

The effect of AT₁ receptor blockade on bax and bcl-2 expression in bleomycin-induced pulmonary fibrosis

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Received 10 May 2008; Revised 8 Oct 2008; Accepted 12 Oct 2008

ABSTRACT

Background and the purpose of the study: Recent studies have indicated the role of apoptosis and angiotensin in the pathogenesis of bleomycin induced-pulmonary fibrosis. Losartan, an angiotensin type 1 receptor (AT₁R) antagonist, has ameliorated apoptosis and fibrosis from bleomycin. In this study, alterations in the expression of apoptosis-regulatory genes (bcl-2 and bax) were investigated in different cells of lung tissue of mice treated with bleomycin in the presence of losartan.

Methods: Losartan (10 mg/kg, i.p.) was given to mice two days before administration of bleomycin (3 U/kg) and throughout the test period. After two weeks, lung tissues of mice were evaluated for fibrosis by biochemical measurement of collagen deposition and semiquantitative analysis of pathological changes of the lung. The expression of bcl-2 and bax was assessed by immunohistochemical assay using biotin-streptavidin staining method on paraffin-embedded lung tissues.

Results and major conclusion: Pre-treatment with losartan significantly ($P < 0.05$) reduced the increase in lung collagen content and also inhibited the histological changes induced by bleomycin. Immunohistochemical studies showed that losartan significantly ($P < 0.05$) reduced the bax/bcl-2 expression ratio in the alveolar epithelial cells, lymphocytes, macrophages and interstitial myofibroblasts. Losartan also inhibited the bcl-2 upregulation which was induced by bleomycin in neutrophils. By reduction of bax/bcl-2 ratio as a determinant of susceptibility of a cell to apoptosis, losartan exerted protective effects on the alveolar epithelial cells that may be important in the amelioration of pulmonary fibrosis. These results may help to better understanding of the role of angiotensin II and apoptosis in pulmonary fibrosis.

Keywords: bcl-2, bax, bleomycin, pulmonary fibrosis, losartan

INTRODUCTION

Apoptosis, a stereotypic program of cell death, is suggested to have a critical role in the pathogenesis of fibrotic lung diseases. Recent studies indicate that excessive apoptosis of alveolar epithelial cells (AEC) and reduction to fibroblast/myofibroblasts apoptosis contribute in progression of lung tissue injury into fibrosis (1-3). Angiotensin II (Ang II), the most important peptide of the renin-angiotensin system, contributes to the development of fibrotic response to tissue injury via its growth factor properties and induction of fibroblast proliferation and collagen deposition (4, 5). Ang II is a potent inducer of apoptosis in different cells such as

AEC (6), glomerular epithelial cells (7), cardiac myocytes and endothelial cells (8). It has been also reported that AEC apoptosis in response to various stimulus such as Fas ligand, tumor necrosis factor- α (TNF- α) or bleomycin requires production of Ang II and subsequent activation of Ang II receptors (9- 11). Furthermore, Ang system antagonists including Ang II converting enzyme inhibitors (ACEI) and Ang II type 1 receptor (AT₁R) blockers have shown inhibition of apoptosis of lung epithelial cells and fibrogenesis in several investigations (12, 13). Although the molecular mechanisms of apoptosis in different cell type of the lung is largely unknown, it has been suggested that apoptosis-

regulatory genes such as bcl-2 and bax may participate in epithelial cell apoptosis in pulmonary fibrosis (14, 15). Bcl-2 protein, an anti-apoptotic member of this family, is an intercellular associated protein, which promotes cell survival (16). Bax protein, a pro-apoptotic member, is a homologue of bcl-2 which may bind to bcl-2 to form bax/bcl-2 heterodimers and antagonizes the pro-survival activity of bcl-2. The imbalance between pro- and anti-apoptotic activities of these family members may determine the cellular decision to undergo apoptosis or not (17). AT₁R blockade and changes in bcl-2 and bax expression have been studied in some pathological condition such as hypertension, pancreatic and cardiac fibrosis (18-20), but to our knowledge there is no report on the role of AT₁R in the expression of bcl-2 and bax in pulmonary fibrosis.

