

図 2 移植膵島障害におけるNKT細胞の役割

ストレプトゾトシン糖尿病マウス(C57BL/6)を作成し、膵島移植レシビエントに用いた(A)。同種同系単離膵島を野生型(B: 400個, C: 200個), あるいは $V\alpha 14$ $^{-/-}$ マウス(D: 200個, E: 100個)の経門脈的肝内移植した。膵島 200個移植 6時間後の野生型(G)または $V\alpha 14$ NKT $^{-/-}$ (H)マウスより肝単核球を単離し、FACSで解析した。

ンが生理的に第1に肝臓に灌流する、局所麻酔で容易に移植できる、動物実験で効果が確認できているなどにより、肝臓が移植部位として選択され、ドナー膵島は経門脈の肝内に移植される。移植後に膵島は門脈末端に塞栓し、末梢側肝臓に虚血性変性が生じ、それに伴い炎症などの自然免疫反応が惹起され、移植膵島が破壊される。とくに肝臓は生体防御の第一線臓器として機能しており、強い自然免疫反応が起こりうる。

著者らは、その際に肝臓内に豊富に存在するNKT細胞が移植膵島障害に関与しているのではないかと想定した。その仮説を直接的に検証するために、 $V\alpha 14$ NKT細胞欠損マウス⁴⁾を実験に使用した。ストレプトゾトシン糖尿病野生型マウス(C57BL/6)の経門脈の肝内に同種同系マウス1匹分の膵島200個を移植した場合、移植後レシピエントの血糖は正常化せず、高血糖で推移した(図2-C)。一方、2匹分400個の膵島移植後には正常血糖になった(図2-B)。このことはマウスにおいてもヒトと同様に、1匹の糖尿病レシピエントを膵島移植後にインスリン離脱するには2匹のドナーが必要であることを示している。驚いたことに、糖尿病 $V\alpha 14$ NKT細胞欠損マウスをレシピエントに用いた場合、200個(図2-D)のみならず100個(図2-E)の膵島移植で血糖は正常化した。さらに、細胞移入の実験においても $V\alpha 14$ NKT細胞が移植早期膵島障害に必須の役割を担っていることが明らかになった。

FACS解析で、膵島移植後6時間の野生型マウス肝臓内にはIFN- γ を産生するCD1 α GalCer-tetramer⁺CD3⁺(NKT)細胞が出現。さらにはIFN- γ ⁺Gr-1⁺CD11b⁺細胞が肝内に集積することが判明した(図2-G)。さらには200個の膵島移植レシピエントに抗Gr-1抗体、抗CD11b抗体、あるいは抗IFN- γ 抗体を投与すると移植後正常血糖になった。 $V\alpha 14$ NKT細胞欠損マウスへの移植後では、Gr-1⁺CD11b⁺細胞が野生型と同様に肝内に集積するものの、IFN- γ 産生はみられなかった(図2-H)。以上の知見は、NKT細胞に依存したGr-1⁺CD11b⁺細胞によるIFN- γ 産生が移植早期膵島障害に必須の役割を果たしていることを示している。

つぎに、NKT細胞の合成リガンドである α -galactosylceramide(α -GalCer)⁵⁾を用い、NKT細胞を標的にした治療法を試みた。NKT細胞は α -GalCerの1回投与でIFN- γ を大量に放出するが、繰り返し投与後にはIFN- γ 産生は抑制されることが知られている。野生型糖尿病マウスに移植前 α -GalCerを繰り返し投与(-15, -10, -7d)し、ストレプトゾトシンで糖尿病作成後に200個の膵島を移植すると血糖は正常化した。これらの知見は、NKT細胞を標的にした治療法で移植早期膵島障害が制御できることを示している。

移植免疫におけるNKT細胞の役割

NKT細胞にはエフェクター細胞、ならびに調節性細胞としての機能があることが報告されている⁶⁾。著者らは移植免疫におけるNKT細胞の機能について解析し、マウス肝臓内CD4⁺NKT細胞が調節性細胞としてラットからマウスへの異種膵島移植拒絶反応の制御にかかわっていることを明らかにした⁷⁾。また、マウス同種膵島移植ではNKT細胞は拒絶反応の発現に関与しており、NKT細胞欠損マウスでは野生型と比較してグラフトの長期生存が得られることを報告した⁸⁾。

今後の展望

臨床膵島移植の成績向上には、マウス実験系で得られた成果がヒトに応用できるかどうかを見極めることが急務と考え、現在ヒト肝リンパ球を用いた*in vitro*実験系で検討している。さらに、著者らが見出したNKT細胞を介した移植膵島障害の機序をもとに、臨床ですでに使用されている薬剤で制御できるものがないか、マウス実験系でスクリーニングしている。その結果、現在までにいくつかの有望な薬剤が判明しており、現在その臨床導入を検討している。また、著者らは膵島移植を通して、NKT細胞の動態・機能を解析してきたが、今後の研究によりいまだ明らかではないNKT細胞の内因性リガンドの発見につながる可能性があるのではないかと考え、研究を進めている。

文献

- 1) Shapiro, A. M. J. et al. : Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N. Engl. J. Med.*, **343** : 230-238, 2000.
- 2) Ryan, E. R. et al. : Five-year follow-up after clinical islet transplantation. *Diabetes*, **54** : 2060-2069, 2005.
- 3) Yasunami, Y. et al. : V α 14 NKT cell-triggered IFN- γ production by Gr-1⁺CD11b⁺ cells mediate early graft loss of syngeneic transplanted islets. *J. Exp. Med.*, **202** : 913-918, 2005.
- 4) Cui, J. et al. : Requirement for V α 14 NKT cells in IL-12-mediated rejection of tumors. *Science*, **278** : 1623-1626, 1997.
- 5) Kawano, T. et al. : CD1d-restricted and TCR-mediated activation of V α 14 NKT cells by glycosylceramides. *Science*, **278** : 1626-1629, 1997.
- 6) Taniguchi, M. et al. : The regulatory role of V α 14 NKT cells in innate and acquired immune response. *Ann. Rev. Immunol.*, **21** : 483-513, 2001.
- 7) Ikehara, Y. et al. : CD4⁺V α 14 natural killer T cells are essential for acceptance of rat islet xenografts in mice. *J. Clin. Invest.*, **105** : 1761-1767, 2000.
- 8) Toyofuku, A. et al. : Natural killer T-cells participate in rejection of islet allografts in the liver of mice. *Diabetes*, **55** : 34-39, 2006.

