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Inflammation Research

Neutrophil elastase inhibitor (sivelestat) reduces the Levels of inflammatory mediators by inhibiting NF-κB

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Abstract. *Objective:* Sivelestat sodium hydrate (sivelestat) is a specific synthetic inhibitor of neutrophil elastase (NE). Various studies suggest that sivelestat treatment reduces inflammation. In this study, we tested the hypothesis that sivelestat acts as an inhibitor of inflammatory mediators and prevents nuclear factor-κB (NF-κB) activation.
Methods: In the presence and absence of sivelestat, the mouse macrophage cell line RAW 264.7 was stimulated with lipopolysaccharide (LPS) and the levels of inflammatory mediators (TNF-α, IL-6 and high mobility group box 1 (HMGB1)) and nitrite in the cell supernatant were measured, along with inducible nitric oxide synthase (iNOS) expression.
Results: While LPS administration increased the secretion of inflammatory mediators and nitric oxide (NO), sivelestat decreased the secretion of these mediators. Cell signaling studies demonstrated that sivelestat decreased NF-κB activation by inhibiting IκB phosphorylation.
Conclusion: Sivelestat may inhibit the various inflammatory mediators through NF-κB inhibition.

Key words: Lipopolysaccharide – Sivelestat sodium hydrate – HMGB1 – Neutrophil elastase

Introduction

Acute respiratory distress syndrome (ARDS) commonly occurs in cases of severe sepsis and yields a mortality rate of 25–31 and few effective therapeutic strategies are currently available [1]. Despite recent improvements in our understanding of the molecular underpinnings of ARDS, acute lung injury (ALI) remains refractory to treatment.

A specific neutrophil elastase (NE) inhibitor, sivelestat, has been developed in Japan [2]. This agent is characterized as having no effects on proteases other than NE [2]. Previous studies reported that sivelestat reduced lung injury associated with systemic inflammatory response syndrome (SIRS) in humans [3] and decreased endotoxin-induced lung injury in animal models [4]. Indeed, plasma NE levels were significantly elevated in patients with ALI and ARDS compared to healthy volunteers [5]. In addition, clinical studies demonstrated that sivelestat improved the arterial oxygen tension-to-inspired oxygen fraction (PaO2/FiO2) and decreased the length of stay in the intensive care unit and days on a ventilator for patients with ALI and ARDS [6]. Moreover, treatment of ARDS with sivelestat reduced serum cytokine levels [7]. Sivelestat is also effective in animal models of ARDS/ALI. These results suggest that sivelestat might be useful for the treatment of SIRS and ARDS/ALI.

While the efficacy of sivelestat for ALI patients remains controversial, a Japanese phase III study reported promising results [8]. In that study, sivelestat administration reduced the artificial ventilation period and duration of stay in the intensive care unit. Additionally, we recently demonstrated the effectiveness of sivelestat in animal models of ARDS/ALI [9]. However, the mechanism(s) by which sivelestat confers protection is not well understood.

During severe inflammation, excessive oxidative stress is observed and nitric oxide (NO) has been postulated to be a key regulator. Various inflammatory stimuli, including lipopolysaccharide (LPS), stimulate endogenous NO production by activating inducible nitric oxide synthase (iNOS) [10]. Overproduction of NO subsequently initiates a cascade of inflammatory responses that lead to tissue injury and, eventually, multiple organ dysfunction [11].

Release of various mediators (including cytokines and the high mobility group box 1 (HMGB1) protein) correlates with severity of inflammation, as observed in SIRS and ALI [12, 13, 14]. The cytokines TNF-α and IL-6 play a central role in the pathogenesis of the acute inflammatory response and some studies report that high levels of TNF-α and IL-6 correlate with the severity of disease [15, 16]. Additionally, HMGB1

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enhances the inflammatory response during septic shock [17, 18], and a later release and action may be generally observed compared to that for cytokines such as TNF-α and IL-1β [13]. Therefore, inhibitors of cytokines and HMGB1 might prove beneficial in the treatment of SIRS and ALI because they affect both the early and late inflammatory responses.

The transcription factor NF-κB regulates the expression of many inflammatory genes including TNF-α and IL-6 [19]. NF-κB activation involves the regulation of p65 and p50 subunit transactivation [20]. Aberrant activation of NF-κB is associated with systemic inflammation, as observed in cases of septic shock [21].

Sivelestat may inhibit the secretion of HMGB1 from LPS-stimulated murine macrophages. To test these hypotheses, we investigated the effect of sivelestat treatment on the secretion of various mediators by RAW264.7 cells. We further examined NF-κB activity and IκB phosphorylation to elucidate the mechanism which controlled these effects.

Materials and Methods

Materials

LPS (O127:B8) was obtained from Sigma-Aldrich (St Louis, MO). Sivelestat was donated by Ono Pharmaceutical Co. Ltd. (Osaka, Japan). Antibodies to iNOS (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phosphorylated IκB α (p-IκB α) and IκB α (Cell Signaling Technology, Beverly, MA), and β-actin (Abcam, Cambridge, UK) were purchased and stored at 4 °C or -20 °C.

Cell Culture

The RAW 264.7 macrophage-like cell line obtained from the American Type Culture Collection was maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 5 % heat-inactivated fetal bovine calf serum, penicillin (50 units/ml, Gibco BRL, Grand Island, NY), and streptomycin (50 μg/ml, Gibco BRL, Grand Island, NY) at 37 °C in 5 % CO2. The medium was removed and replaced with RPMI 1640 containing 5 % fetal bovine serum (FBS) (for most experiments) or Opti-MEM (Sigma-Aldrich, St. Louis, MO) (for experiments measuring HMGB1 levels in conditioned media). RAW264.7 cells were simultaneously treated with sivelestat and stimulated with LPS. Samples of the culture supernatant were obtained at various time points, as described in the next section. Cells were lysed using the Mammalian Protein Extraction Reagent Kit (PIERCE Biotechnology, Rockford, IL) or Nuclear and Cytoplasmic Extraction Reagent Kit (PIERCE Biotechnology, Rockford, IL). Homogenates were boiled (5 min) prior to the addition of dithiothreitol. Cytoplasmic and nuclear protein extracts were analyzed by western blot as described below.

Measurement of cytokine and HMGB1 secretion

Samples of culture supernatant were taken at 0, 1.5, 3, 4.5, 6, 9, 12, 18 and 24 h time points. "0 h" refers to the time point immediately prior to LPS administration. HMGB1, IL-6, and TNF-α levels were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (HMGB1, Shino-Test Corporation, Tokyo, Japan; IL-6 and TNF-α, R&D Systems Inc., Minneapolis, MN).

Determination of nitrite concentration

Samples of culture supernatant were taken at 0, 1.5, 3, 4.5, 6, 9, 12, 18 and 24 h time points. "0 h" refers to the time point immediately prior to LPS administration. NO is rapidly oxidized to nitrite in biological fluids. Therefore, nitrite content in samples was measured as a proxy for NO. Nitrite concentrations were determined using a commercial kit according to the manufacturer's protocol (R&D Systems Inc., Minneapolis, MN). This kit measures nitrite concentration using a modification of the Griess assay, which is a colorimetric assay that measures absorbance at 540 nm. Nitrite concentration was calculated using a standard curve and expressed as micromoles per liter.

Western blot

Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were washed with phosphate buffered saline (PBS) containing 0.1 % Tween 20 and 5 % skim milk (PBS-T). The membranes were incubated with primary antibody (1:1000 dilution). After secondary antibody incubation, blots were developed using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK) and exposed on Hyperfilm ECL (Amersham, Buckinghamshire, UK). The film was then scanned and the band concentration was calculated by the quantification of the integrated optical density of the appropriate band using the Image J 1.37v software program (National Institute of Health, Bethesda, MD).

NF-κB Binding Assay

Samples of the cells were taken at 0, 1 and 2 h time points. "0 h" refers to the time point immediately prior to LPS administration. The DNA binding activity of NF-κB (p50/p65) was determined using an ELISA-based nonradioactive NF-κB p50/p65 transcription factor assay kit (Chemicon, Temecula, CA).

Statistical analysis

For descriptive purposes, all continuous data were presented as the mean ± SD. The data were compared using nonparametric tests and analyzed by the Mann–Whitney U-test for comparison between two independent groups. The Kruskal-Wallis test was used for comparison between all groups. P-values less than 0.05 were considered statistically significant.

Results

Effect of sivelestat on HMGB1 secretion

The secretion of HMGB1 into the culture supernatant was measured 24 h following LPS administration. HMGB1 levels in the culture supernatant increased after the LPS administration, but the secretion was significantly inhibited by the administration of 100 μg/ml sivelestat (Figure 1). The most effective dose of sivelestat was 100 μg/ml, and this dose was used for subsequent experiments.

Effect of sivelestat on cytokine secretion

TNF-α levels in the culture supernatant increased 3 h after LPS administration (Figure 2A). Sivelestat significantly inhibited the secretion of TNF-α in response to LPS administration. Similarly, IL-6 levels in the culture supernatant also increased following LPS administration (Figure 2B), and its increase was inhibited by sivelestat (Figures 2 A, B).

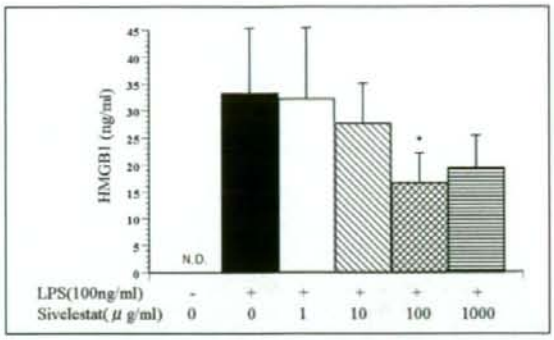

Fig. 1. Effect of sivelestat on HMGB1 secretion by LPS-stimulated murine macrophages.
Murine macrophages treated with or without sivelestat (1, 10, 100, and 1000 μg/ml) were stimulated with LPS (100 ng/ml) for 20 h. Supernatants were prepared and HMGB1 levels were examined by ELISA. Results are expressed as the mean ± SD. *denotes a significant difference compared to LPS-only treated cells ($p<0.05$). N.D.: not detected.

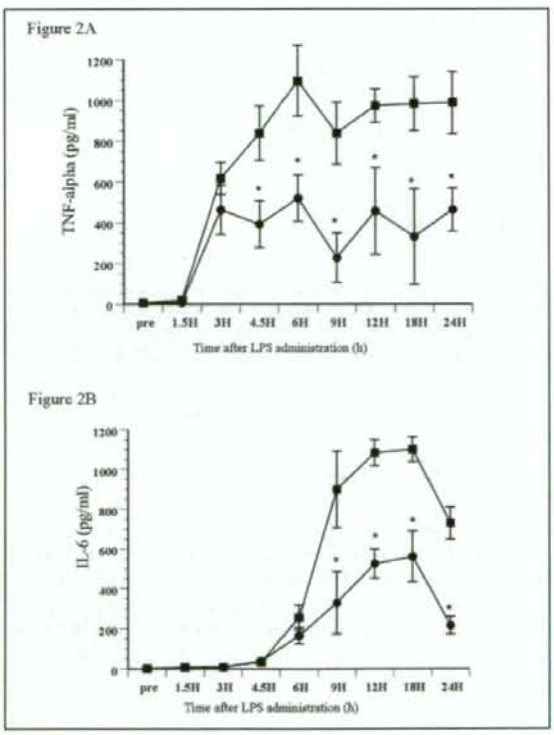

Fig. 2. Effect of sivelestat on TNF-α and IL-6 production by LPS-stimulated murine macrophages.
Murine macrophages treated with or without sivelestat (100 μg/ml) were stimulated with LPS (100 ng/ml) for the indicated durations. Squares represent LPS treatment without sivelestat and circles represent LPS treatment with sivelestat. Supernatants were collected and TNF-α(**A**) and IL-6 (**B**) levels were determined by ELISA. Results are expressed as the mean ± SD. * denotes a significant difference compared to cells treated with only LPS ($p<0.05$).