In this study, with regard to essential role of AT₁R signaling in pulmonary apoptosis, it was hypothesized that changes in expression of apoptosis-regulatory genes, bcl-2 and bax, may be involved in the anti-apoptotic effects of losartan, as an AT₁R antagonist, in bleomycin-induced pulmonary apoptosis and fibrosis.

MATERIAL AND METHODS

Animals

Female NMRI mice, 8-10 weeks of age and weighing 25-30 g (Pasteur Institute, Tehran, Iran) were used. Animals were kept at a temperature of 21 ± 3 °C with a 12h light/dark cycle and had access to water and rodent laboratory chow *ad libitum*. The mice were acclimated to the laboratory conditions for at least 1 week prior to the experiments. Animals were weighed at the beginning and at the end of the experiments. All experiments were carried out according to the internationally accepted guidelines of the care and use of laboratory animals.

Induction of pulmonary fibrosis and treatments

To induce pulmonary fibrosis, animals were treated by intratracheal (i.t.) administration of bleomycin. Under anesthesia by intraperitoneal (i.p.) injection of 75 mg/kg of ketamine (Rotexmedica Co., Germany), animals were tracheostomized and bleomycin hydrochloride (Nippon Kayaku Co., Japan) was instilled at a single dose of 3 U per kg of body weight in 50 μ l of sterile saline (Daru Pakhsh Co., Iran). Control mice received the same volume of sterile saline intratracheally. Six animals were used in each control and experimental groups. Two groups of mice received losartan. In one group, animals received daily intraperitoneal injections of

losartan at a dose of 10 mg/kg in sterile saline after administration of bleomycin and in another group; losartan was given two days before administration of the bleomycin and was continued throughout the test interval (2 weeks). Control mice of the losartan group received daily sham injections of the saline alone.

Two weeks after bleomycin instillation, all groups of animal were sacrificed by high dose of ketamine. Lungs were removed and then weighed. From each mouse, right lobe of lung tissue was taken for biochemical analysis of collagen content. The left lobe of lung was perfused with 10% neutral-buffered formalin via the trachea and kept in formalin solution for 24 hrs and then embedded in paraffin. The 4- μ m-thick paraffin sections were used for immunohistochemical, haematoxylin-eosin (H&E) and Masson's trichrome staining. Semiquantitative morphological study of pathological changes in lung sections was carried out to assess the severity of pulmonary fibrosis and graded according to the method described by Ashcroft and coworkers (21).

Biochemical analysis

Collagen content of the lung tissues was estimated by determination of hydroxyproline in hydrolysates of tissues. In brief, lung samples were hydrolyzed in the presence of hydrochloric acid and processed according to the previously described method (22).

Immunohistochemistry (IHC) for bcl-2 and bax

IHC was performed on formalin-fixed and paraffin-embedded tissues using a biotin-streptavidin technique for bcl-2 (rabbit anti-mouse polyclonal antibody, A18, 1:100, Delta Biolabs, CA) and bax (rabbit anti-mouse polyclonal antibody, 1:100, Dako, CA) (23). Briefly, deparaffinized lung sections were incubated for 30 min at room temperature with bcl-2 and bax antibodies. After rinsing with PBS solution, the sections were incubated with biotinylated goat anti-rabbit IgG (1:200, Dako) for 10 min and then labeled by streptavidin-peroxidase complex (1:200, Dako) for 5 min. The normal lymph node of the lung tissue was used as an internal positive control for bcl-2 and sections of normal breast tissue were used as a positive control for bax. For negative controls, the primary antibodies were replaced by rabbit serum (24).

The intensity of immunohistochemical staining was graded semiquantitatively as follows: grade 0 = no staining present or less than 10% of the cells are positive; grade 1 = 10% of the cells are positive; grade 2 = more than 10% and less than 50% of the cells are positive; and grade 3 = more than 50% of the cells are positive (15).