◆お知らせ◆

■平成 20 年度(財)持田記念医学薬学振興財団
研究助成金および留学補助金公募

応募期間：2008 年 4 月 1 日～6 月 27 日

対象研究分野：①生命科学と医療応用の研究、②薬物科学と医療応用の研究、③情報科学と医療応用の研究

助成内容：研究助成金 80 件(総額 16,000 万円)、留学補助金 20 件(総額 1,000 万円)

※詳細につきましては、4 月 1 日よりホームページ (<http://www.mochida.co.jp/zaidan>)にて公開いたしますのでご参照下さい。

問合せ先：(財)持田記念医学薬学振興財団
〒160-0003 東京都新宿区本塩町 7-6 四谷ワイズビル
TEL : 03-3357-1282, FAX : 03-3357-1264
E-mail : zaidan@mochida.co.jp

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

S. Hagiwara, H. Iwasaka, S. Hidaka, A. Hasegawa and T. Noguchi

Department of Anesthesiology and Intensive Care Medicine, Oita University Faculty of Medicine, 1-1 Idaigaoka-Hasamamachi, Yufu City, Oita 879-5593, Japan, Fax +81-97-586-5949, e-mail: saku@med.oita-u.ac.jp

Received 14 June 2008; returned for revision 11 July 2008; received from final revision 4 September 2008; accepted by H. Katori 9 September 2008

Published Online First 24 February 2009

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- κ B (NF- κ B) 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- κ B activation by inhibiting I κ B phosphorylation.

Conclusion: Sivelestat may inhibit the various inflammatory mediators through NF- κ B 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

Correspondence to: S. Hagiwara

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-1 β [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- κ B regulates the expression of many inflammatory genes including TNF- α and IL-6 [19]. NF- κ B activation involves the regulation of p65 and p50 subunit transactivation [20]. Aberrant activation of NF- κ B 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- κ B activity and I κ B 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 I κ B α (p-I κ B α) and I κ B 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 μ g/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. "0h" 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. "0h" 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- κ B Binding Assay

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

Statistical analysis

For descriptive purposes, all continuous data were presented as the mean \pm 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 μ g/ml sivelestat (Figure 1). The most effective dose of sivelestat was 100 μ g/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).

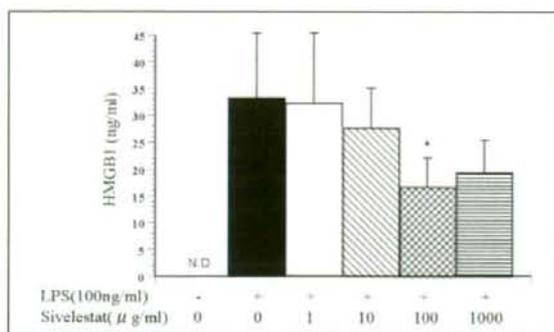


Fig. 1. Effect of sivelestat on HMGB1 secretion by LPS-stimulated murine macrophages.

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.

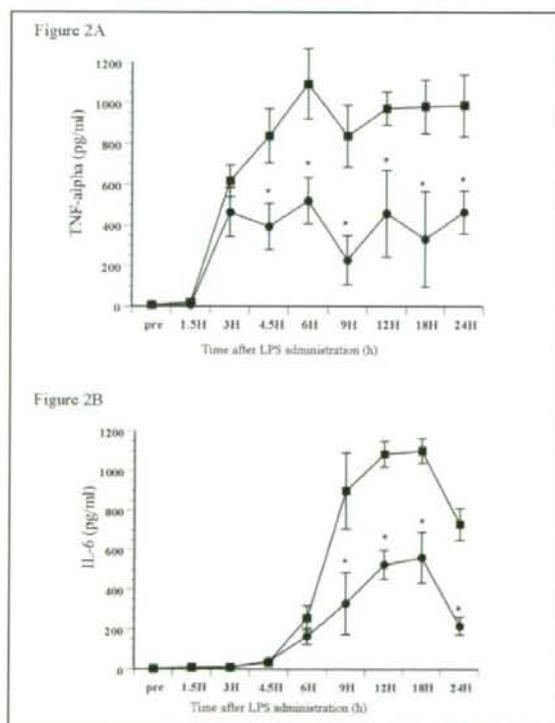


Fig. 2. Effect of sivelestat on TNF- α and IL-6 production by LPS-stimulated murine macrophages.

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- κ B

Since the NF- κ B pathway plays a crucial role in the secretion of cytokines and NO, we measured the activation of the NF- κ B 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 I κ B kinase (IKK) pathway was examined as another potential regulator of NF- κ B. Sivelestat inhibited I κ B α degradation resulting from LPS stimulation (Figure 6). In addition, I κ B α phosphorylation (p-I κ B) 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- κ B activation. This lack of NF- κ B activation may be due to inhibition of I κ B phosphorylation by sivelestat.

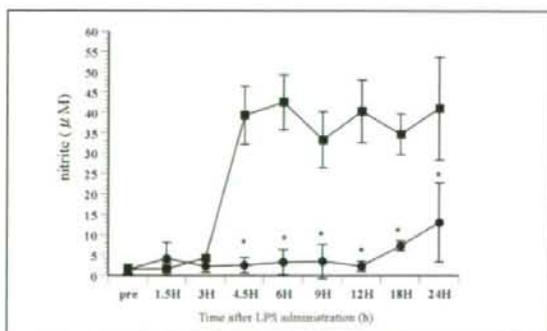


Fig. 3. Effect of sivelestat on serum nitrite levels after LPS stimulation of murine macrophages.

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 ($p < 0.05$).

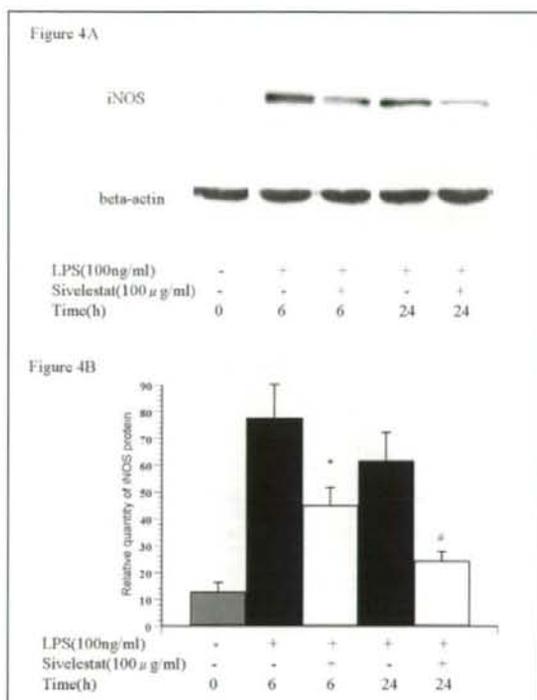


Fig. 4. Changes in iNOS protein expression after LPS stimulation of murine macrophages.

