N-Acetylcysteine Downregulation of Lysyl Oxidase Activity Alleviating Bleomycin-Induced Pulmonary Fibrosis in Rats

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Key Words

Fibrosis · Glutathione · Idiopathic pulmonary fibrosis · N-acetylcysteine · Lysyl oxidase · Pulmonary fibrosis · α -Smooth muscle actin · Transforming growth factor- β_1

Abstract

Background: Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal lung disease without beneficial therapy, except for lung transplantation. A high oral dose of N-acetylcysteine (NAC) added to prednisone and azathioprine has been found to improve lung function in IPF patients, though the mechanism of action remains poorly understood. **Objective:** Based on our previous findings showing elevation of glutathione (GSH) content associated with downregulation of lysyl oxidase (LOX) activity, which is essential for collagen deposition, the aim of the present study was to test the hypothesis that NAC alleviates IPF by regulating LOX function. **Methods:** We firstly analyzed the time course of collagen deposition in lung tissue, hydroxyproline content, LOX activity, GSH levels, and transforming growth factor- β_1 (TGF- β_1) and α -smooth muscle actin (α -SMA) expression in bleomycin (BLM)-induced pulmonary fibrosis in a rat model. Then, we focused our studies on NAC modulation of LOX activity. **Results:** LOX activity was increased on day 9 and peaked 14 days after BLM administration, while TGF- β_1 protein peaked

on day 9. Interestingly, NAC treatment for 14 days from day 0 reversed LOX activity to normal levels and increased GSH levels in the lung of BLM-dosed rats. Consistently, NAC partially attenuated pulmonary fibrosis and inhibited TGF- β_1 and α -SMA expression in this model. **Conclusions:** Our study supports a novel mechanism of NAC alleviating IPF by inhibition of LOX activity via elevation of lung GSH in BLM-induced pulmonary fibrosis. The TGF- β_1 / α -SMA pathway may also play an important role in modulation of LOX activity.

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and lethal lung interstitial disorder, which is characterized by the loss of alveolar structures and functions following apoptosis of epithelial and endothelial cells, proliferation of fibroblasts and excessive deposition of extracellular matrix [1]. Unfortunately, the etiology and mechanisms for IPF pathogenesis remain unclear. Though clinical trials of some agents have suggested a possible benefit, such as pirfenidone [2, 3], interferon, colchicines, bosentan, penicillamine, losartan [4], doxycycline [5] and nilotinib [6] etc., evidence suggests that there is no proven pharmacological therapy for IPF to date [7, 8].

An oxidant-antioxidant imbalance may play a critical role in the pathogenesis of IPF. The level of glutathione (GSH), a major antioxidant, was decreased to approximately one third of the normal concentration in IPF [9]. N-acetyl-L-cysteine (NAC), a precursor of GSH acting as a free radical scavenger, has been shown to restore pulmonary GSH levels in patients with IPF [10] and attenuate early inflammatory responses and late fibrotic lesions in bleomycin (BLM)-dosed animals [11–14]. In a double-blind, randomized, placebo-controlled multicenter clinical trial (the IFIGENIA trial), high-dose, oral NAC in combination with prednisone and azathioprine markedly improved lung function in IPF patients [15, 16]. A recruiting clinical trial (the PANTHER-IPF study, NCT00650091) showed that the three-drug regimen of prednisone + azathioprine + NAC led to a significant increase in mortality and serious adverse events, and therefore this arm was stopped, but the two remaining arms of the study, NAC alone against placebo, are still under investigation with results expected in the third quarter of 2013. Still, NAC may be a promising treatment of IPF and its mechanisms of action upon the lung remain unresolved.