Statistical analysis

Statistical analysis was made by one-way ANOVA followed by Dunnett analysis. Data were presented as mean \pm SEM except for pathological findings. For grading analyses, data were presented as semiquantitative grades and evaluated by the non-parametric Kruskal-Wallis method followed by Mann-Whitney tests. P value < 0.05 was considered statistically significant.

RESULTS

Hydroxyproline content of the lung

Administration of bleomycin significantly increased hydroxyproline level after two weeks by 47% ($P < 0.05$). Daily administration of losartan, at a dose of 10 mg/kg, for 2 weeks after administration of bleomycin failed to attenuate the hydroxyproline content of the fibrotic lungs but pre-treatment with losartan significantly ($P < 0.05$) reduced the increase in hydroxyproline level by 22.3% (figure 1).

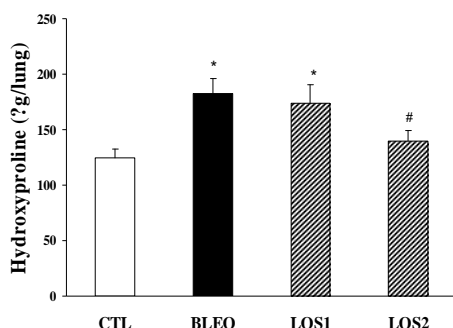


Figure 1. The effects of treatment and pre-treatment with losartan (10 mg/kg, i.p.) (LOS1 and LOS2, respectively) on lung hydroxyproline content. Two weeks after exposure to either intratracheal saline (CTL) or bleomycin (BLEO) (3 U/kg) in the presence or absence of losartan, hydroxyproline content of lung tissue of mice were measured. Data are presented as mean \pm SEM of $n = 6$; * $P < 0.05$ versus control, # $P < 0.05$ versus BLEO.

Lung weight

Total wet lung weight was measured as an indicator of lung inflammation. Bleomycin significantly increased wet lung weight after 2 weeks ($P < 0.05$). The wet lung weight of the mice treated with losartan was significantly ($P = 0.001$) lower than that of bleomycin-induced fibrotic lungs (figure 2). There was no significant difference in body weight of animals at the beginning and at the end of the experiments.

Histological findings

Morphological examination of lung tissues 2 weeks after exposure to bleomycin revealed

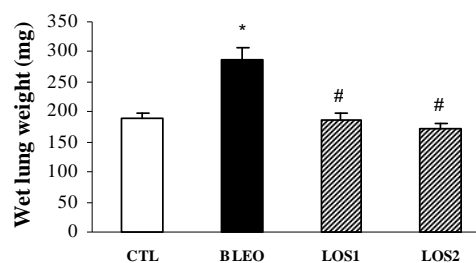


Figure 2. The effects of treatment and pre-treatment with losartan (10 mg/kg, i.p.) (LOS1 and LOS2, respectively) on wet lung weight of mice. Animals were exposed to either intratracheal saline (CTL) or bleomycin (BLEO) (3 U/kg) in the presence or absence of losartan. Data are presented as mean \pm SEM of $n = 6$; * $P < 0.05$ versus control, # $P < 0.05$ versus BLEO.

histological changes such as thickening of the alveolar walls, infiltration of inflammatory cells into the interstitium and increased amount of collagenous fibers in the interstitial areas (Fig 3B). No pathological change was observed in the lungs of control animals (Fig 3A). Pre-treatment with losartan partially reduced the histological changes induced by bleomycin. In some tissues, there was less infiltration of inflammatory cells in alveolar spaces, no changes in alveolar wall and less collagen deposition (Fig 3C). The severity of changes varied from slight to moderate. The Ashcroft fibrotic score for losartan-treated mice was 1 (median grade). The semiquantitative morphological changes of the lung tissue as fibrosis median grades are shown in figure 4.