(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 6h ($p < 0.05$). # denotes a significant difference compared with the LPS-only group at 24h ($p < 0.05$).

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

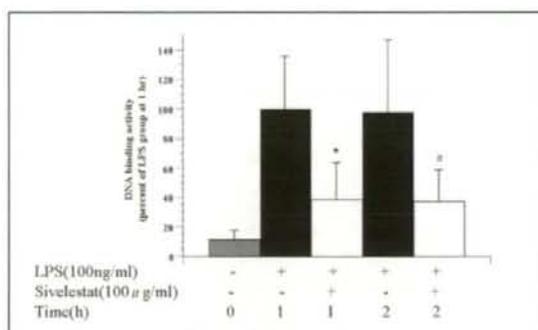


Fig. 5. Effect of sivelestat on the LPS-induced increase of p50/p65 DNA binding activity.

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 1h ($p < 0.05$). # denotes a significant difference compared with the LPS-only group at 2h ($p < 0.05$).

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.

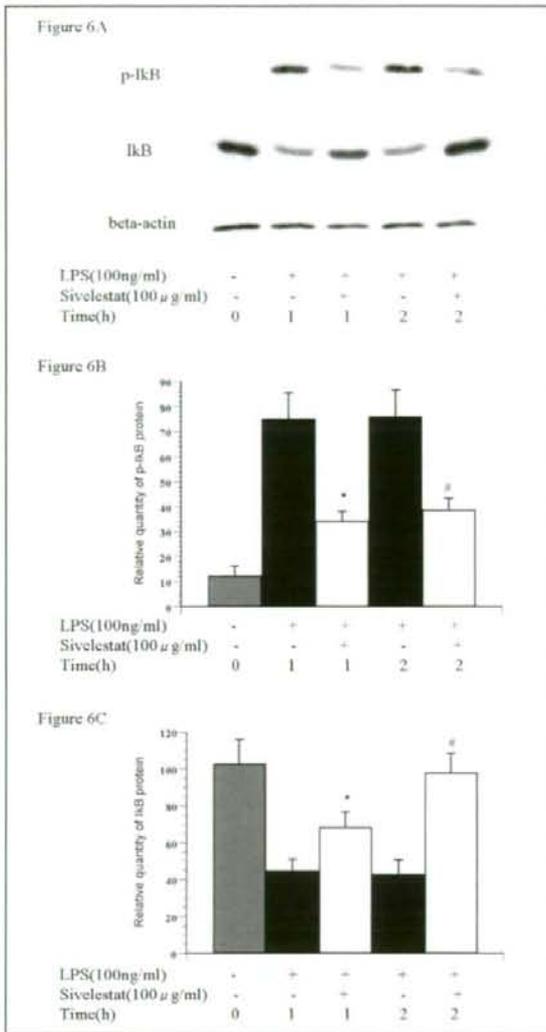


Fig. 6. Effect of sivelestat on LPS-induced IκB phosphorylation.

(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.

References

- [1] Hudson LD, Milberg JA, Anardi D, Maunder RJ. Clinical risks for development of the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1995; 151: 293–301.
- [2] Kawabata K, Suzuki M, Sugitani M, Imaki K, Toda M, Miyamoto T. ONO-5046, a novel inhibitor of human neutrophil elastase. *Biochem Biophys Res Commun* 1991; 177: 814–20.
- [3] Bellomo R, Uchino S, Naka T, Wan L. Hidden evidence to the West: multicentre, randomised, controlled trials in sepsis and systemic inflammatory response syndrome in Japanese journals. *Intensive Care Med* 2004; 30: 911–7.
- [4] Sakamaki F, Ishizaka A, Urano T, Sayama K, Nakamura H, Terashima T, et al. Effect of a specific neutrophil elastase inhibitor, ONO-5046, on endotoxin-induced acute lung injury. *Am J Respir Crit Care Med* 1996; 153: 391–7.
- [5] Kodama T, Yukioka H, Kato T, Kato N, Hato F, Kitagawa S. Neutrophil elastase as a predicting factor for development of acute lung injury. *Intern Med* 2007; 46: 699–704.
- [6] Okayama N, Kakiyama Y, Setoguchi D, Imabayashi T, Omae T, Matsunaga A, et al. Clinical effects of a neutrophil elastase inhibitor, sivelestat, in patients with acute respiratory distress syndrome. *J Anesth* 2006; 20: 6–10.
- [7] Kadoi Y, Hinohara H, Kunimoto F, Saito S, Goto F, Kosaka T, et al. Pilot study of the effects of ONO-5046 in patients with acute respiratory distress syndrome. *Anesth Analg* 2004; 99: 872–7.
- [8] Tamakuma S, Ogawa M, Aikawa N, Kubota T, Hirasawa H, Ishizaka A, et al. Relationship between neutrophil elastase and acute lung injury in humans. *Pulm Pharmacol Ther* 2004; 17: 271–79.
- [9] Hagiwara S, Iwasaka H, Togo K, Noguchi T. A neutrophil elastase inhibitor, sivelestat, reduces lung injury following endotoxin-induced shock in rats by inhibiting HMGB1. *Inflammation* 2008; 31: 227–34.
- [10] Feihl F, Waeber B, Liaudet L. Is nitric oxide overproduction the target of choice for the management of septic shock? *Pharmacol Ther* 2001; 91: 179–213.
- [11] Szabo C. Alterations in nitric oxide production in various forms of circulatory shock. *New Horiz* 1995; 3: 2–32.
- [12] Ueno H, Matsuda T, Hashimoto S, Amaya F, Kitamura Y, Tanaka M, et al. Contributions of high mobility group box protein in experimental and clinical acute lung injury. *Am J Respir Crit Care Med* 2004; 170: 1310–6.
- [13] Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999; 285: 248–51.
- [14] Bhatia M, Mochhala S. Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. *J Pathol* 2004; 202: 145–56.
- [15] Medzhitov R, Janeway CA, Jr. Decoding the patterns of self and nonself by the innate immune system. *Science* 2002; 296: 298–300.
- [16] van der Poll T, van Deventer SJ. Cytokines and anticytokines in the pathogenesis of sepsis. *Infect Dis Clin North Am* 1999; 13: 413–26.
- [17] Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, Strassheim D, et al. High mobility group box 1 protein (HMGB1) interacts with multiple Toll like receptors. *Am J Physiol Cell Physiol* 2006; 290: 917–24.

