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Effects of hyperglycemia and insulin therapy on high mobility group box 1 in endotoxin-induced acute lung injury in a rat model*

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Objective: **Hyperglycemia and insulin resistance are commonly seen in septic patients and are associated with increased morbidity and mortality. High mobility group box 1 (HMGB1) protein has been shown to play a key role as a significant factor in sepsis pathogenesis. This study investigated the increase in lung damage because of hyperglycemia and HMGB1 increase in a lipopolysaccharide-induced septic rat model and the potential for insulin therapy to reduce this lung damage by decreasing the serum level of HMGB1.**

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

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

Subjects: **Male Wistar rats.**

Interventions: **Septic hyperglycemia was induced by infusion of glucose immediately after administration of lipopolysaccharide in rats.**

Measurements and Main Results: **Animals were monitored for blood glucose. Separate cohorts were killed at 12 and 24 hrs postlipopolysaccharide administration and analyzed for HMGB1 and lung damage. The effects of insulin treatment were also examined. Hyperglycemic septic animals had significantly higher blood glucose and enhanced lung damage. In addition, HMGB1 was increased in the serum of hyperglycemic rats. On the other hand, insulin treatment for hyperglycemia resulted in significantly lower blood glucose and decreased both the lung damage and the serum level of HMGB1. In an *in vitro* study, insulin treatment inhibited the activation of NF-kappaB.**

Conclusions: **Hyperglycemia is associated with higher HMGB1 levels and lung damage in sepsis. Insulin therapy significantly reduced lung damage, suggesting that management of hyperglycemia with insulin might decrease HMGB1 levels in the serum and lung tissue. One of the mechanisms that could contribute to the inhibition of HMGB1 secretion might be related to the inhibition of NF-κB. (Crit Care Med 2008; 36:2407–2413)**

Key Words: lipopolysaccharide; lung injury; sepsis; hyperglycemia; insulin; high mobility group box 1

Sepsis is a significant cause of morbidity and mortality worldwide. This is partly due to complications, such as acute respiratory distress syndrome, which are difficult to treat (1–3). Hyperglycemia and insulin resistance are commonly observed in septic patients and have proinflammatory effects, including increased production of reactive oxygen species (4). Indeed, a study concluded that treating hyperglycemia with intensive insulin therapy reduced morbidity and mortality among critically ill patients in a surgical intensive care unit (5). However, a recent trial investigating the effects of insulin intensive therapy on patients with severe sepsis was terminated early because the risk of adverse effects was significantly higher in the intensive-therapy group (6). Researchers and clinicians currently are debating the effects of intensive insulin therapy on patients with severe sepsis.

High mobility group box 1 (HMGB1) protein plays a key role in the pathogenesis of sepsis as a late-phase mediator of systemic inflammation (7). HMGB1 is an intranuclear protein that was originally identified as a regulator of chromatin structure (8). Once released from necrotic cells or secreted by activated monocytes/macrophages, HMGB1 can bind to receptors for advanced glycation end-products (RAGE). The proinflammatory effects of HMGB1 are mediated partially by signaling through RAGE (9, 10). HMGB1 contributes to lethality in septic patients and activates downstream cytokine release (11). Thereafter, release of HMGB1 to the extracellular space is controlled by cytokines (12). Extracellular HMGB1 seems to play a role in both clinical, acute respiratory distress syndrome, and experimental models. The concentrations of HMGB1 were elevated in the plasma and lungs of patients with acute respiratory distress syndrome and in a mouse model of lipopolysaccharide (LPS)-induced lung injury (13).

Hyperglycemia up-regulates various inflammatory mediators (14). One reason for this enhanced inflammation is the activation of NF-kappaB after glucose intake (15). On the other hand, insulin has an anti-inflammatory effect. Dandona et al. (16) reported that this effect includes the inhibition of NF-kappaB. A recent study demonstrated that tight control of blood glucose by insulin therapy protected the endothelium of critically ill patients (17). Controlling hyperglycemia is acknowledged as an important component of sepsis treatment.

We hypothesized that hyperglycemia might increase the serum and tissue HMGB1 levels, thereby contributing to LPS-induced lung injury. Insulin therapy may be able to prevent LPS-induced lung injury by regulating hyperglycemia and inhibiting the levels of HMGB1 in the serum and tissue. To test this hypothesis, we investigated the effects of hyperglycemia and insulin therapy on LPS-induced lung injury in rats.

