Lysyl oxidase promotes bleomycin-induced lung fibrosis through modulating inflammation

Tao Cheng¹,†, Qingbo Liu¹,†, Rui Zhang¹, Ying Zhang¹, Jianfeng Chen¹, Ronghuan Yu²³, and Gaoxiang Ge¹,³,*

¹State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

²Department of Respiratory Medicine, Shanghai Xu-Hui Central Hospital, Shanghai 200031, China

³Cancer Research Center, Shanghai Xu-Hui Central Hospital, Shanghai Clinical Center, Chinese Academy of Sciences, Shanghai 200031, China

*Correspondence to: Gaoxiang Ge, Tel: +86-21-54921102; Fax: +86-21-54921633; E-mail: gxge@sibcb.ac.cn

†These authors contributed equally to this work.
Abstract

Enzymes involved in collagen biosynthesis, including lysyl oxidase (LOX), have been proposed as potential therapeutic targets for idiopathic pulmonary fibrosis. LOX expression is significantly upregulated in belomycin (BLM)-induced lung fibrosis, and knockdown of LOX expression or inhibition of LOX activity alleviates the lung fibrosis. Unexpectedly, treatment of the mice with LOX inhibitor at the inflammatory stage, but not the fibrogenic stage, efficiently reduces collagen deposition and normalizes lung architecture. Inhibition of LOX impairs inflammatory cell infiltration, TGF-β signaling, and myofibroblast accumulation. Furthermore, ectopic expression of LOX sensitizes the fibrosis-resistant Balb/c mice to BLM-induced inflammation and lung fibrosis. These results suggest that LOX is indispensible for the progression of BLM-induced experimental lung fibrosis by aggravating the inflammatory response and subsequent fibrosis process after lung injury.

Keywords: lysyl oxidase, lung fibrosis, inflammation, bleomycin, animal models, extracellular matrix
Introduction

Lung fibrosis, characterized by replacement of normal tissue with scar tissue, destruction of tissue architecture, and organ malfunction, is a progressive chronic interstitial lung disease with poor outcome (King et al., 2011; Wynn, 2011; Noble et al., 2012). Lung fibrosis can develop after exposure to radiotherapy, chemotherapy, or environmental toxins (Wynn, 2011). After injury of pulmonary epithelial and endothelial cells, the damaged tissues are repaired by precisely coordinated inflammatory cell infiltration, fibroblast/myofibroblast proliferation, migration, and activation, epithelial cell proliferation and migration, as well as extracellular matrix (ECM) remodeling (Schafer and Werner, 2008; Araya and Nishimura, 2010; King et al., 2011; Wynn, 2011). Dysregulation of such wound healing process leads to the development of permanent fibrotic scar with excess accumulation of ECM components at the site of injury (Schafer and Werner, 2008; Araya and Nishimura, 2010; King et al., 2011; Wynn, 2011). Although debate exists whether inflammation is required for the progression of lung fibrosis, it is believed that the fibrosis is at least initially induced by inflammatory response after epithelial and endothelial cell injury (Lopez-Novoa and Nieto, 2009; Araya and Nishimura, 2010; du Bois, 2010; Wynn, 2011; Noble et al., 2012).

As the endpoint of lung fibrosis, excess deposition of ECM components and replacement of cellular tissue with acellular ECM severely compromise tissue structure and function. Therefore, the fibrosis process should be controllable by suppressing the production and deposition of ECM components, especially the interstitial collagens (Kagan, 2000). Interstitial collagen precursors undergo a serial post-translational enzymatic modifications before their deposition into ECM,
including proline hydroxylation catalyzed by prolyl 4-hydroxylase (PHD) (Gorres and Raines, 2010), proteolytical removal of N- and C-propeptides catalyzed by procollagen N-proteinases (PNPs) ADAMTS-2, 3, and 14 and procollagen C-proteinases (PCPs) bone morphogenetic protein-1 (BMP-1)/tolloid (TLD) family metalloproteinases (Greenspan, 2005; Ge and Greenspan, 2006), and collagen oxidation and crosslinking catalyzed by copper-dependent lysyl oxidase (LOX) family oxidases (Lucero and Kagan, 2006; Xiao and Ge, 2012). The primary function of LOX family oxidases is to oxidize amine substrates to reactive aldehydes, which results in the crosslinking of collagen and elastin (Lucero and Kagan, 2006; Xiao and Ge, 2012). LOX expression is markedly elevated in lung fibrosis, liver cirrhosis, atherosclerosis, scleroderma, and desmoplastic tumors featured by prominent symptom of fibrosis (Murawaki et al., 1991; Chanoki et al., 1995; Kagan, 2000). LOX blocking antibody treatment prevented bleomycin (BLM)- or irradiation-induced lung fibrosis (Cox et al., 2013). LOXL2 blocking antibody treatment also successfully impeded BLM-induced lung fibrosis (Barry-Hamilton et al., 2010). However, it requires further investigation to determine whether LOX indeed impact the progression of these fibrotic diseases by facilitating collagen crosslinking and deposition or other mechanisms.