- [18] Kokkola R, Andersson A, Mullins G, Ostberg T, Treutiger CJ, Arnold B, et al. RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages. *Scand J Immunol* 2005; 61: 1–9.
- [19] Blackwell TS, and Christman JW. The role of nuclear factor- κ B in cytokine gene regulation. *Am J Respir Cell Mol Biol* 1997; 17: 3–9.
- [20] Hayden MS, Ghosh S. Signaling to NF- κ B. *Genes Dev* 2004; 18: 2195–224.
- [21] Liu SF, Malik AB. NF- κ B activation as a pathological mechanism of septic shock and inflammation. *Am J Physiol Lung Cell Mol Physiol* 2006; 290: 622–45.
- [22] Kulmatycki KM, Jamali F. Therapeutic relevance of altered cytokine expression. *Cytokine* 2001; 14: 1–10.
- [23] Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol* 2005; 5: 331–4.
- [24] Chandra A, Enkhbaatar P, Nakano Y, Traber LD, Traber DL. Sepsis: emerging role of nitric oxide and selectins. *Clinics* 2006; 61: 71–6.
- [25] Lodato RF, Khan AR, Zembowicz MJ, Weisbrodt NW, Pressley TA, Li YF, et al. Roles of IL-1 and TNF in the decreased ileal muscle contractility induced by lipopolysaccharide. *Am J Physiol* 1999; 276: 1356–62.
- [26] Adcock IM. Transcription factors as activators of gene transcription: AP-1 and NF- κ B. *Monaldi Arch Chest Dis* 1997; 52: 178–86.
- [27] Schröder NW, Opitz B, Lamping N, Michelsen KS, Zähringer U, Göbel UB, et al. Involvement of lipopolysaccharide binding protein, CD14, and Toll-like receptors in the initiation of innate immune responses by *Treponema glycolipids*. *J Immunol* 2000; 165: 2683–93.
- [28] Palumbo R, Galvez BG, Pusterla T, De Marchis F, Cossu G, Marcu KB, et al. Cells migrating to sites of tissue damage in response to the danger signal HMGB1 require NF- κ B activation. *J Cell Biol* 2007; 179: 33–40.
- [29] Guha M, Mackman N. LPS induction of gene expression in human monocytes. *Cell Signal* 2001; 13: 85–94.
- [30] Kwak HJ, Song JS, Heo JY, Yang SD, Nam JY, Cheon HG. Roflumilast inhibits lipopolysaccharide-induced inflammatory mediators via suppression of nuclear factor- κ B, p38 mitogen-activated protein kinase, and c-Jun NH2-terminal kinase activation. *J Pharmacol Exp Ther* 2005; 315: 1188–95.

To access this journal online:
<http://www.birkhauser.ch/IR>

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 I κ B.

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.

From the Department of Brain and Nerve Science, Anesthesiology, Oita University Faculty of Medicine, 1-1 Idaigaoka-Hasamemachi-Yufu, Oita, Japan.

The authors have not disclosed any potential conflicts of interest.

For information regarding this article, E-mail: saku@med.oita-u.ac.jp

Copyright © 2009 by the Society of Critical Care Medicine and Lippincott Williams & Wilkins

DOI: 10.1097/CCM.0b013e3181958d91

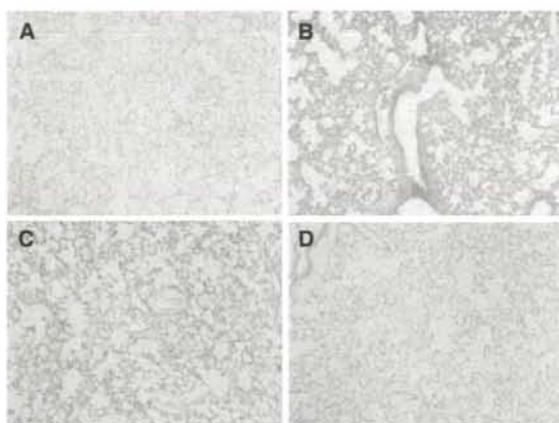


Figure 1. Effects of enalapril on lung injury in lipopolysaccharide (LPS)-treated rats. Rats were treated with saline (control group), LPS (7.5 mg/kg, intravenously; LPS group), LPS and ENA (LPS: 7.5 mg/kg, intravenously and ENA: 50 mg/kg, intraperitoneally; ENA+LPS group), and ENA (50 mg/kg, intraperitoneally;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

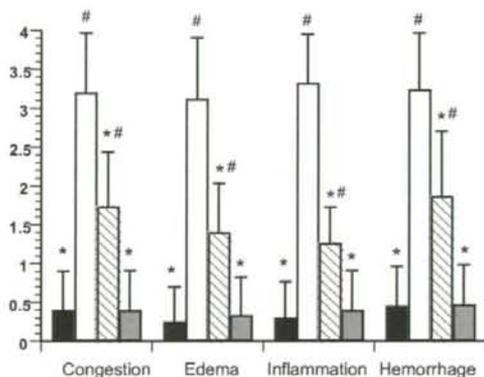


Figure 2. Changes in lung histology score. The identified histologic changes included congestion, edema, inflammation, and hemorrhage 12 hours after the administration of lipopolysaccharide (LPS): control (black bars), LPS (white bars), ENA+LPS (hatched bars), and ENA (gray bars). The data are expressed as the mean \pm 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 mech-

anism 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 Principles 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 μ L of supernatant and 120 μ L of reaction buffer containing 530 nM *o*-dianisidine and 150 nM H₂O₂ (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-

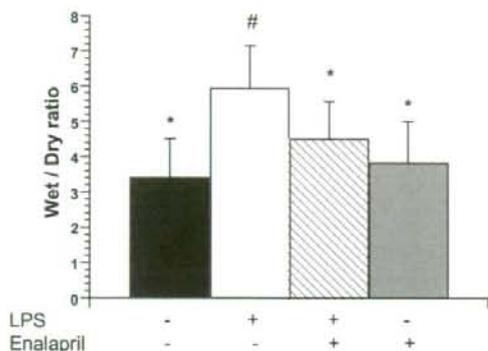


Figure 3. Effects of enalapril on wet-to-dry weight ratio of lipopolysaccharide (LPS)-treated rats. The wet-to-dry weight ratio in lungs was determined 12 hours after LPS treatment for each group ($n = 6$ for each group): control (black bar), LPS (white bar), ENA+LPS (hatched bar), and ENA (gray bar).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