*See also p. 2475.

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METHODS

Materials. Insulin was obtained from Novo Nordisk A/S (Copenhagen, Denmark). LPS (O127:B8) was obtained from Sigma (St Louis, MO). All other reagents were of the highest available analytical grade. We used commercially available antibodies to HMGB1 (Shino-Test Corporation, Tokyo, Japan), phosphorylated IkapperB alpha (p-IkapperB alpha), IkapperB alpha (Cell Signaling Technology, Beverly, MA), and beta-actin (Abcam, Cambridge, UK).

Animals. All protocols conformed to the National Institute of Health guidelines and all animals received humane care 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. All animals were housed with free access to food and water *ad libitum*.

Animals were randomly assigned to one of five groups (n = 10 in each group): 1) saline group, rats intravenously received 0.9% NaCl solution (2.5 mL/kg/hr); 2) Glu group, rats received 20% glucose solution (2.5 mL/kg/hr) intravenously; 3) LPS group, rats received 0.9% NaCl solution (2.5 mL/kg/hr) intravenously after the intravenous injection of a bolus of LPS (2.5 mg/kg); 4) LPS + Glu group, rats received 20% glucose solution (2.5 mL/kg/hr) intravenously after the intravenous injection of a bolus of LPS (2.5 mg/kg); and 5) LPS + Ins group, rats received 20% glucose solution (2.5 mL/kg/hr) and insulin (20 IU/kg/hr) intravenously after the intravenous injection of a bolus of LPS (2.5 mg/kg). Serum samples of venous blood (0.1 mL) were obtained from the external jugular vein at various time points (0, 4, 6, 8, 10, 12, and 24 hrs). All animals were killed at 12 or 24 hrs and lung tissue specimens were quickly removed and processed as indicated below.

Blood Glucose Measurement. Blood samples (n = 6 in each group) were collected from the external jugular vein of rats for each time point (0, 4, 6, 8, 10, 12, and 24 hrs). The whole blood glucose levels of the rats were monitored using the glucose-oxidase method with a Glu-test-sensor blood glucose monitoring system from Sanwa Kagaku Kenkyosho (Nagoya, Japan).

Histologic Analysis. Left lungs (n = 4 in each group) were obtained from animals in the LPS, LPS + Glu, and LPS + Ins groups under sevoflurane anesthesia. The lung tissue specimens were fixed with 10% formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin. A pathologist unaware of the group assignment analyzed the samples. An evaluation of lung injury was done according to Murakami's technique (18). Briefly, 24 areas in the 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, inflammatory cells, and hemorrhaging. The mean score for each of the parameters was calculated.

Measurements of HMGB1. Serum samples (n = 6 in each group) were assayed using the sandwich enzyme-linked immunosorbent assay (ELISA) method. Ninety-six well plates were precoated with monoclonal antibodies specific to rat HMGB1 (Shino-Test, Tokyo, Japan). The samples, negative controls, and diluted HMGB1 standard markers were added into their respective wells. Detection of HMGB1 in the samples was performed according to the manufacturer's protocols. The A450 values were determined using an ELISA reader (Bio-Rad Laboratories, Hercules, CA).

Immunohistochemical Analysis. Lung tissue (n = 4 in each group) samples were fixed immediately in 4% paraformaldehyde at 4°C overnight and then embedded in O.C.T. Compound (Sakura Finetechnical, Tokyo, Japan). Sections were incubated with mouse anti-rat-HMGB1 polyclonal antibody (1:1000 dilution) overnight at 4°C. The sections were rinsed with phosphate buffered saline (PBS) and then incubated with peroxidase-conjugated anti-mouse-IgG (1:1000 dilution). After rinsing with PBS, the slides were stained using the LSAB2 kit (Dako, Carpinteria, CA), including the biotin-avidin-peroxidase complex system. After development, the slides were counterstained with Mayer's hematoxylin and mounted.