IHC for bcl-2 and bax

Bcl-2 protein was detected in bronchiolar and AEC, macrophages, lymphocytes, neutrophils and interstitial myofibroblasts 2 weeks after bleomycin instillation but was not found in neutrophils and interstitial myofibroblasts of the control animals. Bax protein was positive in bronchiolar and AEC, macrophages, lymphocytes, and interstitial myofibroblasts of the normal lung tissues but was not detected in lymphocytes of the bleomycin-instilled mice (figure 5A-5D).

The semiquantitative results of IHC staining for bcl-2 and bax are shown in tables 1 and 2. Bcl-2 expression was significantly ($P < 0.05$) upregulated in myofibroblasts and neutrophils after bleomycin administration. The expression of bax protein was significantly ($P < 0.05$) upregulated in AEC and downregulated in lymphocytes of the lung tissues of the mice 2 weeks after bleomycin instillation.

Pre-treatment with losartan significantly ($P < 0.05$) reduced the expression of bcl-2 in myofibroblasts and neutrophils in comparison

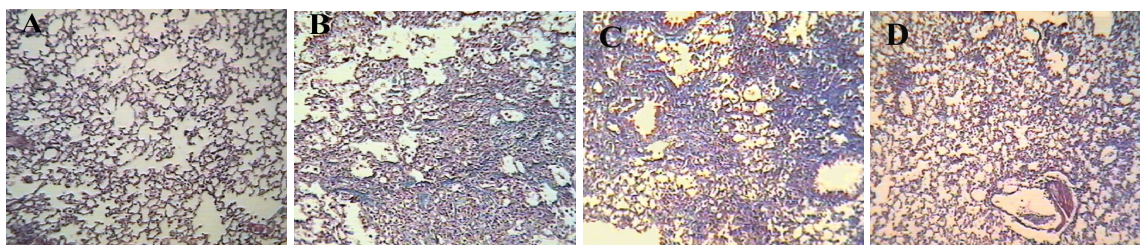


Figure 3. Representative Masson's trichrome histological sections of the lung tissue of mice after intratracheal bleomycin (3 U/kg) (B) or treatments with saline (A) or losartan treatment (10 mg/kg, i.p.) following bleomycin instillation (C) or pretreatment with losartan (D) ($\times 100$ magnification). After 2 weeks, less infiltration of inflammatory cells, partial changes in alveolar wall and less collagen deposition are observed in losartan-pre-treated mice in comparison with bleomycin-treated mice.

Table 1. Frequency and staining grade of bcl-2 expression in the mouse lung tissues

Type of cells	Groups					
	Control		Bleomycin		Losartan	
	F	G	F	G	F	G
Bronchiolar epithelial cells	10.5 \pm 3.5	1	9.5 \pm 2.5	1	12.5 \pm 4.5	1
Alveolar epithelial cells	48 \pm 2.5	2	45 \pm 6	2	43 \pm 8	2
Interstitial myofibroblasts	5 \pm 2.5	0	22.5 \pm 3*	2	7.5 \pm 2 [#]	0
Alveolar macrophages	75.3 \pm 6	3	75.5 \pm 4	3	55.5 \pm 9	3
Lymphocytes	56 \pm 4	3	59 \pm 2.5	3	50 \pm 5	2
Neutrophils	5 \pm 1.5	0	13 \pm 2.5*	1	6 \pm 1.5 [#]	0

F, Frequency, expressed as percentage of immunoreactive cells; G, Staining grade. Data are presented as mean \pm SEM for frequency and as the median grade of the immunoreactivity for six mice. * $P < 0.05$ versus control; [#] $P < 0.05$ versus bleomycin; ^a $P < 0.05$ versus control and bleomycin.