Lysyl oxidase (LOX), a copper-dependent enzyme, oxidizes specific lysine residues in collagen and elastin leading to the formation of inter- or intramolecular cross-links essential for stabilization of the extracellular matrix [17]. Strong evidence supports an association of pulmonary fibrosis with elevated LOX activity [18, 19]. In a study by Counts et al. [20], elevation of LOX activity preceded the maximal increase in total lung hydroxyproline (HYP) elicited by BLM in rats. LOX was upregulated in cultured human fetal lung fibroblasts exposed to BLM [21]. β -Aminopropionitrile [22], an LOX inhibitor, as well as taurine and niacin [23] prevented BLM-induced pulmonary fibrosis in hamsters. Thus, LOX may be a critical target of antifibrosis [24].

Our previous studies indicated that enhancement of cellular GSH by GSH monoethyl ester decreased levels of LOX protein species and catalytic activity in vitro [25]. Elevation of cellular GSH induced reduction of LOX activity in cells exposed to cigarette smoke condensate or cadmium [26]. These findings support the possibility that NAC may ameliorate IPF at least in part via downregulation of LOX. In this study, we examined whether NAC attenuates pulmonary fibrosis by modulation of LOX activity in the BLM animal model in an effort to provide critical information beneficial for IPF treatment.

Materials and Methods

Reagents

BLM hydrochloride A2 was purchased from Nippon Kayak, Japan; NAC from Sigma-Aldrich, USA, and polyclonal antibodies to TGF- β_1 (sc-146) and α -smooth muscle actin (α -SMA; ab5694) from Santa Cruz Biotechnology, USA, and Abcam, USA, respectively.

Animal Model and Experimental Protocol

All experiments were performed according to international and institutional guidelines for animal care and were approved by the Sun Yat-sen University Animal Care and Use Committee. All surgery procedures were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Pathogen-free, male Sprague-Dawley rats weighing 200–250 g were from the Experimental Animal Center of the Southern Medical University (Guangzhou, China) and kept with water and standard rodent chow ad libitum. To start experiments, BLM was dissolved in sterile saline to the final concentration of 5 mg/ml. Animals received a single intratracheal instillation of BLM at a dose of 5 mg/kg body weight on day 0. Control animals were instilled with the same volume of saline only.

To investigate the time course of pulmonary LOX activity in the rat BLM model, treated animals were sacrificed on days 9, 14, 28 and 35 in experiment 1. Since we focused on the late fibrotic phase of the model, based on the literature published [27], day 9 was selected as the starting time point and day 14 was a time point at which lung fibrotic lesions and LOX activities were tested after BLM administration. Animals in the control group were sacrificed on day 14 ($n = 5/\text{group}$).

Secondly, to investigate the effect of NAC on LOX activity in this model, animals were randomly divided into three groups ($n = 5/\text{group}$) in experiment 2, i.e. the control group (saline + deionized water), the B+W group (BLM + deionized water) and the B+N group (BLM + NAC). Treatments (deionized water or NAC) were administered orally by gavage on a daily basis (at 9:00 am) from day 0. All animals were sacrificed on day 14. The oral dose of NAC was 490 mg/kg per day. NAC was dissolved in deionized water to the final concentration of 122.5 mg/ml for the animals, as described [14, 28].

Left lungs in all animals were used for histological assessment. Right lungs were removed en bloc after clearing the major visible bronchi, blotted dry, weighed, homogenated in 2 volumes of Tris-HCl buffer (50 mM, pH 7.4), stored at -80°C for biochemical assays, such as HYP content, protein expression of transforming growth factor- β_1 (TGF- β_1), a profibrotic factor, and α -SMA, a marker of myofibroblast differentiation, GSH concentration and LOX activity.

Histology

For histological studies, the left lungs were perfused via the main bronchus with 10% neutral-buffered formalin and fixed in this fixative solution for 24 h. Tissue blocks were dehydrated in a graded series of ethanols, embedded in paraffin, cut into 4- μm -thick serial sections and processed for hematoxylin-eosin (HE) and Masson's trichrome staining.