In this report, we provide evidence supporting that LOX is the most significantly upregulated collagen post-translational modifying enzyme in BLM-induced experimental lung fibrosis and inhibition of LOX activity alleviates the fibrosis progression. Unexpectedly, blocking LOX activity at the fibrogenic stage can not efficiently prevent further progression of lung fibrosis, while inhibition of LOX activity at the inflammatory stage impaired inflammatory cell infiltration, TGF-β signaling, and lung fibrosis. Ectopic LOX expression enhances the inflammatory response
and sensitizes the fibrosis-resistant Balb/c mice to BLM-induced lung fibrosis, corroborating that LOX promotes lung fibrosis mainly through modulating inflammation.

**Results**

_Elevated LOX expression during BLM-induced lung fibrosis_

After BLM challenge, C57BL/6 mice displayed collapse of the alveolar spaces and extensive collagen production and deposition in the lung (Supplementary Figure S1A and B). mRNA levels of the enzymes involved in collagen biosynthesis, including PHD, PNPs, PCPs, and LOX family oxidases, in lung tissues after 14 days were determined. BLM substantially induced the expression of LOX, LOXL2, prolyl 4-hydroxylase subunit α2 (P4HA2), and ADAMTS2, among which, LOX was most significantly upregulated (~8-fold) (Supplementary Figure S1). Further analyses showed that _Lox_ mRNA level steadily increased from Day 3 to 21 (Figure 1A), compared with _Loxl2_ expression that was only evident on Day 14 and 21 (Supplementary Figure S2A). LOX protein level increased on Day 7 (~4-fold) and 14 (~9.5-fold) (Figure 1B) and the protein was broadly expressed in the alveolar and bronchiolar epithelium and fibroblasts (Supplementary Figure S2B and C), whereas LOXL2 is predominantly expressed in myofibroblasts (Supplementary Figure S2B).

_LOX is indispensible for BLM-induced lung fibrosis_

To investigate the role of LOX expression in BLM-induced experimental lung fibrosis, the LOX knockdown experiment was performed on C57BL/6 mice by administration of
adenovirus-based *Lox* shRNA at 3 days before BLM challenge (Figure 1C). The adenoviruses infected the lung with high efficiency (Supplementary Figure S3) and Ad-sh*Lox* successfully reduced *Lox* mRNA (Figure 1D) and LOX protein levels (Figure 1E) in mouse lungs harvested on Day 14. Mice received Ad-sh*Lox* showed significantly less body weight loss after BLM challenge than those received Ad-sh*Ctrl* (Figure 1F). Histological analysis revealed BLM-induced tissue architecture destruction and dense collagen deposition in lungs from Ad-sh*Ctrl*-treated mice, which were markedly reduced in Ad-sh*Lox*-treated mice (Figure 1G). The extent of fibrosis, determined as Ashcroft score, was significantly decreased in both Ad-sh*Lox* groups (Figure 1H). The collagen content, determined by measuring hydroxyproline level in the lungs, was significantly lower in Ad-sh*Lox* groups (Figure 1I). Concurrently, mRNA levels of collagens and fibronectin were lower in Ad-sh*Lox*-treated mouse lungs (Figure 1J).