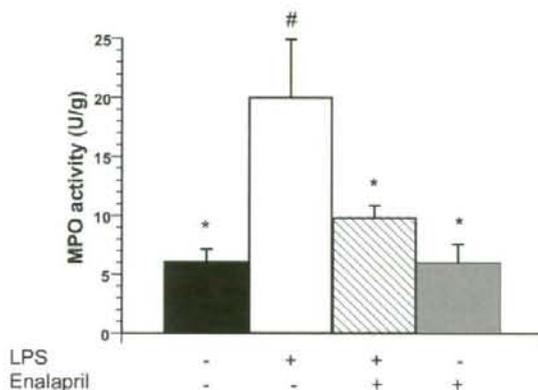


Figure 4. Effects of enalapril on myeloperoxidase (MPO) activity in lipopolysaccharide (LPS)-treated rats. Myeloperoxidase activity in whole lungs was determined 12 hours after LPS treatment for each group ($n = 6$ for each group): control (black bar), LPS (white bar), ENA+LPS (hatched bar), and ENA (gray bar). The data are expressed as the mean \pm 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- α (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 sevoflurane 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 [Ab-

cam, 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% CO₂. 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 μ M) and LPS (100 ng/mL); and ENA group, treated with enalapril (50 μ M) 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- κ B) (p50/p65) was determined using an ELISA-based nonradioactive NF- κ B 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

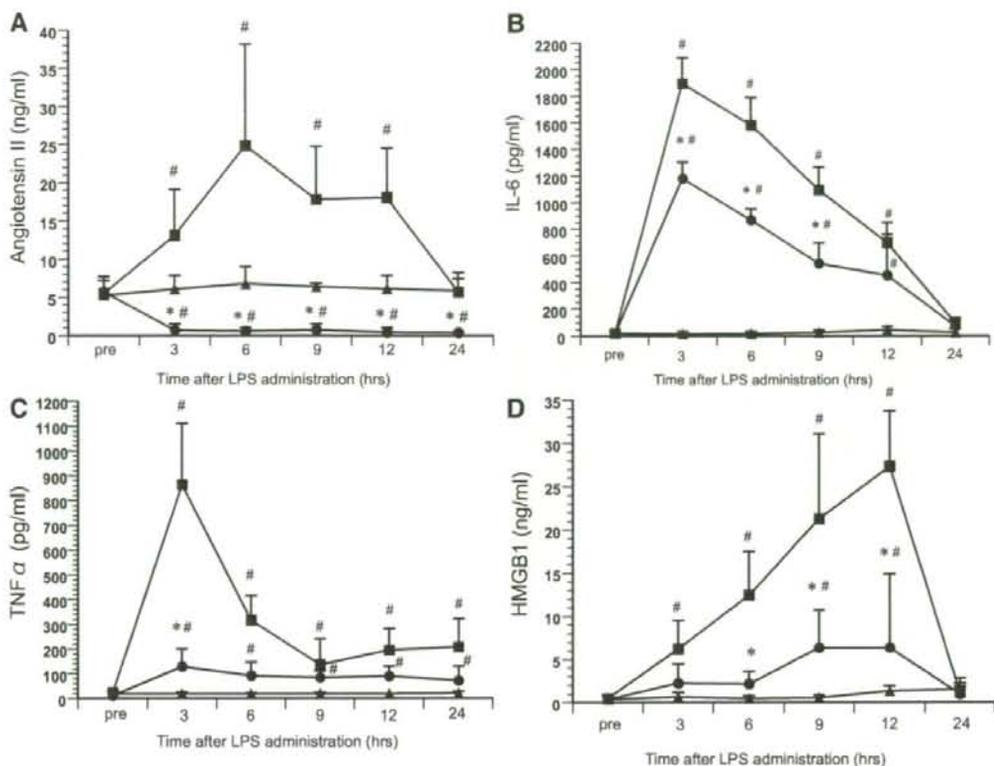


Figure 5. Temporal changes in angiotensin II, interleukin (IL)-6, tumor necrosis factor (TNF)- α , and high-mobility group box 1 (HMGB1) serum concentration after lipopolysaccharide (LPS) administration in rats. Concentrations of the proinflammatory factors were determined in serum taken from animals 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 \pm 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 I κ B α [p-I κ B α], I κ B 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 \pm 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. 1A), marked interstitial edema and inflammatory cell infiltration were seen in the LPS group (Fig. 1B). Interstitial edema and inflammatory cell infiltration were markedly reduced in the ENA+LPS group in comparison to the LPS group (Fig. 1C). Enalapril alone caused no histologic changes (Fig. 1D). 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. 5A). The serum levels of ANG II in the LPS group increased at 3 hours after LPS administration and peaked at 6 hours (Fig. 5A). In contrast, pretreatment with enalapril significantly suppressed the LPS effect at all time points (compare ENA+LPS group to LPS group in Fig. 5A).

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

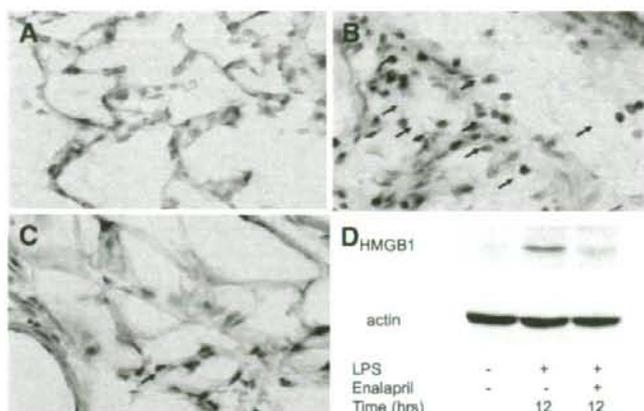


Figure 6. Changes in high-mobility group box 1 (HMGB1) protein expression in lung tissue specimens after lipopolysaccharide (LPS) administration in rats. Immunohistochemical analysis was used to detect HMGB1 in lung sections obtained 12 hours after LPS administration. All photographs are at $\times 400$ 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.