Effect of sivelestat on serum nitrite levels and iNOS expression

Nitrite is a metabolic product of NO that is often used as a marker to indicate NO production. Nitrite concentrations were measured in culture supernatant at the indicated times after LPS administration. We observed a LPS-induced increase in serum nitrite levels that was significantly inhibited by sivelestat treatment (Figure 3). The observed increase in iNOS expression in murine macrophage RAW264.7 cells by LPS administration was also diminished by sivelestat treatment (Figure 4).

Sivelestat inhibits the IKK pathway and modulates NF-κB

Since the NF-κB pathway plays a crucial role in the secretion of cytokines and NO, we measured the activation of the NF-κB subunits p50 and p65 in the nucleus. Treatment of cells with LPS led to a robust activation of p50 and p65. This activation was partially blocked by sivelestat (Figure 5). The IκB kinase (IKK) pathway was examined as another potential regulator of NF-κB. Sivelestat inhibited IκB α degradation resulting from LPS stimulation (Figure 6). In addition, IκB α phosphorylation (p-IκB) in RAW264.7 cells increased after LPS administration, and this phosphorylation was also inhibited by sivelestat (Figure 6).

Discussion

In this study, we demonstrate that sivelestat inhibited HMGB1 secretion and production of cytokines and NO in LPS-stimulated murine macrophages by inhibiting NF-κB activation. This lack of NF-κB activation may be due to inhibition of IκB phosphorylation by sivelestat.

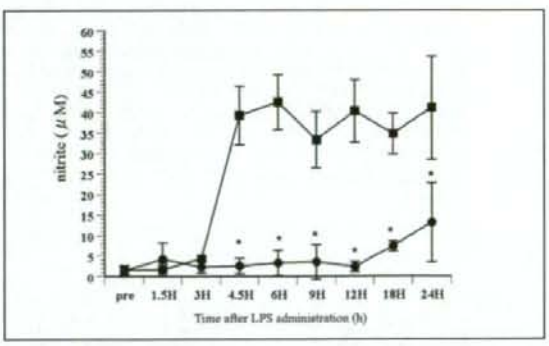

Fig. 3. Effect of sivelestat on serum nitrite levels after LPS stimulation of murine macrophages.
Murine macrophages treated with or without sivelestat (100 μg/ml) were stimulated with LPS (100 ng/ml) for the indicated durations. Squares represent LPS treatment without sivelestat and circles represent LPS treatment with sivelestat. Supernatants were collected and nitrite concentrations were determined by a modification of the Griess assay. Results are expressed as the mean ± SD. *denotes a significant difference compared with cells treated only with LPS ($p<0.05$).

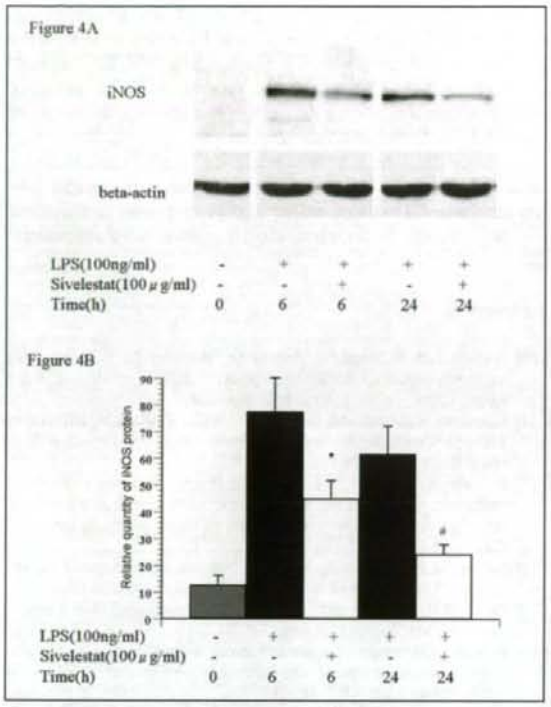

Fig. 4. Changes in iNOS protein expression after LPS stimulation of murine macrophages.
(**A**) Murine macrophages treated with or without sivelestat (100 μg/ml) were stimulated with LPS (100 ng/ml) for the indicated durations. The expression of iNOS after the treatment of cells with LPS, with or without sivelestat, was detected by western blot. The blots were also probed with an antibody against β-actin as a loading control. (**B**) The signal intensities for the density based on an immunoblot analysis of iNOS protein. iNOS was quantified using an image analyzer. The density of the signal intensities was lower for the cells treated with both LPS and sivelestat compared to cells treated only with LPS. Results are expressed as the mean ± SD. * denotes a significant difference compared with the LPS-only group at 6 h ($p<0.05$). # denotes a significant difference compared with the LPS-only group at 24 h ($p<0.05$).

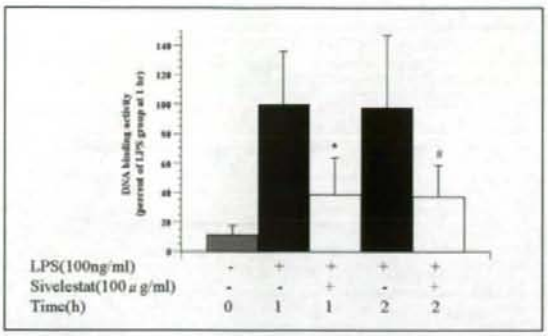

Fig. 5. Effect of sivelestat on the LPS-induced increase of p50/p65 DNA binding activity.
The DNA binding activity assay showed a marked decrease in p50/p65 DNA binding activity in nuclear fractions from RAW264.7 cells treated with LPS and sivelestat compared to cells treated only with LPS. Results are expressed as the mean ± SD. * denotes a significant difference compared with the LPS-only group at 1 h ($p<0.05$). # denotes a significant difference compared with the LPS-only group at 2 h ($p<0.05$).

Previous studies demonstrated that treatment of ALI/ARDS with sivelestat led to reduced cytokine production and improved lung function [6, 7]. One of the proposed mechanisms was NE inhibition by sivelestat [2]. However, other potential mechanisms were not elaborated upon. We demonstrate that sivelestat reduced production of various inflammation mediators through NF-κB inhibition. Therefore, sivelestat inhibits NE production and may also have anti-inflammatory properties.

Cytokines are important mediators of inflammation in various disease states [22]. Previous studies demonstrated the mutual relationship between HMGB1 and cytokines [13] and the proinflammatory properties of HMGB1 [23]. Therefore, cytokines and HMGB1 are thought to play a crucial role in regulating inflammation. We observed that the cytokine and HMGB1 levels were significantly decreased in response to sivelestat treatment (Figures 1, 2). These results indicate that in addition to inhibiting cytokine production, sivelestat inhibits HMGB1 during LPS stimulation of murine macrophages. Therefore, sivelestat may be useful for regulating the inflammatory response.

Inducible nitric oxide synthase (iNOS) and nitrite levels were significantly inhibited by sivelestat treatment (Figure 3, 4). iNOS is typically activated under systemic inflammatory conditions including septic shock [24] and its increased expression promotes nitric oxide (NO) release, leading to organ damage. NO plays a key role in inflammation and has been implicated in a wide variety of disease processes [24]. Furthermore, TNF-α stimulates iNOS activity [25]. These results suggest that the anti-inflammatory actions of sivelestat in response to LPS administration may occur by inhibiting cytokines and iNOS. iNOS inhibition may be related to TNF-α inhibition.

NF-κB coordinates the induction of several genes leading to the production and secretion of pro-inflammatory cytokines when NF-κB is freed from an inhibitory action of IκB [26]. Moreover, iNOS activity in macrophages is first regulated and modulated by cellular receptor molecules via NF-κB pathway activation [27]. In addition, NF-κB activation contributes to HMGB1 secretion after LPS administration [28]. We demonstrate that sivelestat inhibited LPS-induced NF-κB activation (Figure 5). This may, in turn, inhibit the activation of macrophages and the amounts of various inflammatory mediators secreted.

NF-κB associates with inhibitory IκB proteins which sequester NF-κB in the cytoplasm. Phosphorylation of IκB by IκB kinases (IKKs) is required to release NF-κB from this inhibition [29]. Indeed, LPS stimulation of murine macrophages activates several intracellular signaling pathways, including the IκB kinase (IKK)/NF-κB pathway [30]. Our results suggest that sivelestat impinges on the IKK pathway to dampen the inflammatory response by repressing IκB phosphorylation and subsequent NF-κB activation (Figure 5). Accordingly, the inhibition of IκB phosphorylation by sivelestat during LPS-induced inflammation may limit NF-κB activation.

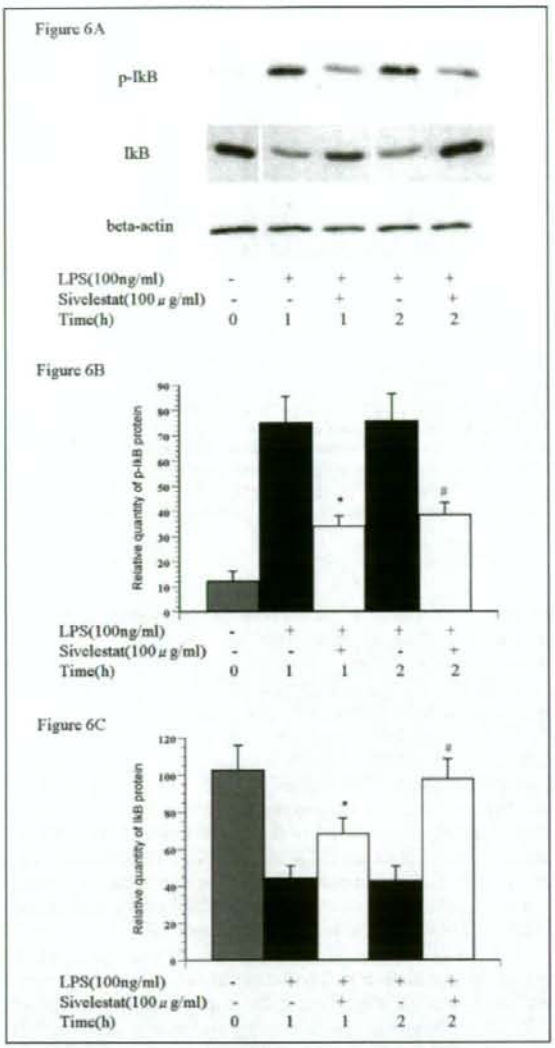

Fig. 6. Effect of sivelestat on LPS-induced IκB phosphorylation.
(**A**) Murine macrophages treated with or without sivelestat (100 μg/ml) were stimulated with LPS (100 ng/ml) for the indicated durations. The cytoplasmic levels of phosphorylated IκB were determined by western blot analysis using antibodies specific for 1) phosphorylated IκB α (p-IκB, upper); 2) total IκB α (IκB, middle); and 3) β-actin as a loading control (lower). (**B**) The signal intensities for the density were based on an immunoblot analysis of p-IκB protein. p-IκB was quantified using an image analyzer. The density of the signal intensities was lower for cells treated with both LPS and sivelestat, compared to cells treated only with LPS. Results are expressed as the mean ± SD. * denotes a significant difference compared with the LPS-only group at 1 h ($p<0.05$). # denotes a significant difference compared with the LPS-only group at 2 h ($p<0.05$). (**C**) The signal intensities for the density were based on an immunoblot analysis of IκB protein. IκB was quantified using an image analyzer. The density of the signal intensities was higher for the cells treated with both LPS and sivelestat compared to cells treated only with LPS. Results are expressed as the mean ± SD. * denotes a significant difference compared with the LPS-only group at 1 h ($p<0.05$). # denotes a significant difference compared with the LPS-only group at 2 h ($p<0.05$).