Table 2. Frequency and staining grade of bax expression in the mouse lung tissues

Type of cells	Groups					
	Control		Bleomycin		Losartan	
	F	G	F	G	F	G
Bronchiolar epithelial cells	36 \pm 6.5	2	20.5 \pm 3	2	15.5 \pm 2	2
Alveolar epithelial cells	32 \pm 2.5	2	60 \pm 3*	3	25.5 \pm 3 [#]	2
Interstitial myofibroblasts	15 \pm 1	2	25.5 \pm 2	2	7 \pm 2 ^a	0
Alveolar macrophages	35 \pm 2.5	2	37 \pm 3	2	10 \pm 1.5 ^a	1
Lymphocytes	10 \pm 1.5	1	5 \pm 1.5*	0	2.5 \pm 1*	0
Neutrophils	1.5 \pm 1.5	0	2.3 \pm 2	0	2 \pm 1	0

Abbreviations: F, Frequency, expressed as percentage of immunoreactive cells; G, Staining grade. Data are presented as mean \pm SEM for frequency and as the median grade of the immunoreactivity for six mice. * $P < 0.05$ versus control; [#] $P < 0.05$ versus bleomycin; ^a $P < 0.05$ versus control and bleomycin.

with bleomycin-induced fibrotic lungs. Losartan also significantly ($P < 0.05$) reduced the expression of bax in the AEC, lymphocytes, myofibroblasts and macrophages.

DISCUSSION

Despite considerable investigations, the precise mechanism(s) of bleomycin-induced pulmonary apoptosis are not clearly known. In this study, pre-treatment with losartan at the dose of 10 mg/kg significantly reduced the histological fibrotic changes and collagen content of the lung by 22.3%. However, there was no significant reduction in fibrotic changes by simultaneous administration of losartan with bleomycin which are in agreement with results of some recent reports. Although there is a report that losartan at the dose of 10 mg/kg which is effective in renal

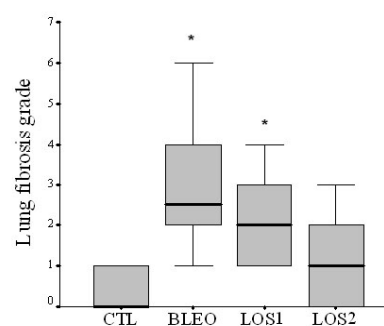


Figure 4. Semiquantitative scoring of lung fibrosis for the groups of mice after intratracheal bleomycin (3 U/kg) (BLEO) or saline treatment (CTL) or losartan treatment (LOS1) or pre-treatment with losartan (LOS2) (10 mg/kg, i.p.). Thick lines represent the median of $n = 6$, boxes show the interquartile range and bars represent the maximum and minimum sample values. * $P < 0.05$ versus control.