Cell Culture Study. The murine macrophage cell line RAW264.7 was maintained in Dulbecco's Modified Eagle's Medium containing 5% heat-inactivated fetal bovine calf serum and antibiotics at 37°C under 5% CO_2. The medium was removed and replaced with high glucose (450 mg/dL) or low glucose (100 mg/dL) Dulbecco's Modified Eagle's Medium containing 5% fetal bovine calf serum. Cells were randomly assigned to the following groups: 1) saline in low glucose medium; 2) LPS in normal glucose medium; 3) LPS in high glucose medium; and 4) LPS and insulin in high glucose medium. Cells were lysed and protein was extracted using nuclear and cytoplasmic extraction reagents (PIERCE Biotechnology, Rockford, IL). Cytoplasmic and nuclear protein fractions were used for Western blot analyses, as described below.

NF-kappaB Binding Assay. The DNA binding activity of NF-kappaB (p50/p65) was determined using an ELISA-based nonradioactive NF-kappaB p50/p65 transcription factor assay kit (Chemicon, Temecula, CA). Detection of NF-kappaB activity in the samples was performed according to the manufacturer's protocols. The A450 values were determined using an ELISA reader (Bio-Rad Laboratories).

Western Blotting. Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% gels and then were transferred onto polyvinylidene difluoride sheets (Millipore, Bedford, MA). The membranes were incubated with the primary antibody at 1/1000 in PBS-T for 1 hr at room temperature. After three washes in PBS-T, peroxidase-labeled secondary antibody was added at 1/1000 in PBS-T and incubated for 1 hr at room temperature. Blots were developed using enhanced chemiluminescence (ECL) reagents and exposed to Hyperfilm ECL (Amersham, Buckinghamshire, UK).

Statistical Analysis. All data are presented as the mean ± SE. The data were compared using nonparametric tests. The Kruskal-Wallis test for comparisons between all groups was used. A p value of <0.05 was considered to be statistically significant.

RESULTS

Effect of Glucose or Insulin Administration on Hyperglycemia

Figure 1 shows the changes in blood glucose concentrations in the five groups of rats during the infusions of saline (saline group), glucose (Glu group), saline after LPS injection (LPS group), glucose after LPS injection (LPS + Glu group), or glucose and insulin after LPS injection (LPS + Ins group). In the saline group, glucose levels were maintained at normal levels over 24 hrs and ranged from 158 ±

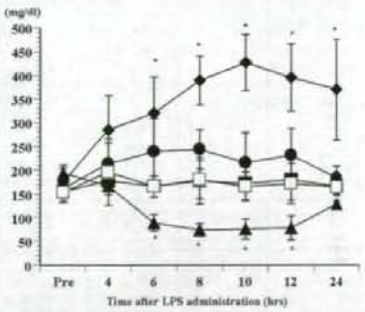


Figure 1. The changes in blood glucose concentration in lipopolysaccharide (*LPS*), LPS + glucose (Glu), and LPS + insulin (Ins) groups after endotoxin challenge. Blood glucose concentration at the indicated hours is shown for each group (n = 6 for each group). *Filled square* (saline group), animals received saline (5 mL/kg/hr) infusion only; *circles* (Glu group), animals received glucose (5 mL/kg/hr) infusion only; *triangles* (LPS group), animals received saline infusion after a 2.5 mg/kg endotoxin injection; *diamonds* (LPS + Glu group), animals received glucose infusion after a 2.5 mg/kg endotoxin injection; *open square* (LPS + Ins group), animals received glucose and insulin infusions after a 2.5 mg/kg endotoxin injection. All data are expressed as the means ± SE. *$p < 0.05$ vs. each point in saline only group.

