulmonary fibrosis (Fig.8). All the results above fully provided the direct proof that Trx nitration modification plays an essential role in BLM-induced pulmonary fibrosis in mice.

**Discussion**

IPF is a chronic inflammatory interstitial lung disease with poor prognosis [2]. Despite significant progress in the understanding of pathological mechanisms of IPF, effective therapeutic interventions are to be revealed. In IPF, apoptosis of AECs has been reported as the main initiator event, though the fibrotic cascade is still unknown. Martinez et al reviewed a connection between ROS overload and the apoptosis rate of AECs in IPF patients [27]. ROS/RNS may induce apoptosis by stimulating caspase 3, inducing the release of cytochrome c and DNA fragmentation, resulting in activation of ASK1-mitogen-activated protein kinase (MAPK) pathway [5]. We attempted to reveal a key protein target susceptible and relevant for nitration. Trx is a small ubiquitous protein which exerts plenty of biological functions [28]. Except for its redox regulating effect, Trx exerts its apoptosis regulating effect by Trx-1/ASK1 interaction mechanism. In resting situations, ASK1 is inhibited by Trx-1 binding. When Trx loses its activity, Trx separates from ASK1, ASK1/p38MAPK apoptosis pathway is stimulated [26].

Trx family includes thioredoxin, thioredoxin reductase, NADPH. Trx contains five cystines, which is essential in maintaining its function. Cysteines 32 and 35 are the main domains in regulating its redox state. When the functional groups of cysteines32 and cysteines35 are oxidized, Trx cannot bind to its target protein, resulting in Trx inactivation. When oxidized, Trx itself is reduced by thioredoxin reductase to keep its redox activity [29]. Moreover, Trx activity decreased when cysteine-73 was glutathionylazed under
oxidative conditions [30]. Tao et al revealed that the S-nitrosylation of Trx at cysteine-73 contributed to the cardioprotective and anti-apoptotic functions of Trx in myocardial-ischemia model of mice [31].

Nitration has been revealed as Trx's fourth post-transcriptional modification. Tao and Zhang reported that Trx nitration at the tyrosine residue caused its inactivation in an ischemia-reperfusion rat model [13]. Yin revealed that changes in Trx nitration might contribute to exaggerated ischemia/reperfusion injury by regulating the apoptosis rate in cardiac cells [32]. These results demonstrated that the inhibition of Trx nitration may attenuate cardiac injury after myocardial ischemia. In our previous study, we observed that in BLM-induced pulmonary fibrosis in rats, the activity of thioredoxin decreased while the level of nitrated thioredoxin increased, we proposed that nitration of thioredoxin resulting in the increasing of AECs apoptosis by p38MAPK-ASK1 pathway, but we did not provide the direct proof [14].

In this research, we revealed that in BLM-induced pulmonary fibrosis in mice, decreased activity of Trx caused the activation of ASK1-p38 MAPK pathway, resulting in lung cells apoptosis. To provide further cause-effect evidence, we examined whether blockade of Trx-1 nitrative inactivation or supplementation with exogenous Trx-1 could affect TRX-ASK1 activity and p38MAPK activity, reduce ACEs apoptosis rate, protect mice against BLM-induced pulmonary fibrosis. We demonstrated that either treatment reduced pulmonary fibrosis to some extent by decreasing AEC apoptosis rate, while N-Trx lost the effect. As administration of oxidized Trx-1 will not lose antiapoptotic effect unless thioredoxin reductase is inhibited concomitantly [33], suggesting that oxidative modification of Trx1 is reversible, which indicates that redox modification of Trx1 may not play essential role in regulating apoptosis. However, nitrative modification of Trx is irreversible [34]. These results provide the first evidence that Trx-1 nitrative inactivation plays a causative role in pulmonary fibrosis. But the BLM-induced pulmonary fibrosis is different from the IPF patients, so the conclusion needed to be in clinical practice.