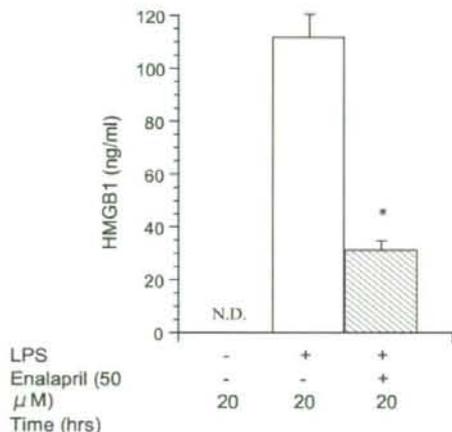


Figure 7. Effect of enalapril on high-mobility group box 1 (HMGB1) release by lipopolysaccharide (LPS)-stimulated murine macrophages. LPS-stimulated (100 ng/mL) murine macrophages were cotreated with enalapril (50 μ M) for 20 hours ($n = 8$ for each group). HMGB1 levels were measured in supernatants by enzyme-linked immunosorbent assay; LPS (white bar) and ENA+LPS (hatched bar) ($n = 8$ for each group). All data are expressed as means \pm 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. 5B). 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. 5C). 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. 5D). 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. 6C). We also determined levels of HMGB1 protein in the lung tissue by western analysis and found that HMGB1 increased after LPS administration (Fig. 6D). 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. 8A). 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. 8B). The cytokine levels in the supernatants of control and ENA groups were undetectable (data not shown).

Enalapril Inhibits I κ B Kinase Pathways and Modulates NF- κ B. Levels of the transcription factor NF- κ B (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 I κ B- α , which was inhibited by enalapril (Fig. 10). In addition, the level of phosphorylated I κ B- α in RAW264.7 cells increased after LPS ad-

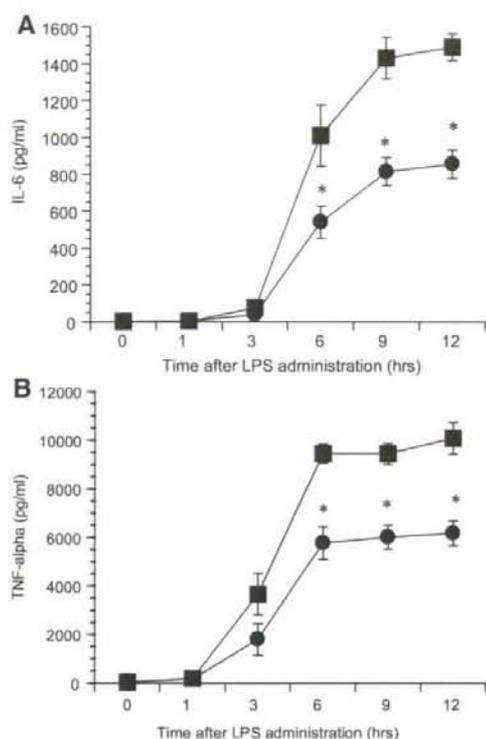


Figure 8. Effect of enalapril on interleukin (*IL*-6) and tumor necrosis factor (*TNF*)- α production by lipopolysaccharide (*LPS*)-stimulated murine macrophages. Murine macrophages were stimulated with *LPS*, with or without enalapril treatment (50 μ M) for the indicated times. Supernatants were collected 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 \pm SD. *Denotes a significant difference compared with *LPS*-treated cells ($p < 0.05$).

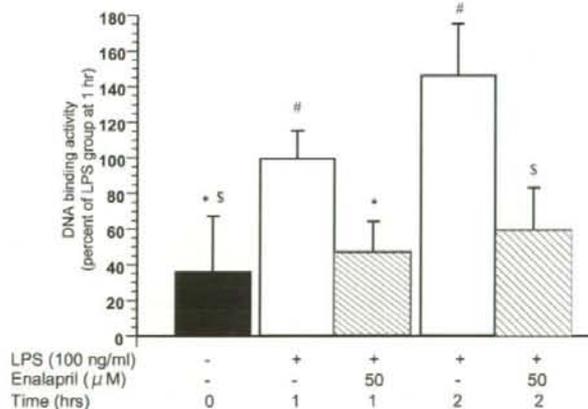


Figure 9. Effect of enalapril on the lipopolysaccharide (*LPS*)-induced increase in p50/p65 binding to DNA. The DNA binding activity assay showed a marked decrease in the p50/p65 binding activity in nuclear fractions from RAW264.7 cells when enalapril at 50 μ M was used to cotreat *LPS*-stimulated cells: control (black bar), *LPS* (white bar), and *ENA* + *LPS* (hatched bar). All data are expressed as the mean \pm 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 ($p < 0.05$).

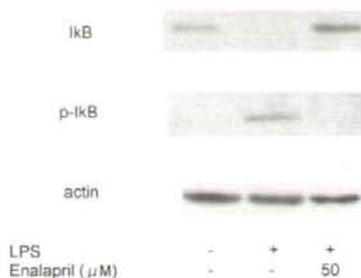


Figure 10. Effect of enalapril on the lipopolysaccharide (*LPS*)-induced phosphorylation of *I κ B*. RAW264.7 murine macrophages were stimulated with lipopolysaccharide, with or without cotreatment with enalapril at 50 μ M. Cells were harvested 1 hour after treatment and the cytoplasmic levels of phosphorylated *I κ B* were determined by Western blot analysis using antibodies specific for *I κ B*- α (*I κ B*), phosphorylated *I κ B*- α (*p-I κ B*), 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- κ B 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- κ B 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- κ B-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- κ B regulates the expression of many inflammatory genes including TNF- α and IL-6 (33), and aberrant activation of NF- κ B is associated with septic shock (34). Suppression of the NF- κ B 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- κ B 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- κ B activation (36). Therefore, the inhibition of NF- κ B 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 I κ B kinase (IKK)-NF- κ B pathway (38). The key step in NF- κ B activation is the rapid degradation of I κ B, which requires I κ B phosphorylation through elevated levels of IKK activity (39). Enalapril seems to suppress NF- κ B activation by preventing phosphorylation of the main driver of the IKK pathway, I κ B.

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 ani-

mals 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.

ACKNOWLEDGMENT

We thank Dr. Tomohisa Uchida for his thoughtful comments and for scoring the lung specimens.