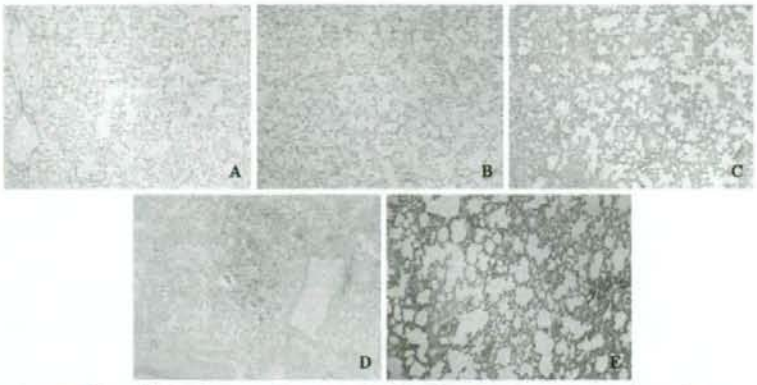


Figure 2. Effects of high glucose on lung injury in lipopolysaccharide (LPS)-treated rats. Rats were given either saline (saline group), glucose (Glu group), saline and LPS (LPS group), glucose and LPS (LPS + Glu group), or glucose, insulin and LPS (LPS + Ins group). Lungs are from the saline (*A*, magnification ×40), Glu (*B*, magnification ×40), LPS (*C*, magnification ×40), LPS + Glu (*D*, magnification ×40), and LPS + Ins (*E*, magnification ×40) groups, respectively. Sections shown are stained with hematoxylin and eosin.

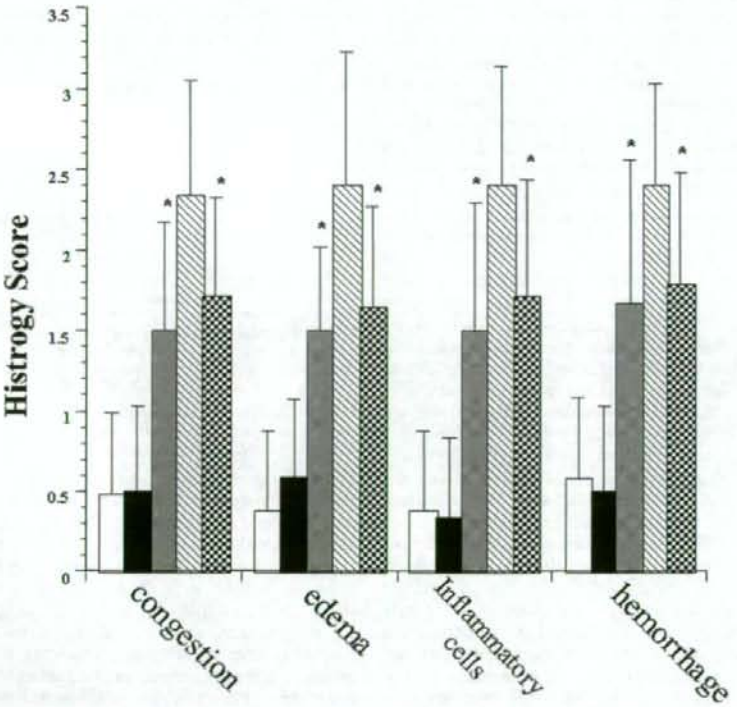


Figure 3. Changes in lung histology score. The identified histologic changes included congestion, edema, inflammation, and hemorrhaging 24 hrs after the administration of lipopolysaccharide (LPS). *White bars* represent the saline-administrated sham animals (saline group), *black bars* represent animals injected with glucose (Glu group), *gray bars* represent animals injected with saline and LPS (LPS group), *slashed bars* represent animals injected with glucose and LPS (LPS + Glu), and *checkered bars* represent animals injected with glucose, insulin and LPS (LPS + Ins group). The data are expressed as the mean ± SE. *$p < 0.05$ vs. LPS + Glu group.

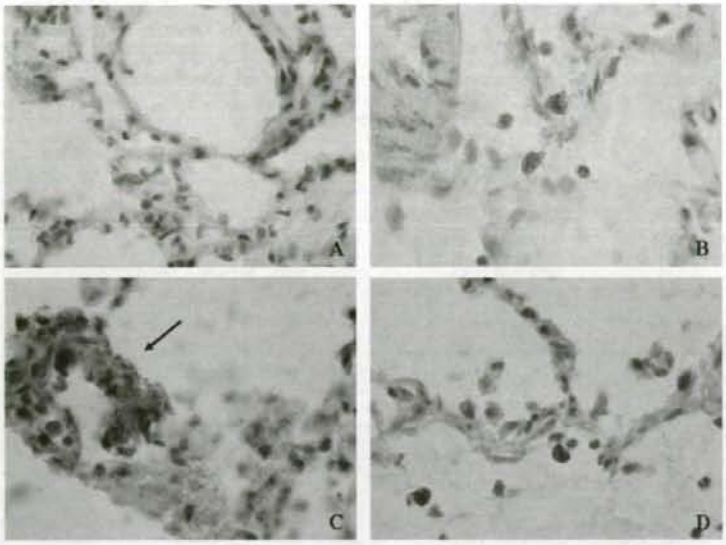
8 to 192 ± 9 mg/dL. In the glucose group, glucose levels were slightly higher than normal levels over 24 hrs, with an average of around 200 mg/dL. In the LPS group, glucose levels were lowered below normal levels by 6 hrs after LPS administration. In the LPS + Glu group, after starting the infusion of glucose, blood glucose levels significantly increased and reached 312 ± 5 mg/dL over the first 6 hrs. In the LPS + Ins group, glucose levels were maintained at normal levels during the experimental period.