**Conclusions**

Our research's novel results strongly suggest that in BLM-induced pulmonary fibrosis in mice, overload of ONOO− formation promote of RNS production, enhancing nitrative inactivation of Trx-1, and thereby sensitizing lung injury by elevated ASK1-p38 MAPK signaling pathway-mediated AEC apoptosis. Therefore, inhibition of thioredoxin nitration might be one of the potential therapeutic targets for the treatment of idiopathic pulmonary fibrosis.

**Declarations**

**Ethics approval and consent to participate**

All animal experiments were approved by the Animal Research Committee of Zheng Zhou University, Zheng Zhou, China.

**Consent for publication**
All the authors have consented to the publication of the research.

**Availability of data and materials**

Data could be obtained upon request to the corresponding author.

**Competing interests**

The authors declare that there is no conflict of interests.

**Funding**

The research was supported by Natural Science Foundation of China (No. U1504801).

**Authors’ contributions**

LW, W-y X, Y-w T and FH undertake main experiments. LW undertook the design of this project and did result analysis.

**Acknowledgements**

None

**References**

1. Rangarajan S, Bone NB, Zmijewska AA, Jiang SN, Park DW, Bernad K, et al. Metformin reverses established lung fibrosis in a bleomycin model. Nat Med. 2018; 24:1121–1127. doi: 10.1038/s41591-018-0087-6.

2. Beers MF, Morrisey EE. The three R's of lung health and disease: repair, remodeling, and regeneration. J. Clin. Invest. 2011; 121: 2065–2073. doi: 10.1172/JCI45961.

3. Tannickal V J, Zhou Y, Gaggar A, Duncan SR. Fibrosis: ultimate and proximate causes. J. Clin. Invest. 2014;124: 4673–4677. doi: 10.1172/JCI74368.

4. Gan CL, Zhang QY, Liu HY, Wang G, Wang LQ, Li YL, et al. Nifuroxazide ameliorates pulmonary fibrosis by blocking myofibroblast genesis: a drug repurposing study. Respiratory Research. 2022;23(1): 32. doi: 10.1186/s12931-022-01946-6.

5. Yoshida K, Kuwano K, Hagimoto N, Watanabe K, Matsuba T, Fujita M, et al. MAP kinase activation and apoptosis in lung tissues from patients with idiopathic pulmonary fibrosis. J Pathol. 2002; 198:388–396. doi: 10.1002/path.1208.

6. Saleh D, Barnes PJ, Giaid A. Increased production of the potent oxidant peroxynitrite in the lungs of patients with idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 1997;155: 1763–69. doi: 10.1164/ajrccm.155.5.9154889.
7. Ricciardolo FLM, Di Stefano A, Sabatini F, Folkerts G. Reactive nitrogen species in the respiratory tract. Eur J Pharmacol. 2006;533: 240–252. doi: 10.1016/j.ejphar.2005.12.057.

8. Samuel SM, Thirunavukkarasu M, Penumathsa SV, Koneru S, Zhan L, Maulik G, et al. Thioredoxin-1 gene therapy enhances angiogenic signaling and reduces ventricular remodeling in infarcted myocardium of diabetic rats. Circulation. 2010; 121: 1244–1255. doi: 10.1161/CIRCULATIONAHA.109.872481.

9. Yu TE, Zhang WL, Lin YS, Li Q, Xue J, Cai ZY, et al. Prognostic value of serum thioredoxin levels in ischemic stroke. Neurol Res. 2017; 39: 988–995. doi: 10.1080/01616412.2017.1359882.

10. Xu J, Li TP, Wu HQ, Xu T. Role of thioredoxin in lung disease. Pulm Pharmacol Ther. 2012; 25:154–62. doi: 10.1016/j.pupt.2012.01.002.

11. Hoshino T, Nakamura H, Okamoto M, Kato S, Araya S, Nomiyama K, et al. Redox-active protein thioredoxin prevents proinflammatory cytokine- or bleomycin-induced lung injury. Am J Respir Crit Care Med. 2003; 168:1075–1083. doi: 10.1164/rccm.200209-982OC.

12. Tanaka R, Watanabe H, Kodama A, Chuang TG, Ishima Y, Hamasaki K, et al. Long-acting human serum albumin-thioredoxin fusion protein suppresses Bleomycin-Induced pulmonary fibrosis progression. J Pharmacol Exp Ther. 2013; 345: 271–83. doi: 10.1124/jpet.112.201814.