REFERENCES

1. Martin GS, Mannino DM, Eaton S, et al: The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 2003; 348:1546–1554
2. Wyncoll DL, Evans TW: Acute respiratory distress syndrome. *Lancet* 1999; 354: 497–501
3. Lehr HA, Bittiger F, Kirkpatrick CJ: Microcirculatory dysfunction in sepsis: A pathogenic basis for therapy? *J Pathol* 2000; 190: 373–386
4. Riedemann NC, Guo RF, Ward PA: The enigma of sepsis. *J Clin Invest* 2003; 112: 460–467
5. Wang H, Yang H, Czura CJ, et al: HMGB1 as a late mediator of lethal systemic inflamma-

- tion. *Am J Respir Crit Care Med* 2001; 164: 1768-1773
6. Bustin M: Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins. *Mol Cell Biol* 1999; 19:5237-5246
 7. Wang H, Vishnubhakata JM, Bloom O, et al: Proinflammatory cytokines (tumor necrosis factor and interleukin 1) stimulate release of high mobility group protein-1 by pituitary cells. *Surgery* 1999; 126:389-392
 8. Wang H, Bloom O, Zhang M, et al: HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999; 285:248-251
 9. Abd el-Aziz MA, Othman AI, Amer M, et al: Potential protective role of angiotensin-converting enzyme inhibitors captopril and enalapril against adriamycin-induced acute cardiac and hepatic toxicity in rats. *J Appl Toxicol* 2001; 21:469-473
 10. Griffin KA, Abu-Amarah I, Picken M, et al: Renoprotection by ACE inhibition or aldosterone blockade is blood pressure-dependent. *Hypertension* 2003; 41:201-206
 11. Stojiljkovic L, Behnia R: Role of renin-angiotensin system inhibitors in cardiovascular and renal protection: A lesson from clinical trials. *Curr Pharm Des* 2007; 13:1335-1345
 12. Suzuki Y, Ruiz-Ortega M, Lorenzo O, et al: Inflammation and angiotensin II. *Int J Biochem Cell Biol* 2003; 35:881-900
 13. Idell S, Kueppers F, Lippmann M, et al: Angiotensin converting enzyme in bronchoalveolar lavage in ARDS. *Chest* 1987; 91:52-56
 14. Jerng JS, Yu CJ, Wang HC, et al: Polymorphism of the angiotensin-converting enzyme gene affects the outcome of acute respiratory distress syndrome. *Crit Care Med* 2006; 34: 1001-1006
 15. Imai Y, Kuba K, Rao S, et al: Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature* 2005; 436: 112-116
 16. Murakami K, McGuire R, Cox RA, et al: Heparin nebulization attenuates acute lung injury in sepsis following smoke inhalation in sheep. *Shock* 2002; 18:236-241
 17. Koike K, Moore FA, Moore EE, et al: Endotoxin after gut ischemia/reperfusion causes irreversible lung injury. *J Surg Res* 1992; 52:656-662
 18. Zhang H, Sun GY: LPS induces permeability injury in lung microvascular endothelium via AT(1) receptor. *Arch Biochem Biophys* 2005; 441:75-83
 19. Cavaillon JM, Adib-Conquy M: Monocytes/macrophages and sepsis. *Crit Care Med* 2005; 33(Suppl 12):S506-S509
 20. Phillips MI, Kagiya S: Angiotensin II as a pro-inflammatory mediator. *Curr Opin Investig Drugs* 2002; 3:569-577
 21. Tham DM, Martin-McNulty B, Wang YX, et al: Angiotensin II is associated with activation of NF-kappaB-mediated genes and downregulation of PPARs. *Physiol Genomics* 2002; 11:21-30
 22. Esteban V, Ruperez M, Vita JR, et al: Effect of simultaneous blockade of AT1 and AT2 receptors on the NFkappaB pathway and renal inflammatory response. *Kidney Int Suppl* 2003; 86:S33-S38
 23. Koyuncuoglu H, Gungör M, Hatipoğlu I, et al: Changes in brain and lung angiotensin converting enzyme activity in various shocks. *Pharmacol Res Commun* 1984; 16: 479-484
 24. Wösten-van Asperen RM, Lutter R, Haitsma JJ, et al: ACE mediates ventilator-induced lung injury in rats via angiotensin II but not bradykinin. *Eur Respir J* 2008; 31:363-371
 25. Jerng JS, Hsu YC, Wu HD, et al: Role of the renin-angiotensin system in ventilator-induced lung injury: An in vivo study in a rat model. *Thorax* 2007; 62:527-535
 26. Kuba K, Imai Y, Rao S, et al: A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat Med* 2005; 11:875-879
 27. Zhao Y, Chen X, Cai L, et al: Angiotensin II suppresses adriamycin-induced apoptosis through activation of phosphatidylinositol 3-kinase/Akt signaling in human breast cancer cells. *Acta Biochim Biophys Sin (Shanghai)* 2008; 40:304-310
 28. Miyoshi M, Miyano K, Moriyama N, et al: Angiotensin type 1 receptor antagonist inhibits lipopolysaccharide-induced stimulation of rat microglial cells by suppressing nuclear factor kappaB and activator protein-1 activation. *Eur J Neurosci* 2008; 27: 343-351
 29. Yayama K, Okamoto H: Angiotensin II-induced vasodilation via type 2 receptor: Role of bradykinin and nitric oxide. *Int Immunopharmacol* 2008; 8:312-318
 30. Sriskandan S, Altmann DM: The immunology of sepsis. *J Pathol* 2008; 214:211-223
 31. Fink MP: Therapeutic options directed against platelet activating factor, eicosanoids and bradykinin in sepsis. *J Antimicrob Chemother* 1998; 41(Suppl A):81-94
 32. Matsuda N, Hattori Y: Systemic inflammatory response syndrome (SIRS): Molecular pathophysiology and gene therapy. *J Pharmacol Sci* 2006; 101:189-198
 33. Li Q, Verma IM: NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2002; 2:725-734
 34. Liu SF, Malik AB: NF-kappa B activation as a pathological mechanism of septic shock and inflammation. *Am J Physiol Lung Cell Mol Physiol* 2006; 290:G22-645
 35. Zingarelli B, Sheehan M, Wong HR: Nuclear factor-kappaB as a therapeutic target in critical care medicine. *Crit Care Med* 2003; 31: 105-111
 36. Park JS, Arcaroli J, Yum HK, et al: Activation of gene expression in human neutrophils by high mobility group box 1 protein. *Am J Physiol Cell Physiol* 2003; 284:C870-C879
 37. Guha M, Mackman N: LPS induction of gene expression in human monocytes. *Cell Signal* 2001; 13:85-94
 38. Kwak HJ, Song JS, Heo JY, et al: Roflumilast inhibits lipopolysaccharide-induced inflammatory mediators via suppression of nuclear factor-kappaB, p38 mitogen-activated protein kinase, and c-Jun NH2-terminal kinase activation. *J Pharmacol Exp Ther* 2005; 315: 1188-1195
 39. Schottelius AJ, Mayo MW, Sartor RB, et al: Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. *J Biol Chem* 1999; 274: 31868-31874
 40. Bhatia M, Mochhala S: Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. *J Pathol* 2004; 202:145-156
 41. Strieter RM, Kunkel SL, Bone RC: Role of tumor necrosis factor- α in disease states and inflammation. *Crit Care Med* 1993; 21: S447-S463
 42. Remick DG, Bolgos GR, Siddiqui J, et al: Interleukin-6 measured 6 h after the initiation of sepsis predicts mortality over 3 days. *Shock* 2002; 17:463-467
 43. Abraham E, Arcaroli J, Carmody A, et al: HMG-1 as a mediator of acute lung inflammation. *J Immunol* 2000; 165:2950-2954
 44. Ueno H, Matsuda T, Hashimoto S, et al: Contributions of high mobility group box protein in experimental and clinical acute lung injury. *Am J Respir Crit Care Med* 2004; 170: 1310-1316
 45. Wang H, Yang H, Tracey KJ: Extracellular role of HMGB1 in inflammation and sepsis. *J Intern Med* 2004; 255:320-331