Since LOX oxidase activity is critical in mediating the crosslinking and deposition of collagens, we further study whether LOX enzymatic activity also impacts BLM-induced lung fibrosis. The inhibition experiment was performed on C57BL/6 mice by daily administration of the LOX inhibitor β-aminopropionitrile (BAPN) or saline as control from one day before BLM challenge (Day -1) till the end of the experiment (Figure 2A). BAPN treatment (Day -1 to 21) resulted in significantly less body weight loss (Figure 2B), reduced tissue destruction and collagen deposition (Figure 2C), as well as decreased Ashcroft score (Figure 2D), hydroxyproline content (Figure 2E), and mRNA levels of ECM proteins (Figure 2F). Thus, LOX oxidase activity is important in the progression of BLM-induced lung fibrosis.
Ectopic LOX sensitizes Balb/c mice to BLM-induced lung fibrosis

Unlike C57BL/6 mice, Balb/c mice are relatively resistant to BLM-induced lung fibrosis (Hoyt and Lazo, 1988; Kolb et al., 2002; Warshamana et al., 2002). In the lungs of Balb/c mice subjected to BLM challenge, only sparse collagen-rich foci were evident (Supplementary Figure S4A), the expression of collagens and fibronectin was mildly upregulated (Supplementary Figure S4B), and notably, LOX expression remained largely unchanged (Supplementary Figure S4C).

Next, the ectopic LOX expression experiment was performed on Balb/c mice by administration of adenoviruses harboring LOX or its mutant at 3 days before BLM challenge (Figure 3A and B). K320A/Y355F mutations disrupt the lysyl tyrosyl quinine cofactor and the oxidase activity of LOX (Kagan and Li, 2003; Gao et al., 2010; Baker et al., 2011). Mice received Ad-LOX, but not Ad-GFP nor Ad-LOX mut that expresses enzymatically inactive LOX, substantially developed lung fibrosis after BLM challenge, accompanied with body weight loss (Figure 3C), tissue architecture destruction, dense collagen deposition (Figure 3D), and increased Ashcroft score (Figure 3E), hydroxyproline content (Figure 3F), as well as mRNA levels of ECM proteins (Figure 3G). The finding that Balb/c mice expressing enzymatically inactive LOX failed to develop lung fibrosis after BLM challenge (Figure 3) reinforces the notion that LOX oxidase activity is essential in promoting BLM-induced lung fibrosis.

LOX is required for TGF-β signaling and myofibroblast accumulation

Myofibroblasts are key cellular effectors responsible for ECM production and remodeling during BLM-induced lung fibrosis (Lopez-Novoa and Nieto, 2009; Araya and Nishimura, 2010).
TGF-β is a major determinant of the conversion from quiescent fibroblasts to myofibroblasts. Knockdown of LOX expression or inhibition of LOX activity in C57BL/6 mice substantially decreased the number of α-smooth muscle actin (α-SMA)-positive myofibroblasts (Figure 4A, B, G, and H), accompanied with significant reduction in bronchoalveolar lavage fluid (BALF) TGF-β level (Figure 4C and I), pSmad2/3-positive cells in lung tissue (Figure 4D, E, J, and K), and the expression of TGF-β target genes Ctgf and Pai1 (Figure 4F and L). On the other hand, ectopic expression of wild-type LOX in Balb/c mice significantly increased the number of α-SMA-positive myofibroblasts (Figure 4M and N), BALF TGF-β level (Figure 4O), pSmad2/3 signal in the lung (Figure 4P and Q), and TGF-β target gene expression (Figure 4R), while the enzymatically inactive LOX failed to induce such TGF-β signaling and myofibroblast accumulation (Figure 4M–R).

Inhibition of LOX activity at the fibrogenic stage does not efficiently alleviate lung fibrosis progression

The BLM model of lung fibrosis has been characterized by an initial influx of inflammatory cells during the inflammatory stage in response to BLM-induced tissue injury, followed by fibrotic reactions during the fibrogenic stage (Shen et al., 1988). Accordingly, interventions during the first 7 days are considered for preventive purpose, while treatments during later stages, i.e. after 7 days, are considered for therapeutic purpose (Moeller et al., 2008). As LOX family oxidases play critical roles in collagen crosslinking and connective tissue homeostasis, we next investigated whether LOX could serve as a therapeutic target. LOX activity was inhibited by
daily administration of BAPN from Day 7 to 21 after BLM challenge (Figure 5A). This treatment alleviated BLM-induced body weight loss (Figure 5B), tissue fibrosis (Figure 5C–F), TGF-β signaling and myofibroblast accumulation (Figure 5G–L), although to lesser extent compared with the long-period BAPN treatment from Day -1 to 21 (Figure 2 and 4G–L).