In conclusion, our results suggest that sivelestat may exert an anti-inflammatory effect due to its ability to inhibit the production and secretion of cytokines and HMGB1, as well as NO secretion. These might be related to the inhibition of NF-κB in sivelestat-treated cells. One important caveat of this study is the high concentration of sivelestat used. Such high concentrations may non-specifically affect intracellular proteases (such as the proteasome) and thereby inhibit NF-κB activation. Further studies will be required to elucidate the mechanisms by which sivelestat regulates inflammation.

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Effects of an angiotensin-converting enzyme inhibitor on the inflammatory response in *in vivo* and *in vitro* models*

Satoshi Hagiwara, MD, PhD; Hideo Iwasaka, MD, PhD; Shigekiyo Matumoto, MD; Seigo Hidaka, MD; Takayuki Noguchi, MD, PhD

Objective: **Sepsis remains a major health threat in intensive care medicine. The renin-angiotensin system (ACE) affects inflammatory responses. In addition, angiotensin-converting enzyme inhibitors act to ameliorate lung injury. To investigate whether the widely used ACE inhibitor enalapril, used to treat hypertension, could inhibit secretion of cytokines and high-mobility group box 1 (HMGB1) protein, thus reducing lung damage in a rat model of lipopolysaccharide (LPS)-induced sepsis.**

Design: **Randomized, prospective animal study.**

Setting: **University medical center research laboratory.**

Subjects: **Male Wistar rats.**

Interventions: **LPS was administered intravenously to rats, with or without intraperitoneal pretreatment with enalapril. In addition, mouse macrophage RAW264.7 cells were stimulated with LPS, with and without simultaneous enalapril treatment.**

Measurements and Main Results: **Histologic examination showed marked reduction of interstitial congestion, edema, inflammation, and hemorrhage in lung tissue harvested 12 hours after treatment with both agents compared with LPS administration alone. Plasma concentration of angiotensin II was strongly induced by LPS; this induction was inhibited by the enalapril pretreatment. Likewise, LPS-induced secretion of proinflammatory cytokines and HMGB1 protein was inhibited by enalapril. The presence of HMGB1 protein in the lung was examined directly by immunohistochemistry; the number of stained cells was significantly lower in LPS-treated animals that also received enalapril. In the *in vitro* studies, enalapril administration inhibited the phosphorylation of IkappaB.**

Conclusions: **The ACE inhibitor enalapril blocked the LPS-induced inflammatory response and protected against the acute lung injury normally associated with endotoxemia in this rat sepsis model. Given these results, enalapril is a strong candidate as a therapeutic agent for sepsis. (Crit Care Med 2009; 37:626–633)**

Key Words: lipopolysaccharides; acute lung injury; high-mobility group box 1; enalapril

Sepsis remains a significant cause of morbidity and mortality throughout the world. (Accompanying complications, such as disseminated intravascular coagulation and acute respiratory distress syndrome, remain refractory to therapy (1–3).) Over the past several decades, various strategies for suppressing the inflammatory response have been tested in clinical trials for the treatment of sepsis. Depending on the ability of the immune system to respond to the infection, however, an anti-inflammatory strategy may not be helpful and could even be harmful. As a result, these clinical trials in sepsis have thus far been unsuccessful (4).

High-mobility group box 1 (HMGB1) protein plays a key role as a late-phase mediator in the pathogenesis of sepsis (5). HMGB1 is an intranuclear protein that was originally identified as an important factor in the regulation of genetic information (6). It is released from necrotic cells or secreted by cells such as activated monocytes/macrophages after cytokine stimulation (7). Secreted HMGB1 then binds to the receptor for advanced glycation end-products, initiating a signal cascade that results in further downstream cytokine release and contributes to lethality associated with endotoxemia (8).

One class of renin-angiotensin system (RAS)–blocking agents is the angiotensin-converting enzyme (ACE) inhibitors. The RAS is essential for maintaining blood pressure homeostasis. A large body of recent studies, both experimental and clinical, has unequivocally shown that pharmacologic blockade of the RAS has a protective effect on several organs, notably the heart and kidneys (9–11). In addition, the involvement of the RAS in the pathogenesis and evolution of inflammatory responses has received attention. On the basis of various experimental studies, the RAS is considered to be a key mediator of inflammation (12).

More recent studies suggest a role of the RAS in pulmonary disease. Levels of ACE are elevated in bronchoalveolar lavage fluid from subjects with acute respiratory distress syndrome (13), and mortality from acute respiratory distress syndrome is positively correlated with genetic polymorphism of ACE (14). The ACE homolog ACE2 can serve to balance the activity of ACE by decreasing levels of angiotensin II (ANG II), and thus providing some protection from acute lung injury (15). These observations suggest that ACE activity may regulate the acute inflammatory response in the lung. ANG II, the product of the proteolytic cleavage of ANG I by ACE, is the main peptide of the RAS and is important for the control of blood pressure and volume in the cardiovascular system. ANG II also produces an inflammatory response by binding to type-1 ANG II receptors (AT1 receptors), thus activating an intracellular signaling cascade that up-regulates several proinflammatory genes (12). ANG II may therefore be an important modulator of inflammation in acute lung disease.

*See also p. 776.

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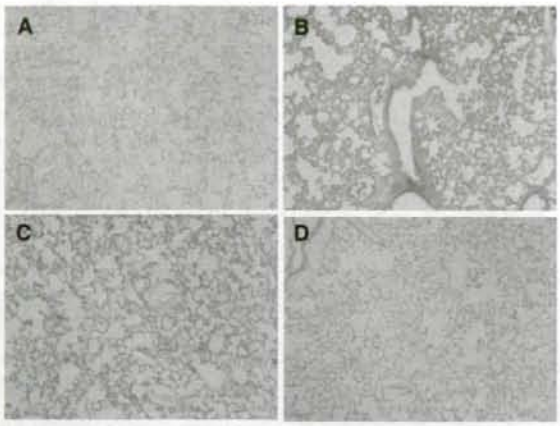

Figure 1. Effects of enalapril on lung injury in lipopolysaccharide (*LPS*)-treated rats. Rats were treated with saline (control group), LPS (7.5 mg/kg, intravenously; LPS group), LPS and ENA (LPS: 7.5 mg/kg, intravenously and ENA: 50 mg/kg, intraperitoneally; ENA+LPS group), and ENA (50 mg/kg, intraperitoneally; ENA group). Shown are representative lung specimens obtained from the control (*A*: ×40 magnification), LPS (*B*: ×40 magnification), ENA+LPS (*C*: ×40 magnification), and ENA (*D*: ×40 magnification) groups, respectively (hematoxylin and eosin staining).

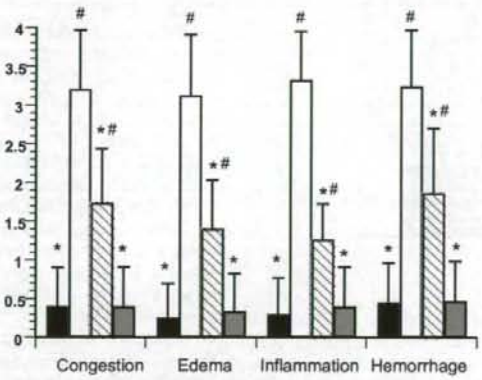

Figure 2. Changes in lung histology score. The identified histologic changes included congestion, edema, inflammation, and hemorrhage 12 hours after the administration of lipopolysaccharide (*LPS*): control (*black bars*), LPS (*white bars*), ENA+LPS (*hatched bars*), and ENA (*gray bars*). The data are expressed as the mean ± SD. #Denotes a significant difference compared with the control group ($p < 0.05$). *Denotes a significant difference relative to the administration of LPS ($p < 0.05$).

The mechanisms behind RAS activation in sepsis, and the concomitant lung injury, are unknown. Enalapril is an ACE inhibitor that is widely used in clinical medicine for the treatment of hypertension. In this study, we tested the effects of enalapril on lung injury in a lipopolysaccharide (LPS)-dependent model of systemic inflammation in rats. We hypothesized that pretreatment with enalapril can down-regulate ANG II and suppress cytokine and HMGB1 levels in serum, thus preventing acute lung disease. In addition, we used a mouse macrophage cell culture system to elucidate the mechanism underlying the potential anti-inflammatory effect of enalapril.

METHODS

In Vivo Study

Animals. All protocols conformed to National Institutes of Health guidelines and animal care was in compliance with the Principals of Laboratory Animal Care. The study was approved by the Ethical Committee of Animal Research at the College of Medicine, Oita University, Oita, Japan. Male Wistar rats weighing 250–300 g (Kyudou, Saga, Japan) were used in all experiments. Animals had access to food and water *ad libitum*.

Drugs. The LPS (O127:B8; Sigma, St Louis, MO) used in this study was derived from *Escherichia coli* (O127) endotoxin, and it was dissolved in sterile saline. Enalapril (donated by Merck, Darmstadt, Germany) was dissolved in sterile saline for injections. Dosage information is described below.

Experimental Protocols. The animals were randomly assigned to one of the following four groups (n = 18 for each group): 1) Control group: rats received intraperitoneal administration of 0.9% NaCl solution 30 minutes before injection into the tail vein of 0.9% NaCl solution; 2) enalapril (ENA)+LPS group: enalapril was administered intraperitoneally (50 mg/kg) 30 minutes before intravenous injection of LPS (7.5 mg/kg) into the tail vein; 3) LPS group: rats received intraperitoneal administration of 0.9% NaCl solution 30 minutes before intravenous injection of LPS (7.5 mg/kg); and 4) ENA group: enalapril was administered intraperitoneally (50 mg/kg) 30 minutes before intravenous injection of 0.9% NaCl solution into the tail vein. Serum samples of venous blood were obtained from the left external jugular vein at the following time points: pre, 3, 6, 9, 12, and 24 hours. All animals were breathing spontaneously during the experimental protocol.

Histologic Analysis. Animals (n = 6 for each group) under sevofluran anesthesia were killed 12 hours after LPS administration; left lungs were quickly removed and processed as indicated below. The lung tissues were stained with hematoxylin and eosin. A pathologist blind to treatment assignment evaluated the extent of lung injury according to Murakami's technique (16). Briefly, 24 areas of lung parenchyma were graded on a scale of 0–4 (0, absent and appears normal; 1, light; 2, moderate; 3, strong; 4, intense) for congestion, edema, inflammation, and hemorrhage. A mean score for each of the parameters was then calculated.

