Role of endogenous peroxynitrite in pulmonary injury and fibrosis induced by bleomycin A_5 in rats

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ABSTRACT

AIM: To observe the role of endogenous peroxynitrite ($ONOO^-$) in pulmonary injury and fibrosis induced by bleomycin A_5 (BLM-A_5) in rats. **METHODS:** Pulmonary injury and fibrosis of rats were evaluated by testing the level of lipid peroxides (LPO) in out-going pulmonary blood (OPB), and by observing histological changes, including type III and type I collagen changes in lung which were examined with Sirius red staining under polarized light. The peroxynitrite expression was detected by immunohistochemistry for nitrotyrosine (NT), a marker of the peroxynitrite production. **RESULTS:** (1) The level of LPO was elevated in OPB of rats on d 14 after intratracheal administration of BLM-A_5. Thickened alveolar wall and macrophage infiltration were seen, and fibroblasts were near by the interstitial macrophages. Increased amounts of type III collagen and type I collagen were deposited in disoriented fashion. (2) High expression of $ONOO^-$ was detected in alveolar epithelial cells and pulmonary interstitial macrophages. (3) The above changes were reduced by aminoguanidine (AG), an inhibitor of nitric oxide synthase (iNOS). **CONCLUSION:** Endogenous $ONOO^-$ mediated BLM-A_5-induced pulmonary toxicity. The therapeutic potential of AG for pulmonary injury and fibrosis was realized partly by reducing $ONOO^-$ formation.

INTRODUCTION

Bleomycin (BLM) is commonly used as a part of the cytostatic treatment of several tumor types, such as germ-cell tumors, lymphomas, and Kaposi's squamous cell carcinomas of head and neck. The application of BLM is featured by the occurrence of some fetal side effects. Pulmonary toxicity is the most serious side effect of BLM. In lung, the toxicity of BLM involved inflammation and fibrosis. So far, agents with therapeutic potential to the side effect are not available.

Our previous study has suggested that the endogenous nitric oxide (NO), produced by inducible nitric oxide synthase (iNOS) in lung, mediates pulmonary toxicity of BLM-A_5, and has demonstrated that aminoguanidine (AG), a preferred iNOS inhibitor, ameliorates the development of pulmonary injury and fibrosis induced by BLM-A_5[1,2].

It is well known that peroxynitrite ($ONOO^-$) is generated by the reaction of nitric oxide (NO) and superoxide. *In vitro* study[3] showed strong expression of peroxynitrite ($ONOO^-$) in alveolar macrophages from mice after BLM injection. Our previous study showed that the high level of NO was partially generated by alveolar macrophages[1]. Beckman *et al*[4] found that $ONOO^-$ could lead to lipid peroxidation and nitration of cell membrane proteins in isolated perfused rat lung. Therefore, two questions arise as to whether the deleterious effect of NO on pulmonary toxicity of BLM-

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A_5 is mediated by formation of $ONOO^-$, and whether the therapeutic potential of AG is produced by reducing $ONOO^-$ expression. Despite the well-accepted fact that many of the cytotoxic effects of NO are mediated by $ONOO^-$ in the pathogenesis of an increasing number of diseases, the above questions remain to be elucidated.

The level of $ONOO^-$ is increased in acute lung injury[5], influenza virus-induced pneumonia[6], oxidant lung injury due to paraquat[7], cystic fibrosis[8], and idiopathic pulmonary fibrosis[9]. However, the effect of peroxynitrite on pulmonary toxicity of BLM is still unclear. Some reports demonstrated that AG could reduce $ONOO^-$ formation by inhibition of iNOS activity in spontaneous hypertensive rats[10], interleukin-1 beta-induced myocardial dysfunction in dogs[11], and LPS-induced acute lung injury in dogs[12]. However, little is known about the influence of AG on $ONOO^-$ formation in pulmonary toxicity induced by BLM-A_5.

The current study was to ascertain the effect of $ONOO^-$, and the protective mechanism of AG, on pulmonary injury and fibrosis induced by BLM-A_5.