HYP Content

Pulmonary collagen deposition was quantitatively assessed by the HYP assay with the HYP detection kit (Nanjing Jiancheng Bio-

engineering Institute, China) according to the manufacturer's instructions. Briefly, Lung tissue samples were homogenized and hydrolyzed at 100°C for 20 min, adjusted to a suitable pH value and added to active carbon. After centrifugation, 500 µl of the supernatant were mixed with the HYP chromogenic agent and measured as absorbance at 540 nm (A_{540}) using an iMark microplate reader (Bio-Rad, USA) [29] and the HYP concentration was determined (expressed as µg/g wet lung).

Western Immunoblotting

Homogenized lung samples were lysed in 6 volumes of ice-cold M-PER® mammalian protein extraction reagent with cocktail proteinase inhibitors and 4 mol/l urea (Pierce Biotechnology, USA). Protein concentration was assessed using the Pierce BCA protein assay kit (Pierce Biotechnology). Equal amounts of protein were separated by 12 (for α -SMA) or 15% (for TGF- β_1) SDS-PAGE. After electrophoresis, the proteins were transferred to PVDF membrane, blocked with 5% skimmed milk in TBS for 1 h at room temperature and reacted with antibodies against TGF- β_1 (1:500) or α -SMA (1:200) at 4°C overnight. After reaction with horseradish peroxidase-labeled secondary antibody, the immune complexes were visualized using the ECL detection reagents following the manufacturer's instructions.

LOX Activity Assessment

Total LOX activity in rat lung tissue was assayed with the methods reported by Palamakumbura [30]. In brief, lung homogenization samples were mixed with 3.5 volumes of extraction buffer containing 1.2 mol/l urea and 50 mmol/l sodium borate (pH 8.2) reacted at 4°C overnight. After centrifugation, supernatant was added to the reaction mixture containing 50 mmol/l sodium borate, 1.2 mol/l urea, pH 8.2, 1 unit/ml horseradish peroxidase (Roche, Switzerland), 10 µmol/l Amplex red (Invitrogen, USA) and 10 mmol/l 1,5-diaminopentane (Sigma) with or without 0.5 mmol/l β -aminopropionitrile (Sigma), an inhibitor of LOX. Samples were incubated at 37°C for 30 min and placed on ice. The difference in fluorescence intensity was recorded at excitation and emission wavelengths of 563 and 587 nm, respectively, using a Hitachi 850 fluorescence spectrophotometer (Hitachi Instruments, Japan). All enzyme activities were expressed as fluorescence at 587 nm, corrected for background levels of fluorescence determined in the reaction mixture supplemented with β -aminopropionitrile. Assays were repeated three times for all samples.

Total Lung GSH Content Assay

Total lung GSH content in rats was determined by the enzymatic recycling method, as described previously [31]. Briefly, lung homogenization samples were added to 3.5 volumes of ice-cold extraction solution containing 0.1% Triton X-100 and 0.6% sulfosalicylic acid in KPE (0.1 mmol/l PBS with 5 mmol/l EDTA disodium salt, pH 7.5) reacted at 4°C for 30 min. After centrifugation, 20 µl supernatant were transferred to a 96-well microtiter plate, and 120 µl reaction mixture containing 0.33 mg/ml NTNB (Roche) and 1.67 units/ml GSH reductase (Sigma) were added, incubated for 30 s, then 60 µl β -NADPH (Roche) were added and absorbance was read at 415 nm (A_{415}) every 30 s for 2 min. GSH content was described as change in absorbance/min corrected for the background determined using KPE only. Assays were repeated three times for each sample.

Statistical Analysis

SigmaPlot v11.1 (Systat Software, USA) was used for statistical analysis. Data are expressed as means $\pm$ SD. Comparisons were made using one-way analysis of variance followed by a least-significant difference post hoc test. $p < 0.05$ was considered statistically significant.