Wet-to-Dry Weight Ratio. Animals (n = 6 for each group) under sevofluran anesthesia were killed 12 hours after LPS administration. The lungs were removed, weighed, and then dried in an oven at 80°C for 48 hours to obtain pulmonary wet-to-dry weight ratios.

Myeloperoxidase Assay. Animals (n = 6 for each group) under sevofluran anesthesia were killed 12 hours after LPS administration. Myeloperoxidase (MPO) activity was assayed using a previously described method adapted for a microplate format (17). Frozen tissue samples were thawed and homogenized. Samples were then centrifuged. To each well, we added 20 μL of supernatant and 120 μL of reaction buffer containing 530 nM *o*-dianisidine and 150 nM H_2O_2 (added immediately before use) in 50 mM potassium phosphate, pH 6.0. Light absorbance at 490 nm was then read.

Measurement of Secreted Cytokines, HMGB1, and ANG II. Interleukin (IL)-6, tumor necrosis factor (TNF)-alpha, HMGB1, and ANG II secretion was assayed by the enzyme-

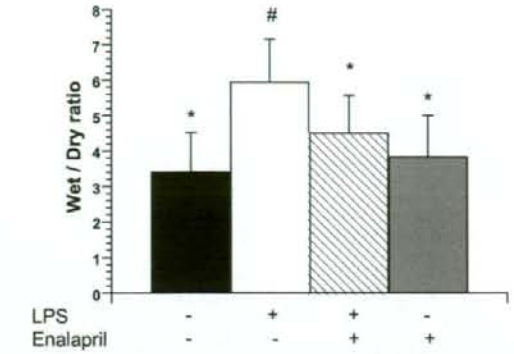

Figure 3. Effects of enalapril on wet-to-dry weight ratio of lipopolysaccharide (*LPS*)-treated rats. The wet-to-dry weight ratio in lungs was determined 12 hours after LPS treatment for each group (n = 6 for each group): control (*black bar*), LPS (*white bar*), ENA+LPS (*hatched bar*), and ENA (*gray bar*). The data are expressed as the mean ± SD. #Denotes a significant difference compared with the control group ($p < 0.05$). *Denotes a significant difference compared with the LPS group ($p < 0.05$).

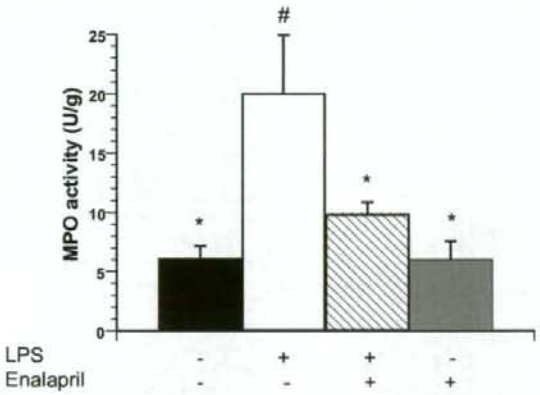

Figure 4. Effects of enalapril on myeloperoxidase (*MPO*) activity in lipopolysaccharide (*LPS*)-treated rats. Myeloperoxidase activity in whole lungs was determined 12 hours after LPS treatment for each group (n = 6 for each group): control (*black bar*), LPS (*white bar*), ENA+LPS (*hatched bar*), and ENA (*gray bar*). The data are expressed as the mean ± SD. #Denotes a significant difference compared with the control group ($p < 0.05$). *Denotes a significant difference compared with the LPS group ($p < 0.05$).

linked immunosorbent assay (ELISA) sandwich method. Ninety-six well plates were precoated with monoclonal antibody specific to rat IL-6 (R&D Systems, Minneapolis, MN), TNF-alpha (R&D Systems), HMGB1 (Shino-Test Corporation, Tokyo, Japan), and ANG II (Phoenix Pharmaceuticals, Burlingame, CA). The secreted factors were detected according to the procedures described in the suppliers' protocols. A450 values were read by an ELISA reader.

Immunohistochemistry Analysis. Lungs were obtained from animals before and 12 hours after LPS administration. Tissue samples were fixed immediately in 4% paraformaldehyde, embedded in optimal cutting temperature Compound (Sakura Finetechnical, Tokyo, Japan), and sectioned. Blocked sections were incubated with anti-HMGB1 (Shino-Test Corporation) polyclonal antibody (1:1000 dilution). The sections were incubated with peroxidase-conjugated anti-mouse IgG. The slides were stained using an LSAB2 kit (Daco, Via Real Carpinteria, CA) as the biotin avidin peroxidase complex system. After development, slides were counterstained with Mayer's hematoxylin and mounted.

Western Blot. Animals under sevofluran anesthesia were killed 12 hours after LPS administration. The lung tissue specimens were homogenized with T-PER (Pierce Biotechnology, Rockford, IL). Proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis in a 12% gel and transferred onto polyvinylidene difluoride sheets (Millipore, Bedford, MA). The membranes were incubated with primary antibodies (HMGB1 [Shino-Test Corporation]; and beta-actin [Abcam, Cambridge, UK]) at 1/1000 in phosphate buffered saline-tween 20. Peroxidase-labeled secondary antibodies (ZYMED, South San Francisco, CA) were then added at 1/1000 in PBS-T and incubated. The blots were developed using ECL (Amersham, Buckinghamshire, UK) and were exposed to Hyperfilm ECL (Amersham).

In Vitro Study

Cells. The murine macrophage cell line RAW264.7 was maintained in RPMI 1640 medium containing 5% heat-inactivated fetal bovine serum and antibiotics at 37°C under 5% CO_2. In experiments designed to measure HMGB1 in conditioned medium, we used Opti-MEM (Sigma). The following treatments were applied: control group, no drug treatment; LPS group, stimulated with LPS (100 ng/mL) only; ENA+LPS group, simultaneously treated with enalapril (50 μM) and LPS (100 ng/mL); and ENA group, treated with enalapril (50 μM) only. Samples of culture supernatant were obtained at the following time points: 0, 1, 3, 6, 9, 12, and 20 hours. "0" refers to a time point immediately before LPS administration.

Nuclear Factor Kappa B Binding Assay. Murine RAW264.7 cells were harvested by scraping the adherent cell population from tissue culture flasks. The cells were collected using the NE-PER (Pierce Biotechnology). The DNA binding activity of Nuclear Factor Kappa B (NF-κB) (p50/p65) was determined using an ELISA-based nonradioactive NF-κB p50/p65 transcription factor assay kit (Chemicon, Temecula, CA). The secreted factors were detected according to the procedures described in the supplier's protocols. A450 values were read by an ELISA reader (Bio-Rad Laboratories, Hercules, CA).

Preparation of Tissue and Cell Culture Protein. Lung tissue homogenates were boiled (5 minutes) followed by the addition of dithiothreitol. In tissue culture studies, murine RAW264.7 cells were harvested by scraping the adherent cell population from tissue culture flasks. The cells were collected using M-PER (Pierce Biotechnology). The homogenates were boiled (5 minutes) followed by the addition of dithiothreitol.

Measurement of Secreted Cytokines and HMGB1. IL-6, TNF-α, and HMGB1 secretion was assayed by the ELISA sandwich method. Ninety-six well plates were precoated with monoclonal antibody specific to rat IL-6 (R&D Systems), TNF-α (R&D Systems), and HMGB1 (Shino-Test Corporation). The secreted factors were detected according to the procedures described in the suppliers' protocols. A450 values were read by an ELISA reader.

Western Blot

Proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis in a 12% gel and transferred onto polyvinylidene

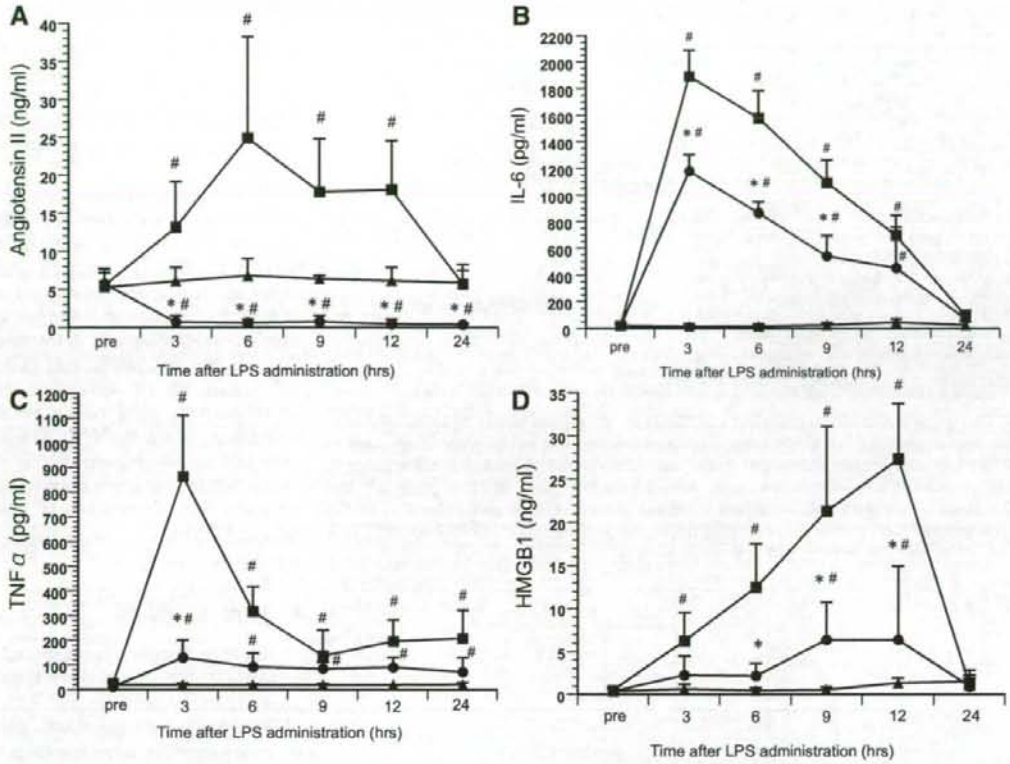

Figure 5. Temporal changes in angiotensin II, interleukin (*IL*)-6, tumor necrosis factor (*TNF*)-α, and high-mobility group box 1 (*HMGB1*) serum concentration after lipopolysaccharide (*LPS*) administration in rats. Concentrations of the proinflammatory factors were determined in serum taken from animals in the control, LPS, and ENA+LPS groups (n = 6 for each group) at the time points indicated. We analyzed angiotensin II, IL-6, TNF-α, and HMGB1 by enzyme-linked immunosorbent assay methods. *Squares* represent the values measured for the LPS group; *circles* represent the values for the ENA+LPS group; *triangles* represent the values for the control group; "pre" refers to a time point immediately before LPS administration. All data are expressed as means ± SD. #Denotes a significant difference compared with the control group ($p < 0.05$). *Denotes a significant difference relative to administration of LPS alone ($p < 0.05$). (*A*) Angiotensin II; (*B*) IL-6; (*C*) TNF-α; and (*D*) HMGB1.

difluoride sheets (Millipore). The membranes were incubated with primary antibodies (phosphorylated IKB α [p-IkB α], IkB α [Cell Signaling Technology, Beverly, MA]; and beta-actin [Abcam]) at 1/1000 in PBS-T. Peroxidase-labeled secondary antibodies (ZYMED) were then added at 1/1000 in PBS-T and incubated. The blots were developed using ECL (Amersham) and were exposed to Hyperfilm ECL (Amersham).