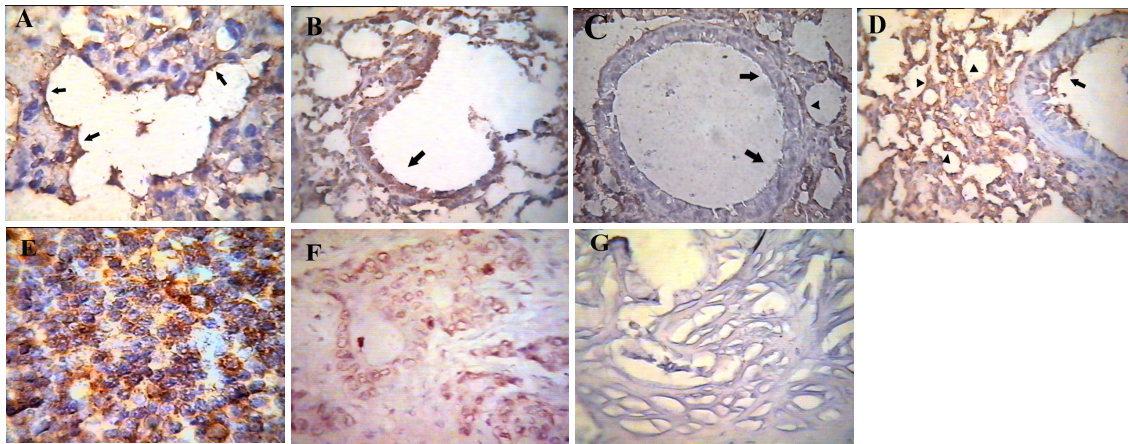


Figure 5. Immunohistochemical localization of bcl-2 and bax protein. The positive signals for bax are observed in the alveolar and bronchiolar epithelial cells of bleomycin-treated mice (arrows in 5A and 5B, respectively) while not in the alveolar and bronchiolar epithelial cells of losartan-treated mice (arrowheads and arrows in 5C, respectively). The positive signals for bcl-2 are observed in the alveolar and bronchiolar epithelial cells of losartan-treated mice (arrowheads and arrows in 5D, respectively). The normal lymph node of the lung tissue as an internal positive control for bcl-2 and sections of normal breast tissue as a positive control for bax are observed in 5E and 5F, respectively. Negative control for bax is observed in 5G. Magnifications: $\times 1000$ (A, E), $\times 400$ (B, C, D, F, G).

and cardiac fibrosis in mice, could not reduce bleomycin-induced pulmonary fibrosis (25), in other studies, simultaneous administration of losartan 10 mg/kg with bleomycin has reduced the AEC apoptosis (13) significantly and at the dose of 20 mg/kg, 24 hrs before administration of bleomycin significantly attenuated lung collagen deposition by 39% (4). It seems that higher doses of losartan are required to ameliorate lung fibrosis in comparison with other fibrotic diseases. For antifibrotic effect of AT₁ antagonists, different potential mechanisms have been described including reduction in vascular tone and permeability, reduction in neutrophil accumulation and chemotaxis, reduction in lung fibroblast proliferation and collagen production, inhibition of transforming growth factor (TGF)- β synthesis and inhibition of apoptosis (13, 4, 5). Our results showed that losartan significantly reduced the bax/bcl-2 expression ratio, as a determinant of susceptibility of a cell to apoptosis, in the AEC. Anti-apoptotic effect in AEC through reduction of caspase 3 activation which plays an essential role in the execution of apoptosis has also been demonstrated for losartan (13). Although losartan inhibited the bcl-2 upregulation in myofibroblasts but it had no desirable effects since it also reduced the bax expression in these cells. Fibroblasts and myofibroblasts have critical roles in the fibrotic changes of the lung and fibroproliferation process may be potential target for treatment of pulmonary fibrosis in future (1, 3). Losartan also inhibited the bcl-2 upregulation in neutrophils induced by bleomycin. Neutrophil

apoptosis and clearance of apoptotic neutrophils by macrophages may play an important role during the resolution of inflammation and suppression of proinflammatory cytokine production in the lung (26). AT₁ antagonists have been also able to reduce neutrophil accumulation in the bronchoalveolar space and an important role for Ang II in the neutrophil chemotaxis (5) has been suggested.

Losartan also reduced the bax/bcl-2 expression ratio in lymphocytes and macrophages. The explanation of losartan effects on lymphocytes is difficult because various subtypes of lymphocytes infiltrate the injured lung and are able to exert both stimulatory and inhibitory effects on fibroblast proliferations (27).

It seems that losartan had no desirable effects on macrophages which play an important role in the development of pulmonary fibrosis. Results of another study have also shown the failure of RAS blockade on reduction of alveolar macrophage accumulation in amiodarone induced-pulmonary fibrosis (28).

It is well recognized that regulation of apoptosis is specific in different cell types exposed to the same stimuli. Additionally, it has been reported that effects of Ang II receptor stimulation is heterogenic in different tissues, cells, and under different experimental conditions (29). AT₁R is involved in the apoptosis process through various molecular pathways including calcium flow, the ratio of bax/bcl-2, activation of NF- κ B and caspase-3 (30).

In summary, findings of this study confirmed the essential role of Ang II in the pathogenesis of

bleomycin-induced pulmonary fibrosis and apoptosis. The present study proposes another mechanism for antiapoptotic effects of AT₁R blockade through changes in expression of

apoptosis-regulatory genes, bcl-2 and bax. However, further studies are required to define the role of Ang II and apoptosis in pulmonary fibrosis more precisely.

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