Results

The Effect of NAC on Lung Histology in the Rat BLM Model

HE-stained lung sections were examined light microscopically. Figure 1a shows normal lung histology (control group). Nine days after BLM administration, alveolar septal thickening was accompanied by marked peribronchiolar and interstitial infiltration with macrophages, lymphocytes, neutrophils and fibroblasts. Such inflammatory cells were also distributed in some alveolar spaces. On day 14, alveolar septal thickening and cellular infiltration became more severe, and fibroblastic foci (the hallmark of IPF) appeared. On days 28 and 35, fibroblastic foci were increased, alveolar architectures collapsed and diffuse fibrosis formed. Notably, at these times bronchi were filled with diapedic neutrophils. In experiment 2, after 14 days of oral NAC, although inflammatory and fibrotic lesions were still present in the lungs, less fibroblastic foci, widening alveolar septa and decreased inflammatory cell infiltration were observed in the B+N group in comparison to those in the B+W group (fig. 1b).

Effect of NAC on Pulmonary Collagen Deposition

Masson's trichrome staining revealed markedly increased collagen deposition from days 9 to 35 (fig. 1a). The increase in collagen deposition was associated with a loss of alveolar structures. Consistently, HYP content, an index for collagen accumulation, was significantly elevated on day 9 ($p < 0.01$ vs. control) and remained increased up to the end of the experiment (fig. 1c). Clearly, BLM causes a strong lung-fibrotic response characterized by a time-dependent collagen deposition. Consistent with the histological results, NAC intervention also resulted in a tendency to a lower HYP content in the lung of NAC-treated rats exposed to BLM, though the difference failed to reach significance (fig. 1d).

Effect of NAC on LOX Activity

As shown in figure 2a, LOX activity in BLM-dosed lungs was increased to 2.3-fold of the control on day 9, reached a peak amounting to 4.7-fold of the control on day 14 ($p < 0.01$) and subsequently returned to the day 9

Fig. 1. Histopathologic changes and collagen deposition in lungs from BLM-treated rats with or without NAC intervention. **a, b** Fibrosis was assessed by HE and Masson's trichrome staining. Each photomicrograph is representative for each group. Scale bars = 500 μ m. **c, d** Pulmonary collagen deposition was quanti-

tatively evaluated by testing HYP content. Each value represents the mean $\pm$ SD in each group (n = 5/group). * $p < 0.001$ vs. control group. **b, d** All animals were sacrificed 14 days after BLM administration.

Fig. 2. LOX activity in lungs from BLM-treated rats with or without NAC intervention. **a** Animals treated with BLM were sacrificed on days 9, 14, 28 and 35. **b** All animals were sacrificed 14 days after BLM administration. Total lung LOX activity was expressed as the mean $\pm$ SD of each group ($n = 5/\text{group}$). * $p < 0.01$ vs. control group, ** $p < 0.05$ vs. B+W group.

level 28 and 35 days after BLM administration. Interestingly, NAC treatment (B+N) almost completely abolished the elevation in LOX activity on day 14 induced by BLM (decrease to 31.7% of the B+W control; $p < 0.05$; fig. 2b).

Effect of NAC on Total Lung GSH Levels

To test the oxidant stress induced by BLM, GSH concentration in lung tissue was assessed. BLM caused a significant reduction in GSH concentration at all experimental time points in experiment 1, as previously reported [14] (fig. 3a).

NAC, a precursor of GSH, is able to restore pulmonary GSH levels. In experiment 2, BLM reduced the GSH concentration in lung tissue of the B+W group to 29.5% of the control ($p < 0.001$). NAC treatment reversed this effect; 70.7% of the control and 239.6% of the B+W group levels of pulmonary GSH were recovered ($p < 0.001$; fig. 3b).

Effects of NAC on TGF- β_1 and α -SMA

TGF- β_1 activation of myofibroblasts plays a central role in the progression of IPF, and this may be involved in the modulation of LOX by BLM. In the control group, TGF- β_1 expression was weak. The expression of TGF- β_1 increased markedly (62.1-fold of control), peaked on day 9 and remained elevated up to 35 days after BLM administration (fig. 4a). The trend of α -SMA, a marker of myofibroblast differentiation, was similar to LOX activity ex-

pression, i.e. it was elevated on day 9 (2.4-fold of control) and reached a plateau on day 14 (4.1-fold of control; fig. 4c), indicating that myofibroblasts may be the main cellular source of LOX. Moreover, the peak of TGF- β_1 preceded that of α -SMA revealing the role of TGF- β_1 in myofibroblast differentiation, as previously reported [32].