Statistical Analysis. All data are presented as mean ± SD. Data were analyzed by Mann-Whitney *U* test for comparisons between two independent groups. A level of $p < 0.05$ was accepted as statistically significant.

RESULTS

In Vivo Study

The Effect of Enalapril on Lung Tissue After LPS Administration. Lung tissue specimens were obtained 12 hours after LPS administration, with or without pretreatment with enalapril. Whereas no histologic alterations were observed in the control (untreated) group (Fig. 1*A*), marked interstitial edema and inflammatory cell infiltration were seen in the LPS group (Fig. 1*B*). Interstitial edema and inflammatory cell infiltration were markedly reduced in the ENA+LPS group in comparison to the LPS group (Fig. 1*C*). Enalapril alone caused no histologic changes (Fig. 1*D*). Likewise, the histology scores were all significantly higher in the LPS group than in the control group; the scores were intermediate in the ENA+LPS group (Fig. 2). The wet-to-dry ratios of the lungs in the LPS group were significantly elevated relative to those of the control animals (Fig. 3). The wet-to-dry ratios of the lungs in the ENA+LPS group were significantly lower than those in the LPS group, i.e., more similar to the values of control and ENA animals. MPO activity was induced after LPS injection, whereas MPO activity was significantly decreased in animals receiving an ENA+LPS injection (Fig. 4).

Effects of Enalapril on the Serum Levels of ANG II After the Administration of LPS. ANG II levels in the serum of control group animals were detectable at baseline (Fig. 5*A*). The serum levels of ANG II in the LPS group increased at 3 hours after LPS administration and peaked at 6 hours (Fig. 5*A*). In contrast, pretreatment with enalapril significantly suppressed the LPS effect at all time points (compare ENA+LPS group to LPS group in Fig. 5*A*).

Effects of Enalapril on the Serum Levels of IL-6, TNF-α, and HMGB1. The se-

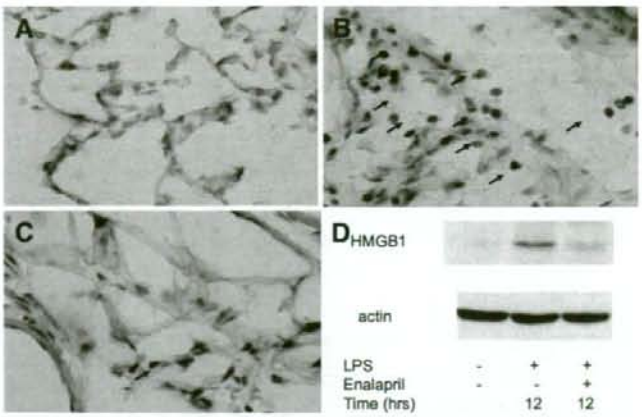

Figure 6. Changes in high-mobility group box 1 (*HMGB1*) protein expression in lung tissue specimens after lipopolysaccharide (*LPS*) administration in rats. Immunohistochemical analysis was used to detect HMGB1 in lung sections obtained 12 hours after LPS administration. All photographs are at ×400 magnification. Representative specimens from the control group (*A*), LPS group (*B*), and ENA+LPS group (*C*) are presented. The *arrows* in (*B*) indicate cells staining positive for HMGB1. (*D*) The expression of HMGB1 protein in the lung 12 hours after administration of LPS in control, LPS, and ENA+LPS groups was detected by Western blot analysis.

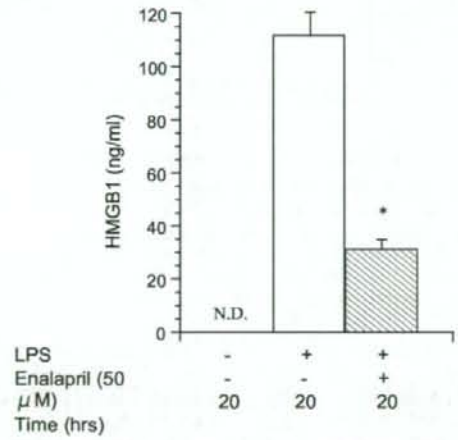

Figure 7. Effect of enalapril on high-mobility group box 1 (*HMGB1*) release by lipopolysaccharide (*LPS*)-stimulated murine macrophages. LPS-stimulated (100 ng/mL) murine macrophages were cotreated with enalapril (50 μM) for 20 hours (n = 8 for each group). HMGB1 levels were measured in supernatants by enzyme-linked immunosorbent assay; LPS (*white bar*) and ENA+LPS (*hatched bar*) (n = 8 for each group). All data are expressed as means ± SD. *Denotes a significant difference compared with LPS group ($p < 0.05$).

rum levels of IL-6, TNF-α, and HMGB1 were undetectable in control group animals (Fig. 5, *B–D*). After LPS administration, with or without pretreatment with enalapril, serum was sampled at various time points over the course of 24 hours. IL-6 levels were elevated at all time points, peaking at 3 hours after LPS administration in both the LPS and the ENA+LPS groups. However, the induction was significantly diminished in the ENA+LPS group (Fig. 5*B*). Likewise, induction of TNF-α peaked 3 hours after LPS administration in both the LPS and the ENA+LPS groups, with enalapril again exerting a suppressive effect on the induction, especially at the 3-hour time point (Fig. 5*C*). Serum levels of HMGB1 increased steadily over time after LPS administration in both the LPS and the ENA+LPS groups, not reaching peak levels until 12 hours after treatment. Again, pretreatment with enalapril resulted in significantly reduced levels of HMGB1; the largest difference was observed at the 12-hour time point (Fig. 5*D*). Serum levels of IL-6, TNF-α, and HMGB1 in the ENA group were undetectable and thereby similar to the control group (data not shown).

Effect of Enalapril on HMGB1 Levels in the Lung. Immunohistochemical analysis revealed that the number of cells expressing HMGB1 increased after LPS administration alone (compare Fig. 6, *A* and *B*). In contrast, the number of cells expressing HMGB1 was dramatically reduced in the enalapril-treated group (Fig. 6*C*). We also determined levels of HMGB1 protein in the lung tissue by western analysis and found that HMGB1 increased after LPS administration (Fig. 6*D*). Consistent with the immunohistochemical analysis, this increase was less pronounced among rats pretreated with enalapril (ENA+LPS).

In Vitro Study

Effect of Enalapril on Secreted Levels of HMGB1. HMGB1 levels in the control supernatants were undetectable. Secreted HMGB1 levels were elevated in culture at 20 hours after the administration of LPS, but were inhibited by cotreatment with enalapril (Fig. 7). HMGB1 levels in the supernatants from the ENA group were undetectable (data not shown).

Effect of Enalapril on Secreted Cytokines. Cultured macrophages secreted elevated levels of IL-6 after LPS administration; this induction was significantly inhibited by enalapril cotreatment (Fig. 8*A*). Likewise, levels of TNF-α in the supernatant increased at 3 hours after the administration of LPS. Cotreatment with enalapril significantly inhibited the secretion of TNF-α in the same manner as IL-6 (Fig. 8*B*). The cytokine levels in the supernatants of control and ENA groups were undetectable (data not shown).

Enalapril Inhibits IkB kinase Pathways and Modulates NF-κB. Levels of the transcription factor NF-κB (p50/p65) in the nucleus of RAW264.7 cells increased by 1 hour after LPS stimulation, as measured by DNA binding activity. However, cotreatment with enalapril partially suppressed this increase (Fig. 9).

Administration with LPS resulted in the degradation of IkB-α, which was inhibited by enalapril (Fig. 10). In addition, the level of phosphorylated IkB-α in RAW264.7 cells increased after LPS ad-

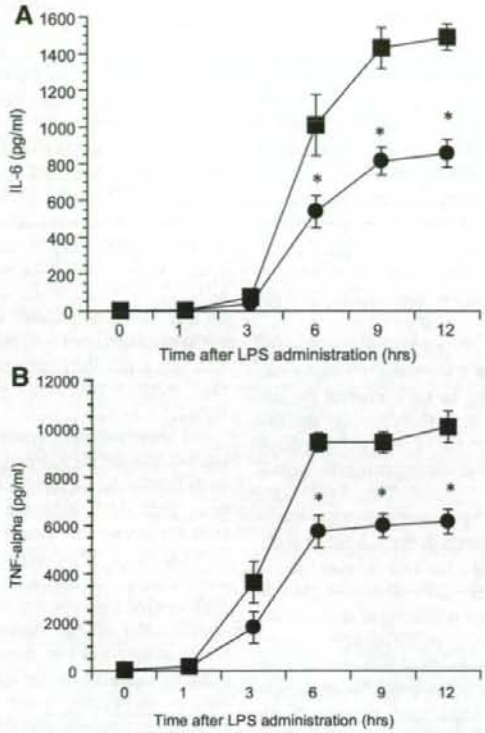

Figure 8. Effect of enalapril on interleukin (*IL*)-6 and tumor necrosis factor (*TNF*)-α production by lipopolysaccharide (*LPS*)-stimulated murine macrophages. Murine macrophages were stimulated with LPS, with or without enalapril treatment (50 μM) for the indicated times. Supernatants were collected and interleukin-6 (*A*) and TNF-α (*B*) levels were determined by enzyme-linked immunosorbent assay (n = 8 for each group): LPS (*squares*) and ENA+LPS (*circles*). All data are expressed as the means ± SD. *Denotes a significant difference compared with LPS-treated cells ($p < 0.05$).

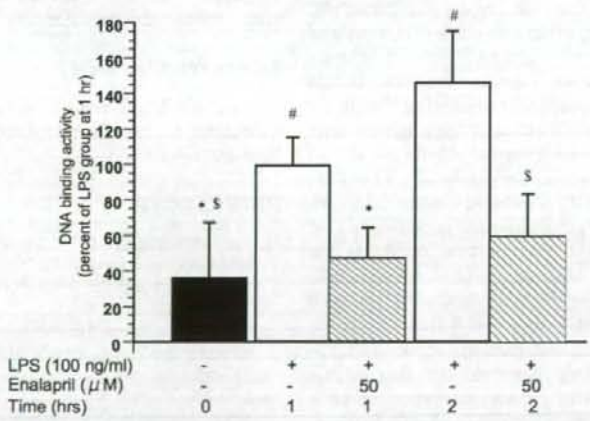

Figure 9. Effect of enalapril on the lipopolysaccharide (*LPS*)-induced increase in p50/p65 binding to DNA. The DNA binding activity assay showed a marked decrease in the p50/p65 binding activity in nuclear fractions from RAW264.7 cells when enalapril at 50 μM was used to cotreat LPS-stimulated cells: control (*black bar*), LPS (*white bar*), and ENA+LPS (*hatched bar*). All data are expressed as the mean ± SD. #Denotes a significant difference compared with the control cells ($p < 0.05$). *Denotes a significant difference compared with LPS treatment alone at 1 hour ($p < 0.05$). $Denotes a significant difference compared with LPS treatment alone at 2 hours ($p < 0.05$).