13. Tao L, Jiao X, Gao E, Lau WB, Yuan Y, Lopez B, et al. Nitrative inactivation of thioredoxin-1 and its role in postischemic myocardial apoptosis. Circulation. 2006; 114: 1395–402. doi: 10.1161/CIRCULATIONAHA.106.625061.

14. Wang L, Song YM, Li XK, Guo HZ, Zhang GJ. Role of thioredoxin nitration in bleomycin-induced pulmonary fibrosis in rats. Can J Physiol Pharmacol. 2016; 94: 59–64. doi: 10.1139/cjpp-2015-0121.

15. Wan XY, Tian XB, Du Y, Lu Y, Xiao YT. Long non-coding RNA H19 deficiency ameliorates bleomycin-induced pulmonary inflammation and fibrosis. Respir Res. 2020; 21(1):290. doi: 10.1186/s12931-020-01534-6.

16. Kim SJ, Cheresh P, Jablonski RP, Ratchek L, Yeldandi A, Piseaux-Aillon P, et al. Mitochondrial 8-oxoguanine DNA glycosylase mitigates alveolar epithelial cell PINK1 deficiency, mitochondrial DNA damage, apoptosis, and lung fibrosis. Am J Physiol Lung Cell Mol Physiol. 2020;318: L1084-L1096. doi: 10.1152/ajplung.00069.2019.

17. Holmgren A, Björnstedt M. Thioredoxin and thioredoxin reductase. Methods Enzymol. 1995; 252: 199–208. doi: 10.1016/0076-6879(95)52023-6.

18. Vadseth C, Souza JM, Thomson L, Seagraves A, Nagaswami C, Scheiner T, et al. Prothrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species. J Biol Chem. 2004; 279: 8820–6. doi: 10.1074/jbc.M306101200.

19. Guo W, Adachi T, Matsu R, Xu S, Jiang B, Zou MH, et al. Quantitative assessment of tyrosine nitration of manganese superoxide dismutase in angiotensin II-infused rat kidney. Am J Physiol Heart Circ Physiol. 2003; 285: H1396-403. doi: 10.1152/ajpheart.00096.

20. Lattaa V D, Cecchettini A, Rya SD, Morales MA. Bleomycin in the setting of lung fibrosis induction: from biological mechanisms to counteractions. Pharmacol. Res. 2015: 97: 122–130. doi:
Hagimoto N, Kuwano K, Miyazaki H, Kunitake R, Fujita M, Kawasaki M, et al. Induction of apoptosis and pulmonary fibrosis in mice in response to ligation of Fas antigen. Am J Respir Cell Mol Biol. 1997; 17(3):272–278. doi: 10.1165/ajrcmb.17.3.2893.

Drakopanagiotakis F, Xifteri A, Polychronopoulos V, Bouros D. Apoptosis in lung injury and fibrosis. Eur Respir J. 2008; 32: 1631–1638. doi: 10.1183/09031936.00176807.

Kruk J, Aboul-Enein HY, Kladna A, Bower JE. Oxidative stress in biological systems and its relation with pathophysiological functions: the effect of physical activity on cellular redox homeostasis. Free Radic Res. 2019; 53(5): 497–521. doi:10.1080/10715762.2019.1612059.

Hsu YC, Wang LF, Chien YW. Nitric oxide in the pathogenesis of diffuse pulmonary fibrosis. Free Radic Biol Med. 2007; 42:599–607. doi: 10.1016/j.freeradbiomed.2006.11.031.

Kuwano K, Hagimoto N, Tanaka T, Kawasaki M, Hara N. Expression of apoptosis regulatory genes in epithelial cells in pulmonary fibrosis in mice. J. Pathol. 2000; 190:221–229. doi: 10.1002/(SICI)1096-9896(200002)190:2<221: AID-PATH495 > 3.0.CO;2-J.

Zhang JM, Duan DZ, Osama A, Fang JG. Natural molecules targeting thioredoxin system and their therapeutic potentials. Antioxid Redox Signal.2021;34(14):1083–1107. Doi:10.1089/ars.2020.8213.