MATERIALS AND METHODS

Materials Bleomycin-A_5 (BLM-A_5) was produced by Tianjin Taihe Pharmaceutical Co, China. Aminoguanidine hemisulfate salt (AG), produced in Germany, was dissolved in sterile normal saline. Mouse anti-rat nitrotyrosine (NT) polyclonal antibody was from Cayman Co (USA). Biotinylated anti-mouse IgG and Histostain-plus kit were obstained from Beijing Zhongshan Biotechnology Co (China). Malondialdehyde (MDA) detecting kit was from Nanjing Jiancheng Biochemical Institute, China.

Experimental animals and groups Sprague Dawley rats (♂, *n*=24, weighing 180-200 g, Grade II, Certificate No 06057) were provided by the Experimental Animal Center of Hebei Province, China. For the duration of the experiment, the animals were fed with commercial rat food and water *ad libitum*. The rats were randomly allocated to 4 groups (6 animals in each group): (1) BLM-A_5 group: rats received intratracheal instillation of a single dose of BLM-A_5 (5 mg/kg, in 0.5 mL of sterile saline)[2] *via* tracheotomy under anesthesia (40 mg/kg, ip) ; (2) BLM-A_5+AG group: rats were given the same amount of BLM-A_5 as BLM-A_5 group, followed by daily ip injection of AG (20 $mg\cdot kg^{-1}\cdot d^{-1}$)[2]; (3) BLM-$A_5$ + NS group: rats were given BLM-A_5, followed by daily ip 0.9 % steril normal saline (NS) instead of AG; (4) Control group: rats received intratracheal injection of the same amount of 0.9 % sterile normal saline (NS) as BLM-A_5 group instead of BLM-A_5. All rats were sacrificed on d 14 after intratracheal injection of BLM-A_5 or NS.

Collection of samples Rats were anesthetized with sodium pentobarbital (40 mg/kg) intraperitoneally. One polyethylene cannula was inserted into the left carotid for out-going pulmonary blood (OPB). The other cannula was introduced into the opening of right atrium through right jugular vein for in-going pulmonary blood (IPB). OPB and IPB were collected for detecting lipid peroxide (LPO) content, evidences of lipid peroxide injury in lung. Lungs were fixed with 4 % paraformaldehyde, embedded in paraffin, sectioned, and stained immunohistochemically for nitrotyrosine (NT), with Sirius red for type I and type II collagens, and with hematoxylin and eosin (HE) for morphological observations.

Measurements of LPO The level LPO was determined as thiobarbituric acid (TBA)-reactive substance (malondialdehyde, MDA) using MDA detecting kit. The concentrations are expressed as μmol/L.

Immunohistochemistry for NT NT is used as an marker of the peroxynitrite production[4]. Tissue sections were deparaffinized and hydrated through graded alcohol to water. Endogenous peroxidase activity was blocked by incubation in 3 % hydrogen peroxide for 10 min. After 3 washes with PBS (NaCl 0.154 mol/L, $NaH_2PO_4\cdot H_2O$ 0.002 mol/L, $Na_2HPO_4\cdot 12H_2O$ 0.002 mol/L, pH 7.5), the sections were blocked with 1:10 goat serum (37 °C, 30 min) to suppress nonspecific background staining. Then, the primary antibody, mouse NT polyclonal antibody, was applied at 1:50 dilution. After incubation at 37 °C for 12 h, the sections were further incubated with biotinylated anti-mouse IgG (working fluid) for 1 h and then with avidin-biotin-peroxidase system for 1 h at room temperature. After a rinse in PBS, the sections were exposed to 0.04 % diaminobenzidine tetrahydrochloride (DAB) for 5-10 min, and were counterstained with hematoxylin. At last, sections were dehydrated in alcohol and xylene, coverslipped, and viewed under a light microscopy.

Sirius red staining Sirius red F2BA was dissolved in saturated aqueous picric acid at a concentration of 1 g/L. Five micrometer-thick sections were stained to identify collagen fibers under polarization microscope. Type I collagen was in yellow or orange color and type III collagen was green under polarized

light.

Statistics Data were expressed as mean±SD and compared by *t*-test.