LANDIOLOL, AN ULTRASHORT-ACTING β_1 -ADRENOCEPTOR ANTAGONIST, HAS PROTECTIVE EFFECTS IN AN LPS-INDUCED SYSTEMIC INFLAMMATION MODEL

Satoshi Hagiwara, Hideo Iwasaka, Hayato Maeda, and Takayuki Noguchi

Department of Brain and Nerve Science, Anesthesiology, Oita University Faculty of Medicine, Yufu City, Oita, Japan

Received 21 May 2008; first review completed 9 Jun 2008; accepted in final form 1 Jul 2008

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 β_1 -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- κ B activity was inhibited by the administration of landiolol. Cotreatment with the selective β_1 -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] phenylpropanoate monohydrochloride), was developed in Japan for emergency treatment of tachyarrhythmias in animals (8, 9). Landiolol hydrochloride is a highly devel-

oped 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- κ B (NF- κ B) is a critical

Address reprint requests to Satoshi Hagiwara, MD, PhD, Department of Brain and Nerve Science, Anesthesiology, Oita University Faculty of Medicine, 1-1 Idaigaoka Hasamamachi, Yufu City, Oita 879-5593, Japan. E-mail: saku@med.oita-u.ac.jp.

No outside financial support was used for the study.

DOI: 10.1097/SHK.0b013e3181863689

Copyright © 2009 by the Shock Society

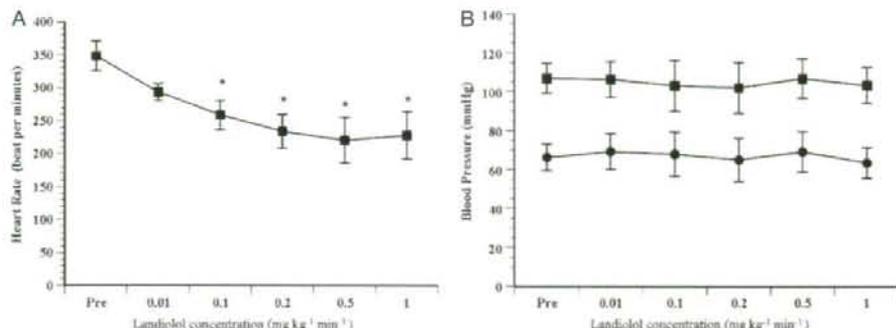


FIG. 1. Dose-response curves of hemodynamic parameters after the administration of each indicated concentration of landiolol. A, Heart rate. B, Blood pressure. Black squares represent systolic blood pressure, and black circles represent diastolic blood pressure ($n = 10$). The data are expressed as the mean \pm 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- κ B 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- κ B 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 \pm 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,

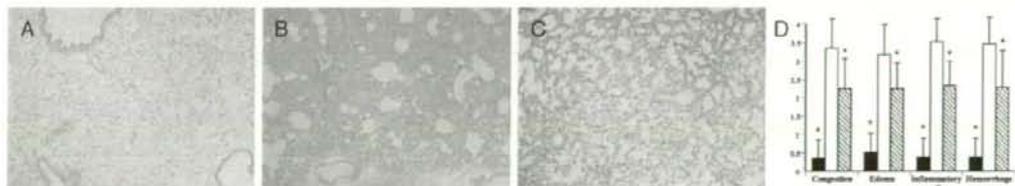


FIG. 2. Effects of landiolol on lung histology in LPS-treated rats. Lung tissues obtained from rats from the control group (A, original magnification $\times 40$), LPS group (B, original magnification $\times 40$), or LPS + landiolol group (C, original magnification $\times 40$) were stained with hematoxylin 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 \pm SD. *Significant difference compared with the LPS group ($P < 0.05$).

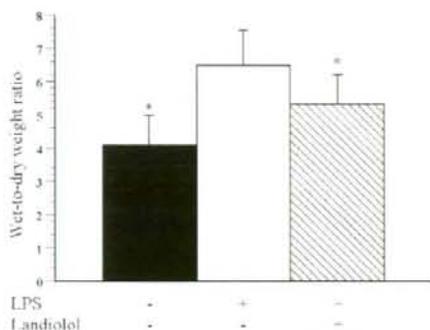


FIG. 3. Effects of landiolol on wet-to-dry weight ratio in lungs of LPS-treated rats. Wet-to-dry weight ratios were determined for each treatment group 12 h after LPS administration. Black bar represents the control group, white bar represents the LPS group, and the slashed bar represents the LPS + landiolol group. The data are expressed as the mean \pm 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 $-dP/dt_{min}$ were continuously recorded using a computerized pressure amplifier-digitizer.

NF- κ B 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- κ B (p50/p65) was determined using an ELISA-based nonradioactive NF- κ B 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 \pm 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 +

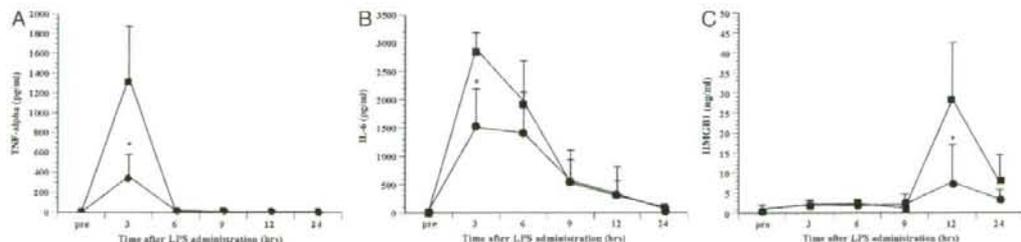


FIG. 4. Temporal changes in serum TNF- α , IL-6, and HMGB-1 concentrations after LPS treatment. Sera were obtained from LPS (squares) and LPS + landiolol (circles) group animals ($n = 10$ per group) at the indicated time points after LPS administration; cytokine levels were measured by ELISA. A, TNF-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