NAC treatment partially inhibited the upregulation of TGF- β_1 (0.7-fold of B+W group) and α -SMA (0.6-fold of B+W group) protein induced by BLM (fig. 4b, d).

Discussion

IPF has been a real challenge for its unknown etiology and pathogenic mechanism, resulting in dimmed therapy and poor prognosis. Three- and 5-year mortality rates of approximately 50–80% were reported for patients after diagnosis [33]. There are no data reporting improved survival or quality of life for patients with IPF with the currently available treatments. In patients receiving lung transplantation, 5-year survival approximates 50–56% [7]. The IFIGENIA trial demonstrated that a high oral dose of NAC, which was added to prednisone and azathioprine, preserves the vital capacity and single-breath carbon monoxide diffusing capacity of patients [15], rendering NAC a promising IPF treatment, but the results of the PANTHER-IPF study will provide more information.

Fig. 3. Total GSH content in lungs from BLM-treated rats with or without NAC intervention. **a** Animals treated with BLM were sacrificed on days 9, 14, 28 and 35. **b** All animals were sacrificed 14 days after BLM administration. Each value represents the mean $\pm$ SD (n = 5/group). * p < 0.001 vs. control group, ** p < 0.001 vs. B+W group.

In animal studies, NAC was found to ameliorate BLM-induced pulmonary fibrosis via the regulation of several key factors, such as attenuation of acute pulmonary inflammation via a decrease in chemokine and lipid hydroperoxide levels, improved pulmonary antioxidant protection, scavenging of reactive oxygen species and detoxification of BLM-generated radicals [11, 13]. However, the antifibrotic mechanism of NAC is still unknown.

LOX is an amino oxidase which catalyzes the oxidative deamination of specific lysine residues in collagen and elastin, leading to inter- or intramolecular cross-links. As a result, LOX may play an important role in the pathogenesis of IPF, as it is essential for the insolubilization and stabilization of extracellular matrix proteins [17], i.e. the hallmark of IPF. Actually, a strong association between pulmonary fibrosis and elevated LOX activity was reported [18–20], and inhibition of LOX activity prevented BLM-induced pulmonary fibrosis [22, 23]. We have found that BLM increased LOX activity in vitro in our previous work [21] and in vivo in this study. Furthermore, we also noted that increasing GSH downregulated LOX catalytic activity in vitro [25]. NAC is able to restore pulmonary GSH levels in IPF [10]. In this study, NAC reversed the content of GSH, inhibited LOX activity induced by BLM and partially alleviated pulmonary fibrosis in a rat model of BLM-induced IPF. Thus, downregulation of LOX activity by NAC may play a critical role in alleviation of BLM-induced pulmonary fibrosis in rats.

GSH, accounting for 90% of total cellular non-protein thiols [26], is shown to inhibit LOX activity. LOX is a metalloenzyme requiring Cu(II) as a cofactor for enzymatic function, which is added to the LOX active center through the secretory pathway and essential for the formation and the maintenance of the carbonyl cofactor identified as lysine tyrosylquinone [34]. Thiol-containing GSH and metallothionein are critical modulators for Cu metabolism. Freedman et al. [35] reported that Cu forms complexes with GSH soon after cell entry which were then transferred to metallothionein, providing temporary storage for cytoplasmic Cu. In fact, the majority of cytoplasmic Cu (>60%) is isolated as GSH complexes [35]. Downregulation of LOX is observed in cultured RFL6 cells exposed to cigarette smoke condensate or cadmium when cells were treated with GSH monoethyl ester, a GSH delivery system [20]. NAC, a cell-permeable sulfhydryl compound, readily enters cells and promotes the production of GSH by furnishing its limiting precursor, L-cysteine [36]. In this study, we demonstrated that oral NAC restores pulmonary GSH levels in BLM-treated rats, which is accompanied by inhibition of LOX activity. In contrast, depletion of pulmonary GSH and upregulation of LOX activity occurs in rats exposed to BLM without NAC treatment. Clearly, a possible mechanism is NAC-induced elevation of GSH in the lung tissue of rats may largely trap cellular Cu, thus limiting its biological availability for LOX leading to downregulation of LOX activity.