RESULTS

Toxicity of BLM-A_5 and the protective role of AG on lung The evidence of pulmonary injury and fibrosis was obtained by testing the LPO level in OPB and IPB, and by observing histological and morphometric changes and type I and type III collagen changes in lung sections. The content of LPO (expressed by MDA, μmol/L) in OPB in BLM-A_5 group was higher than that in control group (P<0.05). The LPO in IPB in BLM-A_5 group had no change compared with that in control group (P>0.05, Tab 1). Alveolar walls were thickened and associated with macrophage infiltration. Fibroblasts were observed near by the interstitial macrophages (Fig 1B). With Sirius red staining and under polarized light observation, increased amounts of type III collagen and type I collagen were observed , which were deposited in disoriented fashion in areas of fibrosis (Fig 2B). Lung sections from control animals did not show fibrosis or injury (Fig 1A, 2A). There was no difference in LPO level and in histological change between BLM-A_5+NS group and BLM-A_5 group (Tab 1, Fig 1B, 1D, 2B, 2D).

The content of LPO in OPB was significantly decreased in BLM-A_5+AG group compared with BLM-A_5+NS group. The content of LPO in IPB of BLM-A_5+AG group did not differ from that of BLM-A_5 +NS group (Tab 1). In BLM-A_5+AG group, the morphological changes of BLM-A_5 group reduced (Fig 1C), and there were a little type III collagen and type I collagen detected in lung interstitum (Fig 2C).

$ONOO^-$ expression in lung induced by BLM-A_5 In BLM-A_5 group, a marked increase in the immunoreaction to 3-nitro-*L*-tyrosine (NT), a footprint of the $ONOO^-$ *in vivo*, was detected in alveolar epithelial cells and in interstitial macrophages,. In addition, masses of fibroblasts were observed nearby (Fig 3B). In control group, no NT positive signal and masses of fibroblasts were detected (Fig 3A).

Effects of AG on $ONOO^-$ expression in lung In BLM-A_5+AG group, the intensity and extent of NT were reduced, and a few fibroblasts and inflammatory cells appeared (Fig 3C). In BLM-A_5+NS group, the expression of NT was similar to that in BLM-A_5 group (Fig 3B, 3D).

Tab 1. Changes of liquid peroxides (LPO) in out-going pulmonary blood (OPB) and in in-going pulmonary blood (IPB). n=6 rats. Mean±SD. ^bP<0.05 *vs* control group; ^eP<0.05 *vs* BLM-A_5 +NS group.

Groups	MDA/μmol·L^{-1}	
	OPB	IPB
Control	8.5±1.5	8.8±2.6
BLM-A_5	11.7±0.8^b	9.6±2.7
BLM-A_5+NS	11.8±1.0^b	9.3±1.6
BLM-A_5+AG	8.7±0.7^e	8.9±2.3

The contents of LPO were expressed by those of MDA.

DISCUSSION

In the present study, high expression of $ONOO^-$ was detected in alveolar epithelial cells and pulmonary interstitial macrophages of rats on d 14 after administration of BLM-A_5. The high level of $ONOO^-$ was associated not only with pulmonary injury, but also with pulmonary fibrosis. In addition, the production of $ONOO^-$ and the severity of lesion and fibrosis in lung were reduced by AG, an inhibitor of iNOS.

It is well accepted that $ONOO^-$ is stable only in alkaline solution. The unusual stability of $ONOO^-$ is due to its being folded into the *cis*-conformation, which can not directly isomerize to the much more stable form, nitrate. After protonation, $ONOO^-$ can isomerize to a *trans*-conformation or *trans*-peroxynitrous acid (ONOOH). Each of *trans*-$ONOO^-$ and *trans*-peroxynitrous acid is a strong oxidant and decays rapidly to hydroxyl radical and nitrogen dioxide[13]. Therefore, *trans*-$ONOO^-$ is toxic by oxidative mechanisms, which results in oxidation of sulphydryls, lipid peroxidation, and nitration of amino acid residues. The nitration of protein tyrosine residues formed 3-nitrotyrosine (NT)[14]. $ONOO^-$ is a stronger oxidant than NO. NO did not directly nitrate tyrosine residues. The demonstration of NT infers the action of ONOO- or related nitrogen-centered oxidants[15]. Therefore, in our present study, the strong expression of NT and the high level of LPO in OPB suggested the presence of *trans*-$ONOO^-$ and *trans*-peroxynitrous acid (ONOOH).