The Effect of Glucose or Insulin Administration on Lung Tissue Specimens

Histologic examinations of lung tissue sections were performed 24 hrs after LPS (2.5 mg/kg intravenous) administration. Observations of microscopic changes in the lung tissue specimens showed edema-like formation and inflammatory cell infiltration in the LPS and LPS + Glu groups compared with the saline and Glu groups (Fig. 2*A–D*). Interstitial edema and inflammatory cell infiltration were markedly reduced in the LPS + Ins group in comparison with the LPS and LPS + Glu groups (Fig. 2*C–E*). We observed no histologic changes after administering saline and glucose alone (Fig. 2*A–B*). The histology scores, based on the number of areas with congestion, edema, inflammation, and hemorrhaging (17) were all significantly higher after the administration of LPS than in the saline group. All of the scores were significantly higher in the LPS + Glu group compared with the saline, LPS, and LPS + Ins groups (Fig. 3).

Changes in HMGB1 Protein Expression in Lung Tissue

At 24 hrs after LPS administration, positive staining cells for HMGB1 were rarely observed in lung tissue from the saline (Fig. 4*A*) and Glu group (data not shown). Positive staining cells for HMGB1 were somewhat increased in lung tissue from the LPS group (Fig. 4*B*) and markedly increased in the LPS + Glu group (Fig. 4*C*). The number of cells expressing HMGB1 was substantially decreased in the LPS + Ins group (Fig. 4*D*) compared with the LPS + Glu group (Fig. 4*C*). At 12 hrs after LPS administration, Western blot analysis showed an increase in HMGB1 expression in the lung tissue from rats receiving glucose after LPS injection. This increase in HMGB1



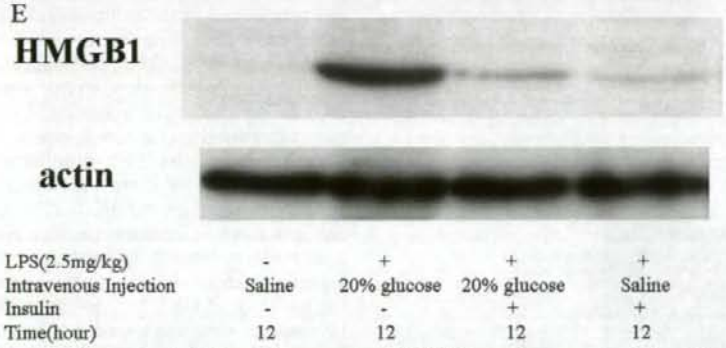


Figure 4. Changes in the high mobility group box 1 (*HMGB1*) protein expression in lung tissue specimens after lipopolysaccharide (*LPS*) administration in rats. The expression of HMGB1 protein 24 hrs after LPS administration was detected by immunohistochemistry in lung tissue specimens from rats in the saline group (*A*), LPS group (*B*), LPS + glucose group (*C*), or LPS + insulin group (*D*); arrows indicate cells that are positively stained for HMGB1. The expression of HMGB1 protein in lung tissue 12 hrs after LPS administration was detected by Western blotting (*E*); the rats were given either saline alone, LPS + glucose, LPS + glucose + insulin, or LPS + saline + insulin.

was not seen in lung tissue from rats that received insulin in addition to glucose after LPS injection (Fig. 4*E*).

Changes in HMGB1 Serum Concentrations

The serum level of HMGB1 increased in all groups at 12 and 24 hrs after the administration of LPS. HMGB1 serum concentration in the LPS + Glu group was significantly higher compared with the LPS group at both time points. On the other hand, in the LPS + Ins group, HMGB1 concentrations were not significantly different from the LPS group at either time point (Fig. 5). The serum level of HMGB1 was below the detection level in all groups before the administration of LPS (data not shown).