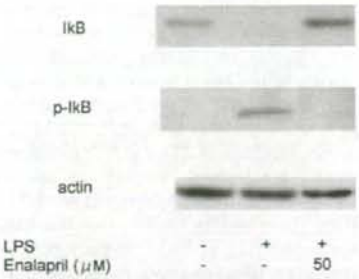

Figure 10. Effect of enalapril on the lipopolysaccharide (*LPS*)-induced phosphorylation of *IkB*. RAW264.7 murine macrophages were stimulated with lipopolysaccharide, with or without cotreatment with enalapril at 50 μM. Cells were harvested 1 hour after treatment and the cytoplasmic levels of phosphorylated IkB were determined by Western blot analysis using antibodies specific for IkB-α (IkB), phosphorylated IkB-α (*p-IkB*), and beta-actin as a control.

ministration; cotreatment with enalapril suppressed this induction (Fig. 10).

DISCUSSION

In this study, we demonstrated that pretreatment with enalapril significantly improved LPS-induced acute lung injury and was associated with a reduction in MPO activity and decreased cytokine and HMGB1 levels. Our results also suggest that the inhibition of cytokine and HMGB1 secretion were the result of an inhibition of NF-κB activity.

The activity of the RAS is reflected by levels of ANG II, which exerts proinflammatory effects by inducing the expression of cytokines, chemokines, adhesion molecules, growth factors, and reactive oxygen species. These effects of ANG II are mediated via the AT1 receptors (12), which were recently shown to be involved in lung permeability in acute lung injury (18). In this study, we showed that a marked increase in serum levels of ANG II accompany LPS-induced lung injury, suggesting that RAS activation plays a role in the pathology of sepsis. Although this study only measured systemic ANG II levels, local production of RAS components may also be important in the pathophysiology. Pretreatment with enalapril led to significantly decreased NF-κB activation and secretion of cytokines by macrophages. Macrophages are one of the main sources of pro- and anti-inflammatory cytokines, and their activation is a critical factor in the development of sepsis and acute lung injury (19). Con-

versely, ANG II exerts immunomodulatory effects that may contribute to various injuries and to the progression of disease (20). ANG II promotes inflammation through NF-κB-mediated induction of proinflammatory genes (20, 21). In addition, blockade of AT1 and AT2 receptors results in a decreased response to inflammatory (monocyte/macrophage) cells (22). These results suggest that the RAS system may be involved in macrophage-mediated inflammatory processes that lead to acute lung injury.

Lung levels of ACE are increased in various shock models (23). ANG II is a key effector peptide of the RAS that induces vasoconstriction and exerts multiple biological functions. Specifically, inflammation and apoptosis in lung injury are due to ACE-mediated ANG II production (24). In this study, we demonstrated that significantly increased serum levels of ANG II accompany LPS-induced systemic inflammation. However, we did not measure ANG II levels in lung tissue or bronchoalveolar lavage fluid, nor did we directly address whether ACE activity is up-regulated in bronchoalveolar lavage fluid during the response. Additional studies will be required to further understand the role of ACE. Recent studies have implicated ACE in the pathology of acute lung injury (15) and have demonstrated that various types of lung injuries are related to ACE and ANG II levels (24–26). We show that pretreatment with the ACE inhibitor enalapril prevents the development of LPS-induced lung injury. In addition, the administration of enalapril suppressed serum levels of ANG II. By blocking ANG II signaling, enalapril seems to suppress the inflammation caused by various inflammatory cells via key cytokines such as TNF-α and the late-phase mediator HMGB1.

ANG II is known to play a role in apoptosis (27), and is also involved in activation protein-1 (AP-1) (28) and bradykinin function (29). Studying the effect that enalapril has on these factors may provide further insight into the pathology of systemic inflammation, given that these factors are important in the development of sepsis (30–32).

The transcription factor NF-κB regulates the expression of many inflammatory genes including TNF-α and IL-6 (33), and aberrant activation of NF-κB is associated with septic shock (34). Suppression of the NF-κB pathway by some drugs may be essential to prevent or treat septic shock (35). We have now shown that enalapril is one such drug that can block NF-κB activation, providing a potential mechanism by which enalapril exerts its anti-inflammatory effect and ameliorates LPS-induced lung injury. In addition, we demonstrated that HMGB1 levels were reduced in our *in vivo* and *in vitro* models. HMGB1 induces acute inflammation in animal models of lung injury and endotoxemia. One mechanism by which this occurs is through NF-κB activation (36). Therefore, the inhibition of NF-κB activation may explain the decreased HMGB1 levels.

LPS is recognized by monocytes and macrophages of the innate immune system. On binding to LPS-binding protein in the plasma, it is delivered to the cell surface receptor CD14 and subsequently transferred to the transmembrane signaling receptor toll-like receptor 4 (37). LPS stimulation of murine macrophages activates several intracellular signaling pathways, including the IκB kinase (IKK)-NF-κB pathway (38). The key step in NF-κB activation is the rapid degradation of IkB, which requires IkB phosphorylation through elevated levels of IKK activity (39). Enalapril seems to suppress NF-κB activation by preventing phosphorylation of the main driver of the IKK pathway, IkB.

Cytokines such as TNF-α and IL-6 are secreted in the early phase of the inflammatory response and play an important role in the development of acute respiratory distress syndrome (40). TNF-α is a primary mediator of inflammation, coordinating the inflammatory response and activating other cytokines (41). Levels of IL-6 positively correlate with mortality in experimental models of sepsis. It has been proposed that measuring IL-6 levels in at-risk patients can accurately predict individuals who are at significant risk of death as a result of sepsis (42). In our rat model of LPS-induced systemic inflammation, we examined serum levels of TNF-α and IL-6 because cytokines are strongly induced by LPS exposure. We found that pretreatment with enalapril significantly suppressed this induction, suggesting that enalapril reduced the inflammatory response in this sepsis model. The clinical relevance of LPS-induced acute lung injury led us to investigate the effect of enalapril specifically in this tissue. Indeed, the histologic changes observed after LPS administration, including interstitial and intra-alveolar inflammation, edema, congestion, and hemorrhage, were ameliorated in animals that received enalapril before LPS administration.

Another molecule of interest in the development of sepsis is HMGB1, a late-phase mediator of the inflammatory response. HMGB1 causes acute lung inflammation when administered intratracheally (43) and plays a critical role in the development of LPS-induced lung injury (44, 45). In this study, we demonstrated that enalapril pretreatment dramatically reduces serum and lung tissue levels of HMGB1 in a LPS-induced septic shock model. The observation that treatment with enalapril also ameliorated LPS-induced acute lung injury suggests a direct relation between lung injury and HMGB1 levels.

In conclusion, enalapril protects against the acute lung injury associated with LPS-induced sepsis in rats. In addition, this ACE inhibitor prevents the HMGB1 induction normally seen after LPS exposure. These effects are related to inhibition of the IKK system. These findings contribute significantly to our understanding of the pathophysiology of acute lung injury in sepsis. Pharmacologic blockade of the actions of ANG II in other conditions, such as congestive heart failure and glomerular diseases, has dramatically improved survival in many individuals. Although this study did not examine the effects of enalapril administered after the onset of disease caused by exposure to live bacteria, yet our results point to the RAS being an important new therapeutic target for the treatment of sepsis-related acute lung injury.

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LANDIOLOL, AN ULTRASHORT-ACTING β1-ADRENOCEPTOR ANTAGONIST, HAS PROTECTIVE EFFECTS IN AN LPS-INDUCED SYSTEMIC INFLAMMATION MODEL

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ABSTRACT—Previous studies suggest that the blockade of β-adrenoceptors augments the release of inflammatory regulators in response to proinflammatory stimuli. High-mobility group box 1 (HMGB-1) is a key mediator in the development of sepsis. We investigated whether landiolol, a short-acting selective β1-adrenoceptor–blocking agent, can attenuate acute lung injury and cardiac dysfunction in a rat model of endotoxin-induced sepsis. We administered LPS i.v. to rats, with or without simultaneous treatment with landiolol (0.1 mg/kg per min). After the induction of sepsis by LPS treatment, we measured cytokine and HMGB-1 levels in the serum and lung tissue. In addition, we performed histopathology, determined wet-to-dry weight ratios, and measured cardiac function and cell signaling in the lung. Cotreatment with landiolol was associated with significantly less severe disease, as assessed by lung histopathology and cardiac function metrics. Serum and lung HMGB-1 levels were lower over time among landiolol-treated animals. Furthermore, nuclear factor-κB activity was inhibited by the administration of landiolol. Cotreatment with the selective β1-adrenoceptor–blocking agent landiolol protects against acute lung injury and cardiac dysfunction in a rat model of LPS-induced systemic inflammation. Treatment was associated with a significant reduction in serum levels of the inflammation mediator HMGB-1 and histological lung damage.

KEYWORDS—Inflammation, sepsis, high-mobility group box 1, β-adrenoceptor–blocking agent, LPS

INTRODUCTION

Severe sepsis accounts for approximately 3% of admissions to hospitals and 10% of admissions to the intensive care unit (ICU), and it is the 10th leading cause of death in the ICU (1). Despite decades of intense therapeutic investigation, the mortality from severe sepsis and septic shock remains between 30% and 60% (2, 3). The high fatality rate of sepsis is caused by the protean complications it can cause throughout the major organ systems.

Abnormalities of cardiac function are clearly associated with many cases of septic shock in humans (4). Acute respiratory distress syndrome and acute lung injury are well defined and readily recognized clinical disorders caused by sepsis in the lung or predisposition to lung injury. These conditions are common in ICUs (5). Septic shock can elicit, to a wide range of intensity, stress-induced activation of the sympathetic nervous system as well as humoral responses to stress (6). The autonomic nervous system, which serves as a link between the nervous and immune systems, plays a role in the pathogenesis of septic shock (7).

The ultrashort-acting β-blocker, landiolol (ONO-1101; (-)-[(S)-2,2-dimethyl-1,3-dioxolan-4-yl] methyl 3-[4-[(S)-2-hydroxy-3-(2-morpholino carbonylamino) ethyl-amino] propoxy] phenylpropionate monohydrochloride), was developed in Japan for emergency treatment of tachyarrhythmias in animals (8, 9). Landiolol hydrochloride is a highly developed cardioselective β-blocker with a potency ratio (β_1/β_2) of 255 compared with 33 for esmolol and 0.68 for propranolol (8). In addition, landiolol has a short duration of activity (a half-life of 4 min), enabling quick recovery after cessation of administration, through rapid hydrolysis of its ester linkage (8). The drug has already been approved as an emergency treatment of supraventricular tachyarrhythmia in patients in Japan.

β-Adrenoceptors play a role in the signaling cascade leading to an inflammatory response and are also involved in preconditioning; β-blockers in turn work by attenuating these signaling pathways (10,11). Indeed, the blockade of β-adrenoceptors has been shown to augment the release of inflammatory regulators in response to proinflammatory stimuli (11). The blockade of β-adrenoceptors might therefore be useful in the treatment of septic shock.

High-mobility group box 1 (HMGB-1) protein enhances the inflammatory response, mediating cell-cell interactions through its binding to multiple Toll-like receptors (12,13). It contributes to the pathology and mortality of sepsis, presumably as a late-phase inflammatory mediator (14). Plasma concentrations of HMGB-1 are elevated in patients with sepsis, and circulating levels of HMGB-1 increase over the hours after LPS administration in experimental models (15). These observations reinforce the putative role of HMGB-1 in contributing to the development of sepsis-induced organ dysfunction. We previously reported that HMGB-1 exerted a negative inotropic effect in the septic rat heart (16).

