Original Article

Thymosin β4 alleviates bleomycin-induced lung damage through inhibiting nitrative thioredoxin-1 inactivation

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Abstract: In this study the action of Thymosin β4 (TB4) on bleomycin (BLM)-induced lung damage and its molecular mechanisms were investigated. Our results showed that exogenous TB4 treatment could attenuate BLM-induced destruction of lung alveoli, inflammatory cells infiltration and thickening of the lung interstitium. Furthermore, TB4 treatment exerted inhibitory effects on the early phase of BLM-induced fibrosis, which was demonstrated by decreased collagen deposition in TB4-treated rat lung tissues. Our findings also showed that thioredoxin (Trx) antioxidant system was involved in the TB4 function. TB4 treatment could increase the Trx activity in the lung tissues of BLM-treated rats. TB4-induced increase of Trx activity was attributable to the increased Trx-1 expression and decreased Trx-1 nitration induced by BLM. Taken together, our results indicated that TB4 exerted protective effects on BLM-treated rat lungs through increasing Trx-1 expression, decreasing Trx-1 nitration, and subsequently maintaining normal Trx activity.

Keywords: Thymosin β4 (TB4), bleomycin, thioredoxin, lung fibrosis, inflammation, protein nitration

Introduction

Thymosin β4 (TB4), a 5 kD small polypeptide, has been proven to harbor multiple functions. It is involved in angiogenesis, inflammation, wound healing and repair, et al [1]. Recent investigations showed that TB4 could attenuate bleomycin (BLM) induced lung damage [2-4]. However, the molecular mechanisms of TB4 action in inhibiting BLM-induced lung damage are still not clear at present and need further investigations.

Thioredoxin (Trx) antioxidant system is an important protective mechanism against oxidative injury [5] which participates in the lung fibrosis [6]. Exogenous recombinant Trx-1 could prevent BLM-induced lung injury and fibrosis [7]. Our previous report [8] showed that Trx activity was modulated by nitration modification in BLM-treated rats. Although the Trx-1 expression level was increased in the lung tissues of BLM treated rats, Trx activity was decreased because of the increased nitrative inactivation [8]. In present study, we wanted to investigate whether TB4 exert its protective effects on the BLM-induced lung injury through modulating Trx activity.

Materials and methods

Animal treatment

Male Wistar rats (SPF grade, 8 weeks old, weighing 200-240 g) were bought from Vital River Laboratory (Beijing, China) and used in this study. Rats were housed in climate-controlled room with a 12-h light/dark cycle and provided with ad libitum access to food and water. Before experiments rats were accustomed to the environment for 7 days. Bleomycin A5 (BLM) (Taihe Pharmaceutical, Tianjin, China) was administrated through intratracheal injection at an dose of 5 mg/kg body weight as described in the previous report [8]. Control rats received the same volume of intratracheal saline. In TB4 treatment group rats received intraperitoneal injection of 100 mg TB4 each
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rat at 0, 2, 4 hours and at day 3, day 6 after intratracheal BLM injection. At day 7 after BLM administration, rats were euthanized by an overdose of chloral hydrate (10%) and the lungs were removed for further histopathological examinations and biochemical analyses. This study was approved by the Animal Research Committee of Zhengzhou University, Zhengzhou, China.

Histology

Left upper lungs were used for histological analyses as previously described [8]. Briefly, paraformaldehyde-fixed lungs were cut into 5 um thick sections and stained with hematoxylin-eosin (H.E) and Masson trichrome according to the standard procedure. Images were captured under a light microscope (Olympus D72, Japan). Lung damages were examined by an independent pathologist blind to this study.

Immunohistochemistry (IHC)

IHC experiments were performed as previously described [8]. 5 um thick sections of lung tissues were probed with monoclonal antibody against nitrotyrosine (Upstate, Charlottesville, Virginia, USA) and stained with a Vectastain ABC kit (Vector Laboratories). The extent of the protein nitration of the slides was examined under a light microscope (Olympus D72, Japan) by an independent pathologist blind to this study.

Determination of Trx activity

Trx activity was determined by spectrophotometric insulin assays [9]. Briefly, 40 mg of tissue homogenate were mixed with 2 ml activation buffer (100 mmol/L HEPES, 2 mmol/L EDTA, 1 mg/ml bovine serum albumin, and 2 mmol/L dithiothreitol) and incubated for 15 min at 37°C. Then added 20 ml reaction buffer (100 mmol/L HEPES, 2.0 mmol/L EDTA, 0.2 mmol/L NADPH, and 140 mmol/L insulin) to the mixture. Incubated the mixture for 30 min at 37°C after addition of mammalian Trx reductase (1 ml, 15 mU, Sigma). Stopped the reaction by adding 125 mL of stopping solution (0.2 mmol/L Tris-HCL, 10 mmol/L guanidine-HCL, and 1.7 mmol/L 3-carboxy-4-nitrophenyl disulfide; DTNB). The absorbance of the solution at 412 nm was measured.