The inflammatory and fibrogenic phases in the BLM model overlap between Day 7 and 9 (Moeller et al., 2008), and the fibrotic foci started to develop on Day 10 after BLM challenge (Supplementary Figure S5). To more accurately define the role of LOX at the fibrogenic stage in BLM-induced lung fibrosis, LOX oxidase activity was inhibited by daily administration of BAPN from Day 10 after BLM challenge (Figure 6A). To our surprise, BAPN treatment at the fibrogenic stage (Day 10 to 21) could not alleviate lung fibrosis progression (Figure 6B–L), as determined by indistinguishable body weight loss, fibrotic alterations, TGF-β signaling, and myofibroblast accumulation between saline and BAPN-treated groups.

Ablation of LOX attenuated inflammatory response

Lung fibrosis in the BLM mouse model is triggered by substantial inflammation. On Day 7 after BLM challenge, abundant inflammatory cells infiltrated into lung parenchyma (Supplementary Figure S6). Meanwhile, LOX was highly expressed at the foci of inflammatory cells (Figure 7A), suggesting that LOX may be involved in recruiting inflammatory cells. Indeed, inhibition of LOX activity with BAPN reduced the number of infiltrating inflammatory cells (Supplementary Figure S6). On the other hand, BLM did not induce robust inflammatory cell infiltration in Balb/c mice (Supplementary Figure S4D), while ectopic expression of wild-type,
but not enzymatically inactive, LOX in Balb/c mice significantly increased the number of inflammatory cells infiltrating into lung on Day 14 after BLM challenge (Figure 7B), reinforcing that LOX enzymatic activity is required for recruitment of inflammatory cells. Differential cell counting in BALF collected on Day 14 indicated that the increased number of macrophages mainly accounted for the increased total inflammatory cell number (Figure 7B). In contrast, marked decrease in macrophage number was observed in BALF collected from Ad-shLox or BAPN (Day -1 to 21)-treated C57BL/6 mice (Figure 7C and D). Consistently, BAPN treatment from Day 7 to 21 reduced the number of infiltrating inflammatory cells as well (Figure 7E), whereas BAPN treatment from Day 10 did not significantly change the number of BALF inflammatory cells (Figure 7F). These findings suggest that LOX may promote lung fibrosis by modulating inflammatory cell infiltration, a mechanism independent of interstitial collagen crosslinking and deposition.

*Inhibition of LOX activity at the inflammatory stage prevented lung fibrosis*

The elevated LOX expression at the inflammatory stage (Figures 1A and B and 7A) promoted us to investigate whether LOX inhibition at the inflammatory stage affected BLM-induced lung fibrosis. C57BL/6 mice received daily administration of BAPN only for the first 7 days after BLM challenge (Figure 8A), which suppressed BLM-induced widespread cell apoptosis in the lung to the baseline (Figure 8B and Supplementary Figure S7). This BAPN treatment substantially reduced the inflammatory cell number (Figure 8C) and resulted in similar protective effects against BLM-induced lung fibrosis, as observed from administration of Lox...
shRNAs or long-period BAPN treatment (Day -1 to 21), which were featured by less body weight loss (Figure 8D), less severe tissue destruction (Figure 8E), reduced collagen deposition and extent of fibrosis (Figure 8E−G), decreased mRNA levels of ECM proteins (Figure 8H), and impaired TGF-β signaling and myofibroblast accumulation (Figure 8I−N). Thus, LOX inhibition at the inflammatory stage sufficiently impaired inflammatory cell infiltration and prevented the development of BLM-induced lung fibrosis.

**Discussion**

Aberrant expression and activity of LOX, the key enzyme mediating collagen crosslinking, have been believed to contribute to the pathological progression of lung fibrosis (Counts et al., 1981; Decitre et al., 1998; Barry-Hamilton et al., 2010; Cox et al., 2013). It has also been proposed that the fibrosis process in principal should be controllable by inhibiting LOX and suppressing the production and deposition of interstitial collagens (Kagan, 2000). In this study, we present evidence that LOX functions more prominently in the initial inflammatory phase rather than later fibrogenic stages, in contrast to the current scenario that LOX plays more crucial roles at the fibrogenic stage, when the collagen production, maturation, and deposition occur.