The release of HMGB-1 from cells was first demonstrated in an LPS-dependent model of sepsis (15, 17). LPS exerts its proinflammatory effects by interacting with Toll-like receptor 4 and inducing a signaling cascade (18). In macrophages, the transcription factor nuclear factor-κB (NF-κB) is a critical

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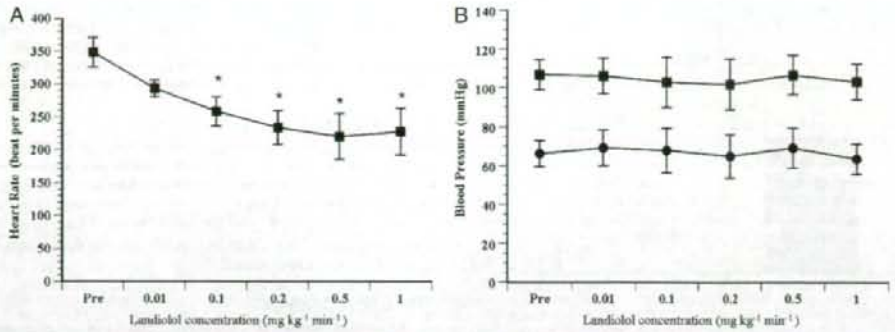

FIG. 1. **Dose-response curves of hemodynamic parameters after the administration of each indicated concentration of landiolol.** A, Heart rate. B, Blood pressure. Black squares represent systolic blood pressure, and black circles represent diastolic blood pressure (n = 10). The data are expressed as the mean ± SD. *Significant difference compared with pretreatment ($P < 0.05$).

mediator of this signaling cascade, regulating the expression of many inflammatory genes including IL-6 and TNF-α (19), which in turn stimulates release of HMGB-1 (20, 21). Aberrant activation of NF-κB is associated with systemic inflammation, such as septic shock (22).

We hypothesized that landiolol could inhibit serum and tissue expression of HMGB-1, thus preventing the acute lung injury and cardiac dysfunction associated with sepsis. We therefore investigated the effect of landiolol administration on serum and lung levels of HMGB-1, as well as on lung histopathology, in an LPS-dependent model of systemic inflammation in rats. To further elucidate the mechanism of landiolol activity, we measured the activity of NF-κB in the lung tissue of these animals.

METHODS

Animals

The study was approved by the Ethical Committee of Animal Research at the College of Medicine, Oita University, Oita, Japan. Male Wistar rats weighing 250 to 300 g (Kyudou, Saga, Japan) were used in all experiments. All protocols conformed to the National Institutes of Health guidelines, and the animals received humane care in compliance with the Principles of Laboratory Animal Care. All animals were housed with access to food and water *ad libitum*.

Drugs

Landiolol was donated by Ono Pharma Co Ltd (Osaka, Japan). LPS was purchased from Sigma Co (St Louis, Mo). Antibodies to the following antigens were purchased: HMGB-1 (Shino-Test Corporation, Tokyo, Japan) and β-actin (Abcam, Cambridge, United Kingdom).

Protocol 1

Surgical procedures—Anesthesia was induced and maintained by 3% sevoflurane. The right femoral artery was cannulated for blood pressure recording. The right external jugular vein was cannulated for drug administration (landiolol, 0.1 mg kg^{-1} min^{-1}, i.v.). Body temperature was maintained at 37°C ± 0.5°C with a thermostatically controlled heated table. We monitored heart rate and blood pressure throughout the procedure.

Recording of arterial pressure and heart rate—To record arterial pressure, the arterial catheter was connected to a pressure transducer (SCK-676, Becton, Dickinson and Company, Franklin Lakes, NJ) attached to a bridge amplifier (BSM-3201, Nihon Kohden Corporation, Shinjuku-ku, Tokyo, Japan). Blood pressure and heart rate were recorded continuously.

Protocol 2

Treatment protocol—Anesthesia was induced by 3% sevoflurane. Animals were randomly assigned to one of three groups: (*a*) LPS group: rats were treated i.v. with LPS (7.5 mg/kg) and received continuous i.v. administration of 0.9% NaCl solution (1.0 mL/h); (*b*) LPS + landiolol group: rats were treated i.v. with LPS (7.5 mg/kg) and received continuous i.v. administration of landiolol (0.1 mg kg^{-1} min^{-1}); and (*c*) control group: rats received continuous i.v. administration of 0.9% NaCl solution (1.0 mL/h). Before and after surgery, animals had unlimited access to food and water.

Wet-to-dry weight ratio

Animals under sevoflurane anesthesia were sacrificed 12 h after LPS administration. The lungs were removed, weighed, and then dried in an oven at 80°C for 48 h to obtain pulmonary wet-to-dry weight ratios.

Histological analysis

Lung sections were stained with hematoxylin and eosin. A pathologist blinded to group assignment analyzed the samples and determined the extent of lung injury according to Murakami's technique (23). Briefly, 24 areas in the lung parenchyma were graded on a scale of 0 to 4 (0, absent and appears normal; 1, light; 2, moderate; 3, strong; 4, intense) for congestion, edema, infiltration of inflammatory cells, and hemorrhaging. The mean score for each of the parameters was then used for analytic purposes.

Measurements of cytokine and HMGB-1 secretion

High-mobility group box 1, IL-6, and TNF-α levels were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (HMGB-1: Shino-Test Corporation; IL-6 and TNF-α: R&D Systems Inc, Minneapolis,

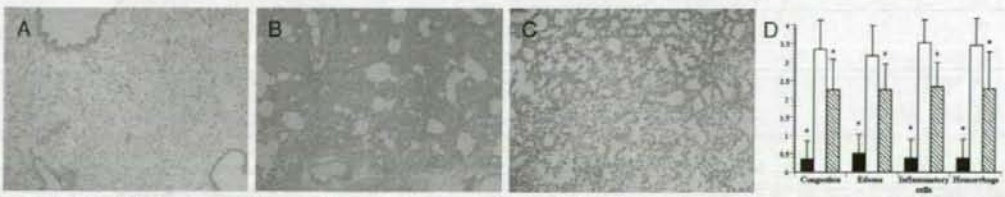

FIG. 2. **Effects of landiolol on lung histology in LPS-treated rats.** Lung tissues obtained from rats from the control group (A, original magnification ×40), LPS group (B, original magnification ×40), or LPS + landiolol group (C, original magnification ×40) were stained with hematoxylin and eosin. Histological changes (D) were scored based on findings of congestion, edema, inflammation, and hemorrhage 12 h after LPS administration. Black bars represent the control group, white bars represent the LPS group, and the slashed bars represent the LPS + landiolol group. The data are expressed as the mean ± SD. *Significant difference compared with the LPS group ($P < 0.05$).

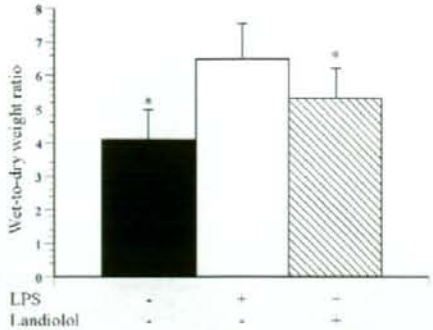

FIG. 3. **Effects of landiolol on wet-to-dry weight ratio in lungs of LPS-treated rats.** Wet-to-dry weight ratios were determined for each treatment group 12 h after LPS administration. Black bar represents the control group, white bar represents the LPS group, and the slashed bar represents the LPS + landiolol group. The data are expressed as the mean ± SD. *Significant difference compared with the LPS group ($P < 0.05$).

Minn). The absorbance at 450 nm was determined using an ELISA reader (Bio-Rad Laboratories, Hercules, Calif).

Immunohistochemical analysis

Lung tissue samples were fixed upon resection in 4% paraformaldehyde at 4°C overnight. Immunohistochemistry was performed after blocking endogenous peroxidase activity. Sections were incubated with anti–HMGB-1 polyclonal antibody (1:1,000 dilution) and visualized with horseradish peroxidase conjugate and diaminobenzidine.

Western blotting

Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, Mass). The membranes were incubated with antibodies to HMGB-1 or β-actin (1:1,000 dilution) for 1 h at room temperature followed by horseradish peroxidase–conjugated secondary antibody (1:1,000 dilution, Invitrogen, Carlsbad, Calif) for 1 h at room temperature. Blots were subsequently developed using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, United Kingdom) and exposed on Hyperfilm enhanced chemiluminescence (Amersham).

Isolated heart perfusion and the assessment of cardiac function

Animal hearts were isolated 12 h and 24 h after LPS administration. Cardiac function was determined by a modified isovolumetric Langendorff technique, as previously described (24–26). Cardiac function was represented in terms of left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), and increased left ventricular pressure development during isovolumetric contraction (dP/dt_{max}) and relaxation (dP/dt_{min}). Hearts were rapidly excised while still beating into oxygenated Krebs-Henseleit perfusion solution containing 11 mM glucose, 2.0 mM $CaCl_2$, 4.3 mM KCl, 25 mM $NaHCO_3$, 118 mM NaCl, 1.2 mM $MgSO_4$, and 1.2 mM KH_2PO_4. Normothermic retrograde perfusion was performed with the same solution in an isovolumetric and nonrecirculating mode. The perfusion buffer was saturated with a gas mixture of 95% O_2 and 5% CO_2 at pH 7.4. Perfusion pressure was maintained at 75 mmHg. A latex balloon was inserted through the left atrium into the left ventricle, and the balloon was filled with water (0.18–0.28 mL). The LVDP, LVEDP, $+dP/dt_{max}$, and $-dP/dt_{max}$ were continuously recorded using a computerized pressure amplifier-digitizer.

NF-κB binding assay

We prepared nuclear extracts from lung tissue. Nuclear protein was extracted by Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Pierce Biotechnology, Rockford, Ill). The DNA-binding activity of NF-κB (p50/p65) was determined using an ELISA-based nonradioactive NF-κB p50/p65 transcription factor assay kit (Chemicon, Temecula, Calif). The absorbance at 450 nm was determined using an ELISA reader (Bio-Rad Laboratories).

Statistical analysis

All data are presented as the mean ± SD. Blood pressure and heart rate data were analyzed using repeated measures followed by *post hoc* testing; the data were analyzed by Mann-Whitney *U* test for comparison between two independent groups. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of landiolol administration on blood pressure and heart rate

Treatment of rats with landiolol alone resulted in small decreases in heart rate. The effect was dose dependent, with the decrease being minimal at a concentration of 0.01 mg kg^{-1} min^{-1}, significant ($P < 0.05$) at a concentration of 0.1 mg kg^{-1} min^{-1}, and maximal at 0.2 mg kg^{-1} min^{-1} (Fig. 1A). In contrast, systolic and diastolic blood pressures remained unchanged by the administration of landiolol (Fig. 1B). Based on these results, we chose the concentration of 0.1 mg kg^{-1} min^{-1} landiolol for subsequent studies.