Fig. 4. TGF- β_1 and α -SMA protein expression in lungs from BLM-treated rats with or without NAC intervention. TGF- β_1 (**a**, **b**) and α -SMA (**c**, **d**) were assessed by Western blot. **b**, **d** All animals were sacrificed 14 days after BLM administration. Experiments were repeated thrice. Data shown are the mean $\pm$ SD (n = 3) of TGF- β_1 / α -SMA normalized to β -actin/tubulin band densities. * p < 0.001 vs. control, ** p < 0.001 vs. B+W group.

LOX is expressed in a variety of cell types, such as fibroblasts, aortic and lung smooth muscle cells, myofibroblasts and corneal endothelial cells [37, 38]. However, the source(s) of LOX in lung tissues, especially during IPF development, is little known. TGF- β_1 , a fibrogenic growth factor regulating collagen synthesis and deposition, fibroblast proliferation and transformation to myofibroblasts, has been found to strongly promote expression of LOX in vitro, e.g. in fibroblasts from neonatal rat lungs [39], and in rat vascular smooth muscle cells [40]. This

study showed that BLM caused strong expression of TGF- β_1 on day 9, and the elevated level of TGF- β_1 was maintained up to day 35. Additionally, the myofibroblast differentiation marker α -SMA started to increase from day 9 and peaked on day 14, followed by a gradual decrease to very low levels. These two findings indicate that the BLM-induced increase in the number of lung myofibroblasts might mainly be mediated by the TGF- β_1 pathway, in agreement with previous reports [32, 41]. Since there was a significant correlation between α -SMA and LOX ex-

pression, we speculate that myofibroblasts may be the main source of LOX in BLM-induced pulmonary fibrosis. Thus, TGF- β_1 and myofibroblasts play a central role in BLM-induced upregulation of LOX. Since oral administration of NAC for 14 days partially inhibited TGF- β_1 and α -SMA protein levels, NAC may attenuate pulmonary fibrosis and regulate LOX activity via downregulation of the TGF- β_1 / α -SMA pathway in this model.

The potential of NAC to attenuate lung injury and subsequent fibrosis is partially dependent on the dose of BLM, the timing and the route of NAC used, the animal species and the strain [14]. In the literature, high doses of oral, aerosolized, intraperitoneal NAC significantly reduced collagen deposition only when NAC was given 1–7 days prior to BLM exposure in rats and mice [11, 13, 14, 28]. Cortijo et al. [14] reported that 490 mg/kg NAC given orally 1 or 7 days before BLM significantly decreased HYP values in rat lungs, but NAC treatment at the same dose starting 7 days after BLM treatment failed to reduce HYP levels [14]. In our study, NAC was given concomitantly with BLM at the same dose and via the same route as in the study by Cortijo et al. [14]. However, results show that this procedure is limited regarding the attenuation of the HYP content in lungs of rats exposed to BLM. Thus, timing of NAC treatment is critical for its antifibrotic effect. Therefore, as the American Thoracic Soci-

ety suggests, therapy should be started at the first identification of clinical or physiological evidence of impairments or documentation of lung function decline in selected cases [7].

Conclusions

Oral high-dose NAC inhibits LOX activity via a decrease in pulmonary GSH levels, which trap the cofactor of LOX, cellular Cu. Furthermore, BLM increases LOX activity possibly via the TGF- β_1 / α -SMA pathway, and myofibroblasts may be one of the main sources of LOX in this animal model. Additional studies are required to further elucidate the precise mechanism(s). NAC is a promising therapy of IPF and largely free of adverse effects. The regulatory potential of NAC on LOX may be a critical basis for its beneficial effect on IPF.

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