LOX mainly affects the number of macrophages in BLM-induced lung fibrosis (Figure 7). LOX and LOXL2 secreted by primary tumors are reported to accumulate at the premetastatic sites, crosslink basement membrane type IV collagen, and recruit CD11b⁺ myeloid cells to form the premetastatic niche, while LOX inhibition prevents CD11b⁺ cell recruitment and metastatic tumor growth (Erler et al., 2009; Park et al., 2011; Wong et al., 2011). Macrophages are
multifunctional in the tissue repair and fibrosis process by promoting inflammation, angiogenesis, fibroblast proliferation, myofibroblast recruitment, and ECM synthesis (Koh and DiPietro, 2011). Depletion of macrophages in mice results in delayed wound closure and lung fibrosis (Duffield et al., 2005; Murray et al., 2011). Macrophage is also the major source of TGF-β, the key cytokine in the development of lung fibrosis (Khalil et al., 1993). As a result of decreased macrophage infiltration, blocking LOX activity attenuated TGF-β signaling in C57BL/6 mice, whereas ectopic LOX expression enhanced the TGF-β signaling in Balb/c mice (Figure 4). Consistently, LOX inhibitor treatment at the fibrogenic stage (Day 10 to 21), which did not reduce the number of infiltrating macrophages, had no obvious effect on the progression of lung fibrosis (Figure 6).

Abundant LOX and LOXL2 expression has been observed in the myofibroblasts (Supplementary Figure S2). Myeloid cell-derived TGF-β induces LOX expression in resident tissue fibroblasts that subsequently promotes breast cancer metastasis (Pickup et al., 2013). Macrophages therefore may secrete TGF-β and induce LOX and LOXL2 expression in the fibroblasts. It is yet unclear why inhibition of LOX activity at the fibrogenic stage (Day 10 to 21) can not prevent further progression of lung fibrosis. The increased expression of LOX family member LOXL2 at the fibrogenic stage may provide redundancy in catalyzing collagen crosslinking (Supplementary Figure S2). Unlike the broad distribution of LOX in injured lung, LOXL2 is primarily expressed in the myofibroblasts (Supplementary Figure S2) (Barry-Hamilton et al., 2010). Myofibroblasts are the key mediators of ECM deposition and structural remodeling during lung injury, thus play a central role in the pathogenesis of lung fibrosis (Lopez-Novoa and Nieto, 2009; Araya and Nishimura, 2010). Targeting LOXL2 with an inhibitory monoclonal
antibody satisfactorily inhibited lung fibrosis, liver fibrosis, breast cancer growth and metastasis (Barry-Hamilton et al., 2010). It is controversial whether LOX inhibitor BAPN indeed inhibits LOXL2 enzymatic activity (Vadasz et al., 2005; Rodriguez et al., 2010). In addition to collagens and elastin, LOX readily oxidizes basic globular proteins, e.g. histone H1 (Kagan et al., 1983), and non-peptidyl amine substrates, e.g. 1,5-diaminopentane (Palamakumbura and Trackman, 2002) in vitro. BAPN potently inhibits the oxidase activity of both LOX and LOXL2 when 1,5-diaminopentane was used as the substrate (Palamakumbura and Trackman, 2002; Erler et al., 2006; Barry-Hamilton et al., 2010; Rodriguez et al., 2010). However, BAPN at the concentration (0.05 mg/ml, 0.7 mM) that completely inhibits LOX activity can not efficiently inhibit the oxidizing activity of LOXL2 when physiological substrate collagen is used in an in vitro assay (Vadasz et al., 2005). Indeed, LOXL2 blocking antibody, but not BAPN, resulted in significant reduction in tumor growth, accompanied with reduced collagen crosslinking, fibroblast activation, and endothelial cell recruitment in the tumor microenvironment (Barry-Hamilton et al., 2010). It is conceivable that LOXL2, but not LOX, plays more crucial roles in promoting collagen crosslinking and deposition at the fibrogenic stage. It is also possible that BAPN is not potent enough to completely inhibit the activity of high level of LOX and LOXL2 at the fibrogenic stage.

In summary, LOX expression is markedly elevated in BLM-induced lung fibrosis. In contrast to its presumed functions in collagen deposition at the fibrogenic stage, this study provides evidence supporting that LOX primarily functions as a critical regulator in the inflammatory response after tissue injury.
Materials and methods

BLM-induced lung fibrosis

All mice were housed in a specific pathogen-free environment at the Shanghai Institute of Biochemistry and Cell Biology (SIBCB) and treated in strict accordance with protocols approved by the Institutional Animal Care and Use Committee of SIBCB. Female C57BL/6 and Balb/c mice (20–25 g) were used. Lung fibrosis was induced by intratracheal instillation with BLM (2.5 mg/kg, Nippon Kayaku). For LOX inhibition, β-aminoproprionitrile (BAPN) (100 mg/kg, Sigma) in 200 μl saline was injected intraperitoneally daily as indicated. Saline was administered via the same manner in the corresponding control group. Body weight was recorded and presented as the percentage to that right before BLM administration (Day 0).