Watson WH, Pohl J, Montfort WR, Stuchlik O, Reed MS, Powis G, et al. Redox potential of human thioredoxin 1 and identification of a second dithiol/disulfide motif. J Biol Chem. 2003; 278: 33408–33415. doi: 10.1074/jbc.M211107200.

Michelet L, Zaffagnini M, Marchand C, Collin V, Decottignies P, Tsan P, et al. Glutathionylation of chloroplast thioredoxin f is a redox signaling mechanism in plants. Proc Natl Acad Sci U S A. 2005;102: 16478–16483. doi: 10.1073/pnas.0507498102.

Tao L, Gao E, Bryan NS, Qu Y, Liu HR, Hu A, et al. Cardioprotective effects of thioredoxin in myocardial ischemia and reperfusion: role of S-nitrosation. PNAS. 2004; 101: 11471–6. doi: 10.1073/pnas.0402941101.

Yin T, Hou RR, Liu SW, Lau WB, Wang HC, Tao L. Nitrative inactivation of thioredoxin-1 increases vulnerability of diabetic hearts to ischemia/reperfusion injury. J Mol Cell Cardiol. 2010; 49: 354–61. doi: 10.1016/j.yjmcc.2010.05.002.

Jiao WX, Bai M, Yin HW, Liu JY, Sun J, Su XX, et al. Therapeutic effects of an inhibitor of thioredoxin reductase on liver fibrosis by inhibiting the transforming Growth factor-β1-Smads Pathway. Front Mol Biosci. 2021; 8, 690170. doi: 10.3389/fmolb.2021.690170.

Yoon S, Eom GH, KangG. Nitrosative stress and human disease: therapeutic potential of denitrosylation. Int J Mol. 2021; 22: 9794. doi: 10.3390/ijms22189794.
Figure 1

(A) H&E staining and (B) Masson staining for pathological changes in C57/BL6 mice lungs caused by bleomycin (BLM). BLM (5.0 mg/kg body mass in phosphate buffered saline, intratracheal) was given once to mice. Sections of pulmonary tissues were prepared on days 7 and 28 after BLM administration, and subjected to histopathological and microscopic examination (×200 magnification). (C) Semiquantified histological analysis of lung alveolitis on day 7 and 28 after BLM administration. (D) Semiquantified histological analysis of pulmonary fibrosis on day 7 and 28 after BLM administration. Data are means ± SD, n = 6-8 in each group. **P < 0.01 vs. control group.
Figure 2

Apoptosis rate of lung cells after BLM administration. BLM (5.0 mg/kg body mass in phosphate buffered saline, intratracheal) was given once to mice. Sections of pulmonary tissues were prepared on day 7 and 28 after BLM administration. (A) Lung cells apoptosis determined by TUNEL staining. Representative photographs of lung sections are shown. (B) Quantification of apoptotic nuclei. TUNEL-positive nuclei are expressed as a percentage of the total number of nuclei, automatically counted and calculated by Image-
Pro Plus software. (C) lung cells apoptosis determined by caspase-3 activity by colorimetric assay kit. **P 0.01 vs. control group. n=6 to 8 in each group.

**Figure 3**

BLM increased protein nitration in the lungs of C57 /BL6mice. BLM (5.0 mg/kg body mass in phosphate buffered saline, intratracheal) was given once to mice. Sections of pulmonary tissues were prepared on day 7 and 28 after BLM administration. Nitrotyrosine content in lungs determined by immunohistochemistry (A) and ELISA assay (B). Representative photographs of lung sections are shown. The iNOS expression in lungs was determined by Western-blotting method(C). *P<0.05 vs. control group,**P<0.01 vs. control group. n=6 to 8 in each group.
Figure 4

Effect of BLM on the activity, expression of Trx and the nitrated Trx in mice. BLM (5.0 mg/kg body mass in phosphate buffered saline, intratracheal) was given once to the mice. Sections of pulmonary tissues were prepared on day 7 and 28 after BLM administration, the activity (4A) and expression of thioredoxin (4B) and nitrated Trx (4C) were examined. Data are the mean ± SD, n = 6-8 C57/BL6 mice in each group; *P < 0.05 compared with the control group, **P < 0.01 vs. control group.
Figure 5