Immunoprecipitation (IP) and Western blot

Trx expression and nitration was determined according to previously published methods [10, 11]. Endogenous Trx-1 in the Lung homogenate was precipitated by monoclonal Trx-1 antibody (Santa Cruz). The precipitated Trx-1 or the lung homogenates were subjected 12% SDS-PAGE electrophoresis and electro-transferred to 0.22 um PVDF membrane. The membranes were probed with a monoclonal antibody against nitrotyrosine (Upstate, Charlottesville, Virginia, USA) or Trx-1 antibody (Santa Cruz) and developed with SuperSignal Western reagent (Pierce). Bands density was analyzed using the free software of Image J (http://rsb.info.nih.gov/ij/).

Statistical analysis

SPSS 21.0 (SPSS Inc., Chicago, Illinois, USA) was utilized to perform statistical analysis in this study. The data were presented as mean ± SD. Two-way ANOVA was used to compare the difference between different groups. P<0.05 was considered as statistically significant.
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Results

Effects of TB4 on BLM-induced lung damage

As shown in the H.E staining results (Figure 1), compared with control, destruction of lung alveoli, inflammatory cells infiltration and thickening of the lung interstitium were observed in BLM-treated rats. However, in TB4-treated rats, all of the changes induced by BLM were attenuated (Figure 1C). Masson staining showed that BLM treatment induced excessive collagen deposition compared with control, which was inhibited by TB4 administration (Figure 2). These results indicated that TB4 could reduce BLM-caused lung damages, which was similar to previous reports [2, 3].

Effects of TB4 on Trx activity

Thioredoxin (Trx) antioxidant system is an important protective mechanism against oxidative injury [5] and previous study has demonstrated that Trx played a protective role in BLM-induced lung damage [7]. Here we want to know the relationship between Trx activity and TB4 treatment. As indicated in Figure 3A, Trx activity was significantly decreased by BLM treatment, which could be reversed to normal level by TB4 treatment. These results indicated that TB4 exerted protective function through modulating Trx activity in the lung tissues.

Effects of TB4 on Trx nitration

In order to elucidate the molecular mechanisms of TB4 function, we further investigated how TB4 modulated Trx activity. Trx activity can be regulated by protein level and posttranslational modification [12]. And previous study indicated that tyrosine nitration could inactivated Trx [13]. So we detected the protein level and nitration modification status of Trx. Firstly, the immunohistochemistry results showed that BLM
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Figure 4. Immunohistochemistry detection of protein nitration in the lung tissues from Control (A), Bleomycin-treated (B) and Bleomycin+TB4 treated (C) rats. (100×) TB4: thymosin β4.

treatment induced increased protein nitration as detected by monoclonal antibody against nitro-tyrosine, which could be attenuated by TB4 treatment (Figure 4). Secondly, Western blots indicated that BLM-induced Trx up-regulation could be further enhanced by TB4 (Figure 3B). Thirdly, results from immune-precipitation experiments and Western blots illustrated that BLM caused increased Trx nitration, which was decreased by TB4 treatment (Figure 3C).

Discussion

In this study, we confirmed the conclusion of previous report that TB4 could attenuated the lung damage and fibrosis at day 7 after BLM administration [2, 3]. Furthermore, our results also showed that TB4 treatment could decrease BLM-induced protein nitration in the lung tissues as demonstrated by immunohistochemistry experiments. TB4 treatment could also increase Trx activity in the lung tissues of BLM-treated rats by upregulation of Trx-1 expression and inhibition of nitrative Trx inactivation.

Trx system is an important antioxidant system, including Trx-1, Trx reductase and NADPH, and has been reported to be involved in regulating cellular redox status, cell survival and apoptosis [14]. Previous studies showed that, in the human disease of asthma, chronic obstructive pulmonary disease (COPD), especially idiopathic pulmonary fibrosis (IPF), Trx-1 expression increased in the lung, which represented an attempt to protect the lung from injury and fibrosis [15, 16]. Animal experiments showed that expression of Trx-1 was strongly induced in the lungs of BLM- or cigarette -treated animals [17, 18]. In 2003, Hoshino et al proved that exogenous recombinant Trx-1 could inhibit the BLM-induced lung fibrosis [7]. In this study, our results demonstrated that TB4 treatment could significantly increase the Trx-1 expression and activity in the lung tissues after BLM administration.

So far, four main types of posttranslational modification including glutathionylation, thiol-oxidation, and S-nitrosylationation were identified for Trx-1 protein [12, 13]. Among the four post-translational modifications of Trx-1, tyrosine nitrative inactivation of Trx-1 was recently identified and has been extensively investigated in cardiovascular system [11, 13, 19, 20]. Our previous study reported that in BLM-treated rats Trx-1 nitration increased in the lung tissues and correlate with the decreased Trx activity, which indicated that loss of Trx activity caused by tyrosine nitration might participate in the BLM-induce lung damage [8]. In this study, our results demonstrated that TB4 treatment could inhibit the BLM-induced Trx nitration in the lung tissues of rats.

In conclusion, our results illustrated that TB4 exerts inhibitory effects on BLM-induced lung injury and early phase of lung fibrosis by decreasing Trx nitration and increasing Trx activity in the lung tissues.

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Disclosure of conflict of interest

None.

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