Pulmonary epithelial cells were sensitive to $ONOO^-$ and easier to be injured[16]. Up to now, there has been no report on $ONOO^-$ expression in alveolar epithelial cells after BLM injection. In the present experiment,

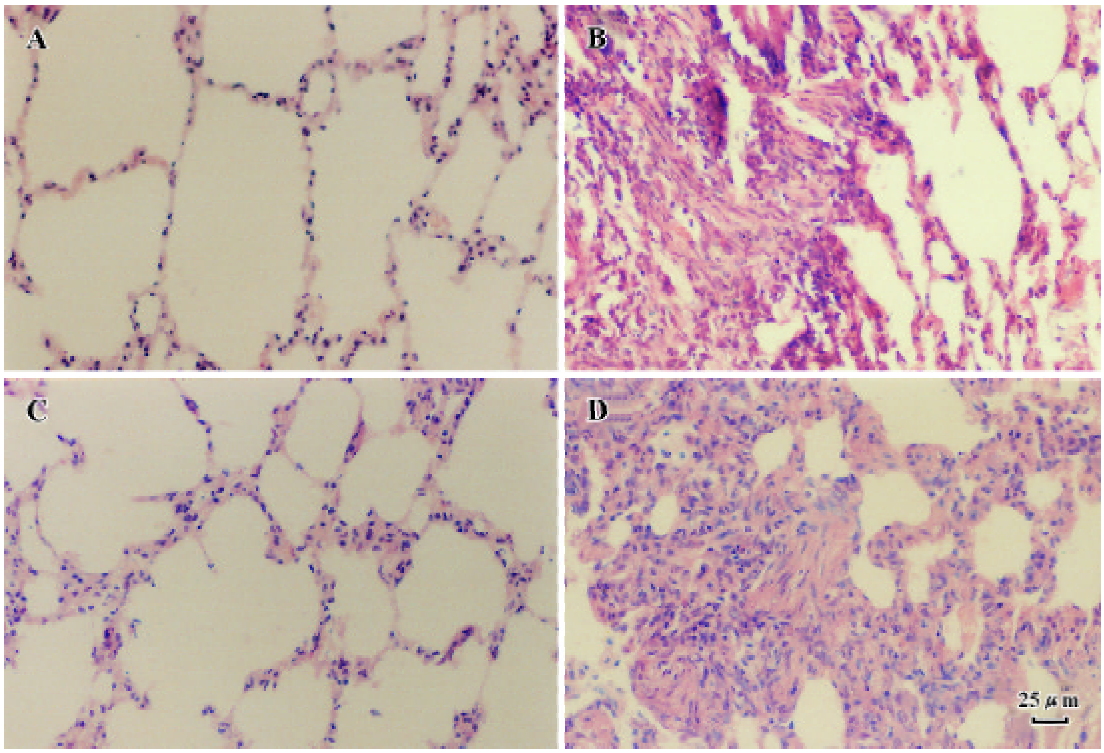

Fig 1. Typical morphology of lung tissue in various groups (HE stain, ×200). A: control group; B: BLM-A_5 group; C: BLM-A_5+AG group; D: BLM-A_5+NS group.