The inhibition of Trx-1/ASK1 interaction and increased p38MAPK activity was involved in BLM-induced pulmonary fibrosis in C57 mice. BLM (5.0 mg/kg body mass in phosphate buffered saline, intratracheal) was given once to the mice. Sections of pulmonary tissues were prepared on day 28 after BLM administration. (A) Trx-1/ASK1 binding in lungs determined by immunoprecipitation. (B) p38 MAPK activity determined by assay kit. Representative immunoblot graphs are shown. Data are the mean ± SD, n = 6-8 C57/BL6 mice in each group; **P < 0.01 vs. control group.
Figure 6

Effect of aminoguanidine (AG) on nitrotyrosine content in lungs of BLM-induced fibrosis in C57 /BL6 mice. BLM (5.0 mg/kg body mass in phosphate buffered saline, intratracheal) was given once to the mice. Before BLM administration, mice were randomized to receive either vehicle (0.9% NaCl), aminoguanidine (AG, a peroxynitrite scavenger, 40 mg/kg) by intraperitoneal injection. Sections of pulmonary tissues were prepared on day 28 after BLM administration. (A) Nitrotyrosine content in lung sections. (B) Trx activity in lung sections in different groups. (C) Nitrated Trx in lung sections in different
Figure 7

Effect of AG on p38-MAPK pathway in BLM-induced lung fibrosis in C57/BL6 mice. BLM (5.0 mg/kg body mass in phosphate buffered saline, intratracheal) was given once to the mice. Before BLM administration, mice were randomized to receive either vehicle (0.9%NaCl), aminoguanidine (AG, a peroxynitrite scavenger, 40 mg/kg) by intraperitoneal injection. Sections of pulmonary tissues were prepared on day 28 after BLM administration. (A) BLM-induced dissociation of Trx from ASK1 in lung sections. (B)p38MAPK activation. Data are the mean ± SD, n = 6-8 C57 mice in each group; **P < 0.01 compared with the control group; #P < 0.05 compared with BLM group, ##P < 0.01 compared with BLM group.
Figure 8

Effect of AG, exogenous rhTrx-1, or nitrated Trx-1 (N-TRX-1) on BLM-induced pulmonary fibrosis. BLM (5.0 mg/kg body mass in phosphate buffered saline, intratracheal) was given once to the mice. Before BLM administration, mice were randomized to receive either vehicle (0.9%NaCl), aminoguanidine (AG, a peroxynitrite scavenger, 40 mg/kg), recombinant human Trx-1 (40ug/20g), or nitrated Trx-1 (please see details) by intraperitoneal injection. Sections of pulmonary tissues were prepared on day 28 after BLM administration. (A) the rate of pulmonary fibrosis. (B) and (C) the apoptosis rate of lung cells in fibrosis mice. Recombinant human Trx-1 was incubated with vehicle or SIN-1 at 37 °C for 30 min in the presence and absence of MnTBAP chloride (500 μM, 5-times of SIN-1 concentration), a cell-permeable SOD mimetic (SODm). Unreacted SIN-1 was removed by ultrafiltration over membranes with a 3-kDa cutoff. Samples were electrophoretically size fractionated on SDS–PAGE and transferred onto a polyvinylidene difluoride (PVDF)-plus membrane, and nitrated Trx-1 was detected with antinitrotyrosine antibody. Data are the mean ± SD, n = 6-8 C57/BL6 mice in each group; **P < 0.01 compared with the control group; #P < 0.05 compared with BLM group, ##P < 0.01 compared with BLM group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.
• Fig.8.jpg
• Fig.7ASK1.jpg
• Fig.7p38activity.jpg
• Fig.8d1.jpg
• Fig3.iNOS.jpg
• Fig4.TRX1.jpg
• Fig4.coIP.jpg
• Fig5.ASK1.jpg
• Fig5.p38.jpg
• Fig6.COIP.jpg