Adenoviruses

Human LOX and GFP were cloned into pShuttle-CMV vector. shRNA sequences were cloned into pLKO.1-puro retroviral vector and the shRNAs with U6 promoter were then cloned into pShuttle vector followed by recombination with pAdEasy vector (AdEasy TM Adenovirial vector system, Stratagene). shRNA target sequences are scramble control:

5′-CAAGATGAAGAGCACCAA-3′; Lox-#1: 5′-GATGGCGATATATGATT-3′; and Lox-#2: 5′-CCAGTGAAACAATGATGAT-3′. The adenoviruses were packaged in HEK-293 cells and purified with CsCl gradient centrifugation. At 3 days before BLM instillation, 1×10⁸ pfu adenoviruses in 30 μl PBS were instilled intranasally to the mice.
**BALF collection, ELISA, and differential cell counting**

Lungs were lavaged with two sequential 1-ml PBS after 14 days of BLM administration. BALF was pooled and centrifuged, and the cell pellet was washed with and resuspended in PBS. Cells were counted with a hemocytometer and differential cell counting was performed after centrifugation in cytospin with May Grunwald/Giemsa staining using Hemacolor® (Merck). TGF-β level in BALF was measured with the ELISA kit (R&D systems).

**Histology, immunohistochemistry, immunofluorescence, and Picro Sirius red staining**

The left lobe of Lung was perfused, fixed with 4% paraformaldehyde in PBS, dehydrated, and embedded in paraffin. Paraffin sections (5 μm) were stained with hematoxylin and eosin A or Picro Sirius red solution. Immunohistochemical staining was performed with Histostain-Plus Kit (Invitrogen) followed by DAB development. Alexa Fluor 555/488-conjugated secondary antibodies (Invitrogen) were used for immunofluorescent staining. Epitope retrieval was performed using sodium citrate buffer. Primary antibodies used are anti-LOX (1:1500) (Sigma), anti-α-SMA (1:3000) (Sigma), anti-CC10 (1:200) (Santa Cruz), anti-CD31 (1:200) (BD Pharmigen), anti-GFP (1:200) (Invitrogen), and anti-pSmad2/3 (1:1500) (Santa Cruz). TUNEL assay was performed following manufacturer’s protocol (Vazyme) to detect apoptotic cells. High power images (40×) were captured and the number of pSmad2/3-positive or TUNEL-positive cells and the percentage of α-SMA-positive area were quantified with ImagePro Plus (v6.0, Media Cybernetics).
**Ashcroft score**

Pictures were taken from 10 random 20× fields in paraffin sections stained by Picro Sirius red. Each specimen was individually assessed for severity of interstitial fibrosis and allotted a score between 0 and 8 using a predetermined scale of severity following the Ashcroft fibrosis scoring system (Hubner et al., 2008). Grade 0: Normal lung; Grade 1: Minimal fibrous thickening of alveolar or bronchiolar walls; Grade 2: Clearly fibrotic changes with knot-like formation but not connected to each other; Grade 3: Contiguous fibrotic walls predominantly in whole microscopic field; Grade 4: Single fibrotic masses; Grade 5: Confluent fibrotic masses; Grade 6: Large contiguous fibrotic masses; Grade 7: Alveoli nearly obliterated with fibrous masses but still with up to five air bubbles; Grade 8: Microscopic field with complete obliteration with fibrotic masses. All specimens were randomly numbered and the grading was performed in a blinded manner by three observers.

**Hydroxyproline quantification**

Total collagen content was measured with a conventional hydroxyproline quantitation method (Huszár et al., 1980).

**Quantitative RT–PCR analysis**

Quantitative RT–PCR was performed as previously described (Gao et al., 2010) to determine mRNA levels of Lox and other genes. The primers used are listed in Supplementary Table S1.
Western blot analysis

Western blot analysis was performed as previously described (Gao et al., 2010) to determine protein levels of LOX and β-actin as loading control.

Statistical analysis

Data are presented as means ± SD and analyzed by Student’s two-tailed t test. P < 0.05 is considered statistically significant.