Effects of landiolol on the lung in the LPS-induced septic shock model

In the control group, no pulmonary histological alterations were observed (Fig. 2A). Lung tissue obtained 12 h after the administration of LPS showed edema formation, interstitial infiltration by neutrophils, and reduced alveolar spaces (Fig. 2B). In contrast, we observed reduced interstitial edema and inflammatory cell infiltration in the lung tissue of the LPS + landiolol group compared with the LPS group (Fig. 2C). Histology scores, based on the number of areas with congestion, edema, inflammatory cells, and hemorrhaging, were all significantly higher in the LPS group than in the control group. All scores were significantly lower in the LPS +

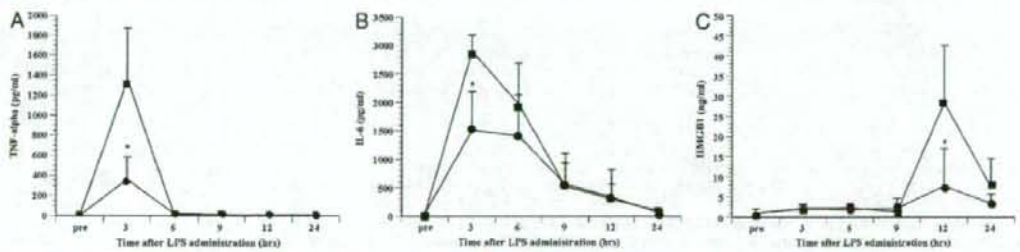

FIG. 4. **Temporal changes in serum TNF-α, IL-6, and HMGB-1 concentrations after LPS treatment.** Sera were obtained from LPS (squares) and LPS + landiolol (circles) group animals (n = 10 per group) at the indicated time points after LPS administration; cytokine levels were measured by ELISA. A, TNF-α. B, IL-6. C, HMGB-1. All data are expressed as the mean ± SD. *Significant difference compared with the LPS group ($P < 0.05$).

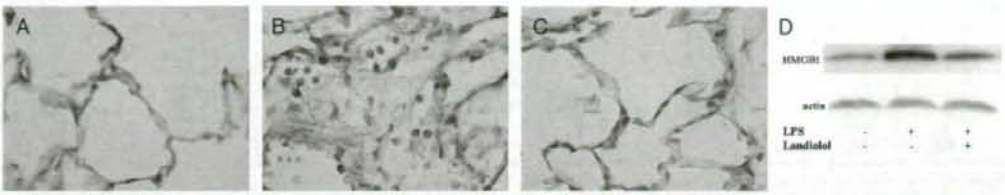

FIG. 5. **Changes in HMGB-1 protein expression in lung tissue upon administration of LPS in rats.** Lung tissue was obtained from control, LPS, and LPS + landiolol group rats 12 h after LPS administration. The HMGB-1 expression was analyzed by immunohistochemistry in the three groups (hematoxylin-eosin, original magnification ×400): control group (A), LPS group (B), and landiolol group (C). Western blot analysis (D) of HMGB-1 expression in lungs. β-Actin was used as a control for loading.

landiolol group compared with the LPS-only group (Fig. 2D). The wet-to-dry weight ratios obtained from the lungs of the LPS group animals were significantly elevated compared with those from control group animals (Fig. 3). Wet-to-dry weight ratios obtained from the LPS + landiolol group were comparable to the control group ratios, that is, significantly lower than those from LPS group animals.

Effects of landiolol on serum levels of TNF-α, IL-6, and HMGB-1

The cytokines IL-6 and TNF-α both showed marked peaks of expression in serum 3 h after LPS administration. Levels of both TNF-α and IL-6 were significantly lower in the LPS + landiolol group compared with LPS group, with the most marked effect seen at the 3-h time point (Fig. 4, A and B). Similarly, serum levels of HMGB-1 increased after the administration of LPS; however, the peak in expression did not occur until 12 h after treatment. This peak was significantly less prominent in LPS + landiolol group rats (Fig. 4C). Serum levels of IL-6, TNF-α, and HMGB-1 remained unchanged at all time points in the control group (data not shown).

Effects of landiolol on lung tissue

Immunohistochemical analysis revealed that the number of cells expressing HMGB-1 increased after LPS treatment alone (Fig. 5B). This increase was dramatically suppressed by landiolol cotreatment (Fig. 5C). Western blot analysis of lung tissue also showed the increase in HMGB-1 expression resulting from LPS treatment. This increase was less pronounced among LPS + landiolol group rats compared with the LPS group (Fig. 5D). We next examined NF-κB signaling in lung tissue by determining the extent of DNA binding activity by the NF-κB transcription factor p50/p65. Administration of LPS led to a robust increase in the DNA binding activity. However, cotreatment with landiolol significantly suppressed this increase (Fig. 6).

Effect of landiolol treatment on myocardial function in the LPS-induced systemic inflammation model

The administration of LPS was associated with a marked increase in LVEDP compared with values from control group animals. This increase was significantly suppressed in LPS + landiolol group animals (Fig. 7A). In contrast, the LPS + landiolol group showed markedly increased LVDP (Fig. 7B), LV $+dP/dt_{max}$ (Fig. 7C), and LV $-dP/dt_{min}$ (Fig. 7D) relative to the LPS group. Of particular note was the 12-h time point, at which all indicated parameters were significantly improved by the cotreatment with landiolol.

DISCUSSION

In this study, we demonstrated that landiolol provided protection against lung injury and reduced the wet-to-dry weight ratio in the lung in a rat model of LPS-induced sepsis. Acute lung injury in acute respiratory distress syndrome is considered an acute inflammatory condition by many clinicians. Although the pathology of this syndrome is poorly defined, Zhang et al. (27) reported that β-adrenoceptor activation can attenuate the acute inflammatory response induced in the lung by LPS. In addition, β-blockers reduce lung injury (28). These results suggest a critical role for β-adrenoceptors in acute lung injury.

The inflammatory response leading to lung and cardiac dysfunction and failure continues to be the major problem after injury in many clinical conditions such as sepsis (29, 30). Our findings showed that serum levels of cytokines and HMGB-1 were significantly inhibited by the administration of landiolol. This is the first report of a landiolol-dependent suppression of HMGB-1 expression in the septic rat model. The results presented here build on the growing body of evidence suggesting that several cytokines, including TNF-α and IL-6, are released during the early phase of systemic inflammatory response syndrome (29, 30). TNF-α and IL-6 mediate cardiac inflammation and contractile dysfunction after sepsis (31, 32). In addition, Ueno et al. (14) reported that HMGB-1 is important in the initiation of acute lung injury.

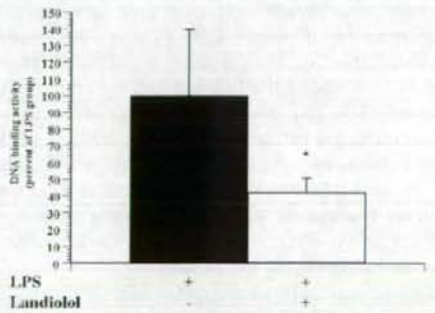

FIG. 6. **Effect of landiolol on the LPS-induced increase of specific binding of p50/p65 to DNA.** Lung tissue cells were obtained from LPS and LPS + landiolol group rats 6 h after LPS administration. Nuclear fractions were prepared and subjected to a DNA binding activity assay specific for the NF-κB (p50/p65) transcription factor. All data are expressed as the mean ± SD. *Significant difference compared with LPS group ($P < 0.05$).

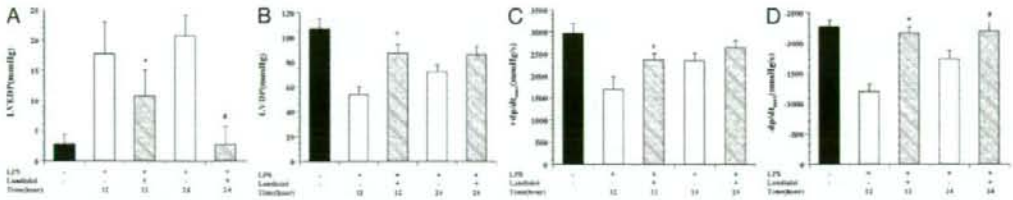

FIG. 7. **Effects of landiolol treatment on heart function in rats from LPS-induced systemic inflammation model.** Myocardial function in rats from the control (black bars), LPS (white bars), and LPS + landiolol (slashed bars) groups (n = 10 per group) was determined at the time points indicated. A, LVEDP. B, LVDP. C, $+dP/dt_{max}$. D, $-dP/dt_{min}$. Data are presented as the mean ± SD. *Significant difference compared with LPS group at 12 h ($P < 0.05$). #Significant difference compared with LPS group at 24 h ($P < 0.05$).

In this study, we analyzed the levels of HMGB-1 in serum and lung tissue in the rat model of LPS-induced lung injury. TNF-α is thought to mediate LPS-induced HMGB-1 release (21); a number of different pathways have been implicated in the inhibition of HMGB-1 secretion. The selective blockade of β1-adrenoceptors has been shown to inhibit cytokine release in septic animals (33). We found that serum levels of TNF-α and IL-6 were significantly lower in animals cotreated with the β-blocker landiolol compared with levels found in rats treated with LPS alone.

In this study, we demonstrated that the release of inflammatory mediators can be inhibited by administration of the β1-adrenoceptor blocker landiolol. On the other hand, previous studies indicated that β-adrenoceptor agonists inhibit the secretion of various cytokines in the LPS-induced inflammatory response (34, 35). Likewise, β2-adrenoceptor stimulation reduces cytokine release and organ damage (36, 37). These results suggest that β1- and β2-adrenoceptors might play different roles in inflammation. Further studies will be required to elucidate the mechanisms of β1- and β2-adrenoceptor function in the regulation of inflammation.

Many signaling events lead to cytokine synthesis and release after LPS exposure. One such key event involves the transcription factor NF-κB. Nuclear factor-κB signaling controls tissue regeneration and early events in inflammation (38), and is a central regulator of septic shock (22). In addition, NF-κB is markedly activated in peripheral mononuclear cells from patients with sepsis, and its activity is significantly higher in nonsurviving patients than in surviving patients (39, 40). Furthermore, numerous NF-κB target genes are implicated in the pathogenesis of sepsis (22, 41). In this study, we demonstrated that NF-κB activation is inhibited by administration of landiolol in the LPS-induced systemic inflammation model. The improvement of LPS-induced systemic inflammation might be attributed to the inhibition of NF-κB activation. However, the mechanism by which landiolol administration inhibits NF-κB remains unclear. One possibility involves free-radical scavenging. Further studies on the anti-inflammatory effects of landiolol are needed to elucidate the basis of its activity in this context.

Previous studies have demonstrated that β-blockers inhibit the activation of NF-κB (42). We showed here that landiolol exerts such an effect in a rat model of LPS-induced lung injury. Nuclear factor-κB is a major driver in the induction of several proinflammatory cytokine genes (22) as well as the secretion of HMGB-1 after LPS administration (43). Our results here indicate that one mechanism by which landiolol administration suppresses cytokine and HMGB-1 secretion may be through the inhibition of NF-κB, suggesting a critical role for β1-adrenoceptor in NF-κB activity in the LPS-induced shock model.

In our rat model of LPS-induced septic shock, cotreatment with landiolol prevented acute lung injury and improved cardiac function. In conclusion, our results suggest that landiolol may bring therapeutic benefit to patients with sepsis through its ability to inhibit the inflammatory response, especially by suppressing HMGB-1 expression, while improving lung injury and cardiac function. Further studies are needed to determine whether treatment with landiolol after the onset of sepsis is similarly efficacious. Additional studies are also required to better understand the mechanism of disease; our results point to the β-adrenoceptors being important new therapeutic targets for the treatment of LPS-induced acute lung injury.

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