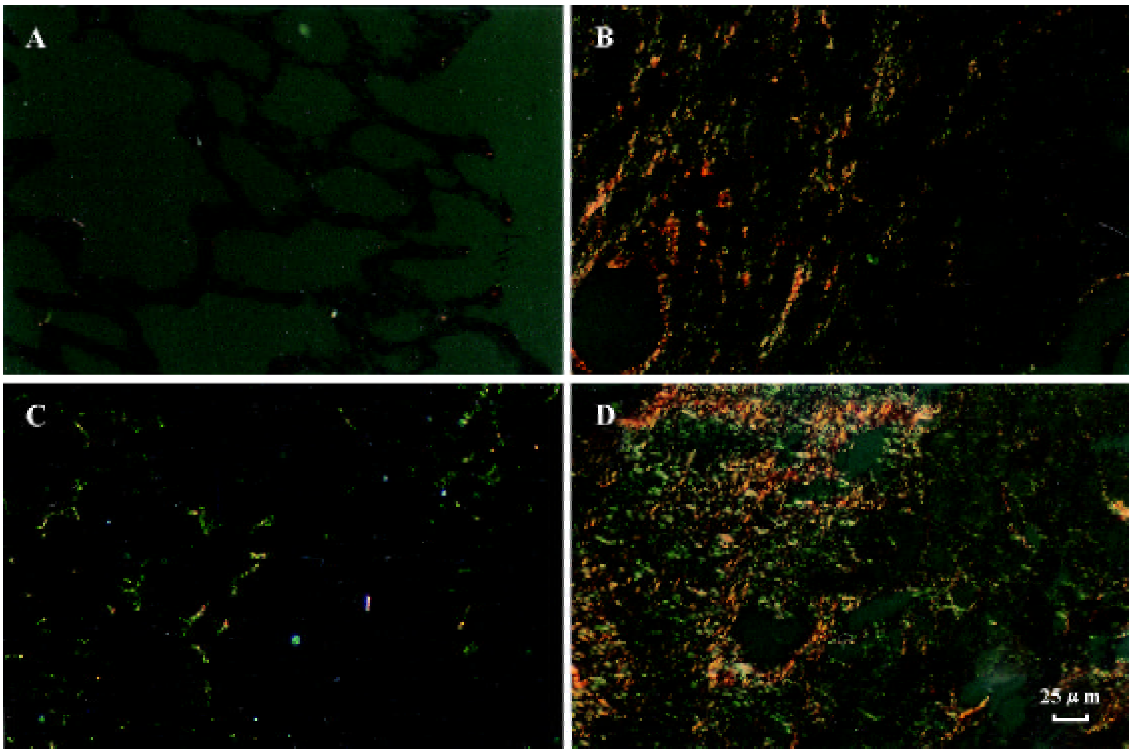

Fig 2. Changes of type I collagen (yellow or orange) and type III collagen (green) in various groups under polarization microscope (Sirius red stain, ×200). A: control group; B: BLM-A_5 group; C: BLM-A_5+AG group; D: BLM-A_5+NS group.