Acknowledgements

The authors acknowledge Drs Baoliang Song, Xiaoyan Ding (Shanghai Institute of Biochemistry and Cell Biology), and Huiping Li (Shanghai Pulmonary Hospital) for sharing reagents and Haojie Chen and Wei Bian (Shanghai Institute of Biochemistry and Cell Biology) for their technical assistance.

Funding

This work was supported by the National Basic Research Program of China (2010CB912102 and 2010CB529703) and the National Natural Science Foundation of China (31190061, 31371408 and 30971495). G.G. is a scholar of the SA-SIBS Scholarship Program.

Conflict of interest: none declared.
References


chemical targets in the control of fibrosis. Acta Trop. 77, 147-152.


and fibrosis after bleomycin is directly related to the severity of acute injury. Am. Rev. Respir. Dis. 137, 564-571.


Figure legends

**Figure 1** LOX expression is required for BLM-induced lung fibrosis. (A-B) Determination of Lox mRNA level (A) and LOX protein level (B) in lung tissues at indicated time points after BLM (2.5 mg/kg) challenge. (C–J) In a LOX knockdown experiment, C57BL/6 mice received a single intranasal administration of adenoviruses harboring scramble control shRNA (Ad-shCtrl) or shRNAs against Lox (Ad-shLox) at 3 days before BLM challenge and were sacrificed on Day 14 (C). Body weight was monitored from Day -3 till the end of the experiment (F). Lung tissues harvested on Day 14 were analyzed for Lox mRNA expression (D), LOX protein level (E), H&E and Picro Sirius red staining (G), Ashcroft score (H), Hydroxyproline content (I), and mRNA levels of Col1a1, Col3a1, Col4a1, and Fn (J). Scale bar, 100 μm. Data are presented as means ± SD. ***P < 0.001. n = 5–8 mice per group.

**Figure 2** Inhibition of LOX activity prevents BLM-induced lung fibrosis. (A) In a LOX inhibition experiment, C57BL/6 mice received daily treatment of BAPN (100 mg/kg) from one day before BLM challenge (Day -1) till the mice were sacrificed on Day 14 or 21. (B) Body weight was monitored throughout the experiment. (C–F) Lung tissues harvested on Day 14 were analyzed for H&E and Picro Sirius red staining (C), Ashcroft score (D), hydroxyproline content (E) and mRNA levels of Col1a1, Col3a1, Col4a1, and Fn (F). Scale bar, 100 μm. Data are presented as means ± SD. *P<0.05, **P<0.01, ***P < 0.001. n = 5–10 mice per group.

**Figure 3** Ectopic LOX expression sensitizes fibrosis-resistant Balb/c mice to BLM-induced lung fibrosis. (A) In an ectopic LOX expression experiment, Balb/c mice received a single intranasal administration of adenoviruses harboring GFP (Ad-GFP), wild-type LOX (Ad-LOX), or LOX
with K320A/Y355F mutations that disrupt the lysyl tyrosyl quinine cofactor and LOX enzymatic activity (Ad-LOX mut) at 3 days before BLM challenge and were sacrificed on Day 14. (B) LOX protein level was determined in lung homogenates harvested on Day 14 after BLM challenge. (C) Body weight was monitored from Day -3 till the end of the experiment. (D–G) Lung tissues harvested on Day 14 were analyzed for H&E and Picro Sirius red staining (D), Ashcroft score (E), hydroxyproline content (F), and mRNA levels of Col1a1, Col3a1, Col4a1, and Fn (G). Scale bar, 100 μm. Data are presented as means ± SD. *P<0.05, **P<0.01, ***P < 0.001. NS, not significant. n = 5–10 mice per group.

**Figure 4** LOX regulates TGF-β signaling and myofibroblast accumulation. Lung tissues were harvested on Day 14 from C57BL/6 mice in the LOX knockdown experiment (A–F, procedure in Figure 1C) and the LOX inhibition experiment (G–L, procedure in Figure 2A), and Balb/c mice in the ectopic LOX expression experiment (M–R, procedure in Figure 3A) for immunohistochemistry of α-SMA (A, G, and M), quantification of α-SMA-positive area (B, H, and N), ELISA analysis of BALF TGF-β level (C, I, and O), immunohistochemistry of pSmad2/3 (D, J, and P), quantification of pSmad2/3-positive cells (E, K, and Q), and quantitative RT–PCR analysis of Pai1 and Ctgf mRNA levels (F, L, and R). Negative controls (Neg) were incubated with secondary antibodies only. High-magnification images for pSmad2/3 staining are also shown. Scale bar, 100 μm. Data are presented as means ± SD. *P<0.05, ***P < 0.001. NS, not significant. n = 5–10 mice per group.