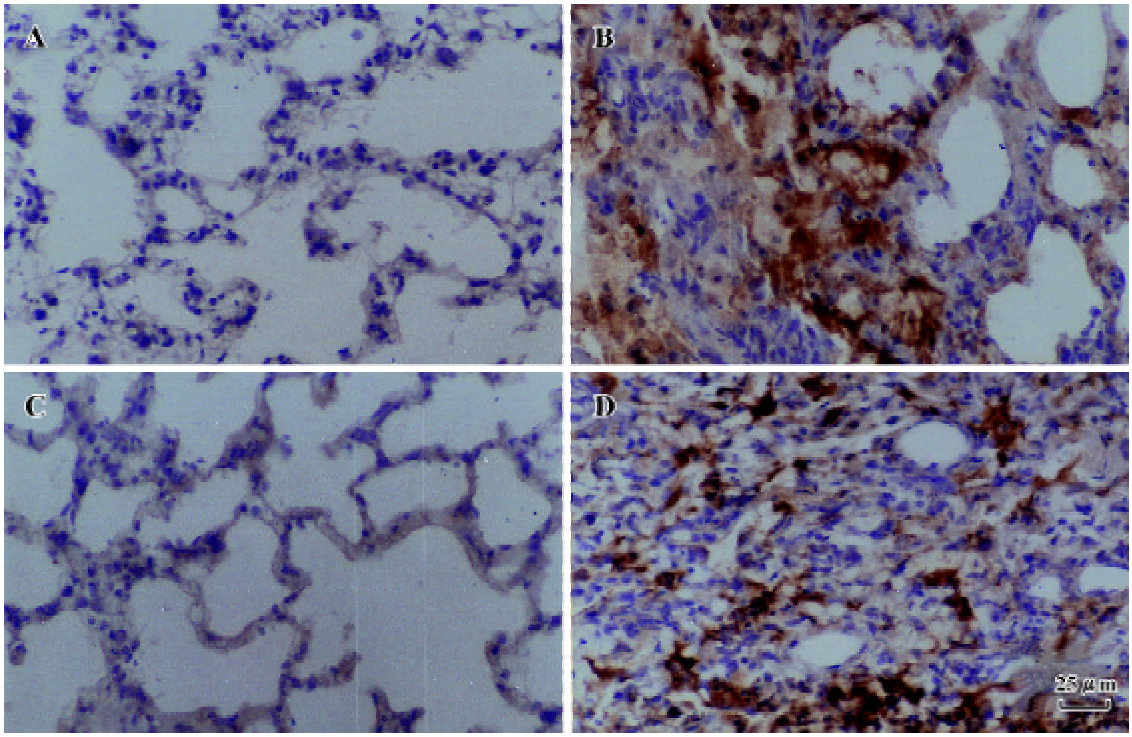

Fig 3. Immunoreactivity to nitrotyrosine (NT) in lung tissue of various groups (Immunohistochemistry stain, ×250). A: control group; B: BLM-A_5 group; C: BLM-A_5+AG group; D: BLM-A_5+NS group.

the evidence of high ONOO- expression in alveolar epithelial cells suggested that ONOO- mediated the injury of pulmonary epithelial cells induced by BLM-A_5.

Strong expression of $ONOO^-$ was seen in alveolar macrophages on d 14 after BLM injection[3]. However, there has been no report on $ONOO^-$ expression in other kind of pulmonary cells after BLM injection. Our present study found that strong expression of $ONOO^-$ also appeared in pulmonary interstitial macrophages. It is well accepted that many mediators, released by macrophages, have been implicated in the pathogenesis of pulmonary fibrosis. Pulmonary interstitial macrophages were superior to alveolar macrophages regarding mechanisms mainly involved in the induction of fibrosis[17]. Therefore, the high $ONOO^-$ expression in interstitial macrophages indicated that $ONOO^-$ might be of significance in the development of pulmonary fibrosis of BLM-A_5. Further studies are to be performed to elucidate the detailed mechanisms.

It is important to note that fibroblasts appear nearby areas of inflammatory interstitial infiltration, and of strong expression of $ONOO^-$. The distribution of type III collagen and type I collagen deposit was consistent with that of fibroblasts. This result has not been reported by others. The detailed mechanisms are unclear. The increased fibroblasts might be related to the injury of pulmonary epithelial cells, or/and might be directly related to $ONOO^-$. The above results suggested that $ONOO^-$ might be one of the critical factors, which mediated BLM-induced pulmonary toxicity.

Our previous study demonstrated that NO formation in lung was also increased on d 14 after intratracheal administration of BLM-A_5[1]. It is well known that ONOO- is formed by near diffusion-limited reaction of nitric oxide (NO) and superoxide anion. The production of $O_2^{\cdot-}$ also increased on d 14 after BLM injection[3]. Therefore, the high expression of $ONOO^-$ might be related to the high level of NO. Our present study also revealed that AG treatment markedly suppressed lung injury, NT production, and fibrosis induced by BLM-A_5. This result suggested that the deleterious role of NO in this mode might be accounted for by a cytotoxic action of $ONOO^-$, and that the effects of AG were predominantly mediated by reducing $ONOO^-$ formation.

In vivo, NO has both physiological and pathophysiological effects. It is important to distinguish toxic consequence of NO from beneficial one, and to envision potential therapeutic strategies. Our present study demonstrated that the deleterious effect of NO on pul-

monary toxicity of BLM-A_5 was mediated by formation of *trans*-$ONOO^-$ or *trans*-peroxynitrous acid (ONOOH). AG acted not only as a selective inhibitor of the iNOS, but also as a scavenger of *trans*-$ONOO^-$ or trans-peroxynitrous acid (ONOOH). The therapeutic potential of AG for pulmonary toxicity of BLM-A_5 was partly mediated by reducing $ONOO^-$ formation. Therefore, AG may be a hopeful chemical reagent to prevent and cure the pulmonary fibrosis induced by BLM-A_5. In addition, other inhibitors of $ONOO^-$ might become effective therapeutic agents.

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