**Figure 5** Inhibition of LOX activity from Day 7 after BLM challenge alleviates lung fibrosis. (A) C57BL/6 mice were challenged with BLM and received daily treatment of BAPN (100 mg/kg)
from Day 7 till the mice were sacrificed on Day 14 or 21. (B) Body weight was monitored throughout the experiment. (C–L) Lung tissues were harvested on Day 14 for histology analysis (C), Ashcroft scoring (D), hydroxyproline content (E), and determination of *Col1a1*, *Col3a1*, *Col4a1*, and *Fn* mRNA expression (F), *α*-SMA expression (G–H), BALF TGF-β level (I), pSmad2/3 expression (J–K), and *Pai1* and *Ctgf* mRNA levels (L). Negative controls (Neg) and high-magnification images are shown for immunohischemistry analysis. Scale bar, 100 μm. Data are presented as means ± SD. *P* < 0.05, **P** < 0.01, ***P** < 0.001. *n* = 5–10 mice per group.

**Figure 6** Inhibition of LOX activity from Day 10 after BLM challenge can not prevent further progression of lung fibrosis. (A) C57BL/6 mice were challenged with BLM and received daily treatment of BAPN (100 mg/kg) from Day 10 till the mice were sacrificed on Day 17 or 21. (B) Body weight was monitored throughout the experiment. (C–L) Lung tissues were harvested on Day 17 for histology analysis (C), Ashcroft scoring (D), hydroxyproline content (E), and determination of *Col1a1*, *Col3a1*, *Col4a1*, and *Fn* mRNA expression (F), *α*-SMA expression (G–H), BALF TGF-β level (I), pSmad2/3 expression (J–K), and *Pai1* and *Ctgf* mRNA levels (L). Negative controls (Neg) and high-magnification images are shown for immunohischemistry analysis. Scale bar, 100 μm. Data are presented as means ± SD. NS, not significant. *n* = 5–10 mice per group.

**Figure 7** Inhibition of LOX impaired inflammatory cell infiltration. (A) C57BL/6 mice were challenged with saline as control or BLM (2.5 mg/kg). Lung tissues were harvested on Day 7 for immunohistochemistry of LOX. Scale bar, 100 μm. (B–F) BALF cells were collected from Balb/c mice in the ectopic LOX expression experiment (B, procedure in Figure 3A), C57BL/6
mice in the LOX knockdown experiment (C, procedure in Figure 1C) and the LOX inhibition experiments with BAPN treatment for the duration of Day -1 to 21 (D, procedure in Figure 2A), Day 7 to 21 (E, procedure in Figure 5A), and Day 10 to 21 (F, procedure in Figure 6A) on Day 14, except those in F were collected on Day 17. Total cell numbers and individual cell type counts were determined. mϕ, macrophages; lymph, lymphocytes; neu, neutrophils. Data are presented as means ± SD. *P<0.05, **P<0.01, ***P<0.001. n = 3–5 mice per group.

**Figure 8** Inhibition of LOX activity at the inflammatory stage prevents BLM-induced fibrosis. (A) C57BL/6 mice received daily treatment of BAPN (100 mg/kg) from one day before BLM challenge (Day -1) till Day 7 and were sacrificed on Day 14 or 21. (B) Box plot of apoptotic (TUNEL-positive) cell numbers in lungs on Day 7 (n=5 mice per group). (C) BALF cells were collected on Day 14 for total cell numbers and individual cell type counts. mϕ, macrophages; lymph, lymphocytes; neu: neutrophils. (D) Body weight was monitored throughout the experiment. (E–N) Lung tissues were harvested on Day 14 for histology analysis (E), Ashcroft scoring (F), hydroxyproline content (G), and determination of Col1a1, Col3a1, Col4a1, and Fn mRNA expression (H), α-SMA expression (I–J), BALF TGF-β level (K), pSmad2/3 expression (L–M), and Pail and Ctgf mRNA levels (N). Negative controls (Neg) and high-magnification images are shown for immunohischemistry analysis. Scale bar, 100 μm. Data are presented as means ± SD. *P<0.05, **P<0.01, ***P<0.001. n = 5–10 mice per group.