Effect of High Glucose on Levels of HMGB1 Secreted by LPS-Stimulated Murine Macrophages

Secreted HMGB1 levels increased after LPS stimulation in high glucose medium compared with low glucose medium, but were inhibited by the addition of insulin in high glucose medium. In addition, the inhibition of HMGB1 by insulin seemed to be dose-dependent, with minimal inhibition at a concentration of 1 μM and the greatest effect observed at the highest dose (100 μM) tested in this study (Fig. 6). Because in this experiment we observed the greatest inhibition of HMGB1 using 100 μM insulin, we used this concentration for subsequent experiments.

Insulin Inhibits the IkappaB Kinase Pathways and Modulates NF-kappaB

The DNA binding activity of the transcription factor NF-kappaB (p50/p65) in the nucleus of RAW264.7 cells increased 1 hr after administration of LPS in both low and high glucose medium. Activation of the NF-kappaB transcription factor p50/p65 after the administration of LPS was higher in high glucose medium than in low glucose medium. However, administration of insulin suppressed this increase (Fig. 7).

Treatment with LPS resulted in the degradation of IkapperB alpha in both the low and high glucose medium, which was inhibited by the addition of insulin to the high glucose medium. In addition, the phosphorylation of IkapperB alpha in RAW264.7 cells increased after LPS administration in both the low glucose medium and high glucose medium, which was also inhibited by the addition of insulin to the high glucose medium (Fig. 8).

DISCUSSION

Our study showed that hyperglycemia induced histologic changes in our LPS-induced septic rat model. Hyperglycemia occurs frequently among critically ill patients, including those with sepsis (19). When hyperglycemia occurs as a complication, it has been shown to be associated with high mortality and poor clinical outcomes in subpopulations of clinically ill patients (20). One of the reasons for this is that hyperglycemia can amplify the inflammatory response (21). These results indicate that hyperglycemia aggravates the condition of a patient in septic shock by increasing the severity of lung injury.

HMGB1 has been shown to play an important role in various types of inflammation. In addition, HMGB1 is an important mediator of cell death, and its expression occurs at a relatively late stage after injury (7). Especially when it is released as a result of acute lung inflamma-

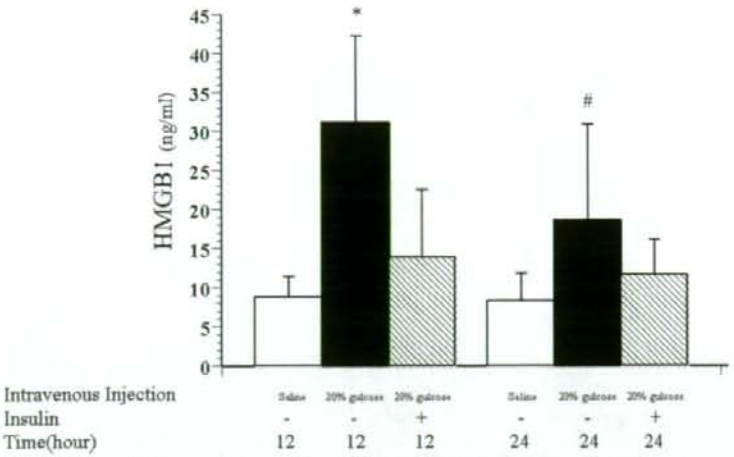


Figure 5. Temporal changes of high mobility group box 1 (*HMGB1*) serum concentration after lipopolysaccharide (*LPS*) administration in rats. Rats were given either saline and LPS (LPS group), glucose and LPS (LPS + Glu group), or glucose, insulin and LPS (LPS + Ins group). HMGB1 serum concentration at 12 and 24 hrs after LPS administration is shown for each group (n = 6 for each group). *White bars* represent LPS group animals, *black bars* represent LPS + Glu group animals, and *slashed bars* represent LPS + Ins group animals. All data are expressed as the means ± SE. *$p < 0.05$ vs. LPS group at 12 hrs. #$p < 0.05$ vs. LPS group at 24 hrs.

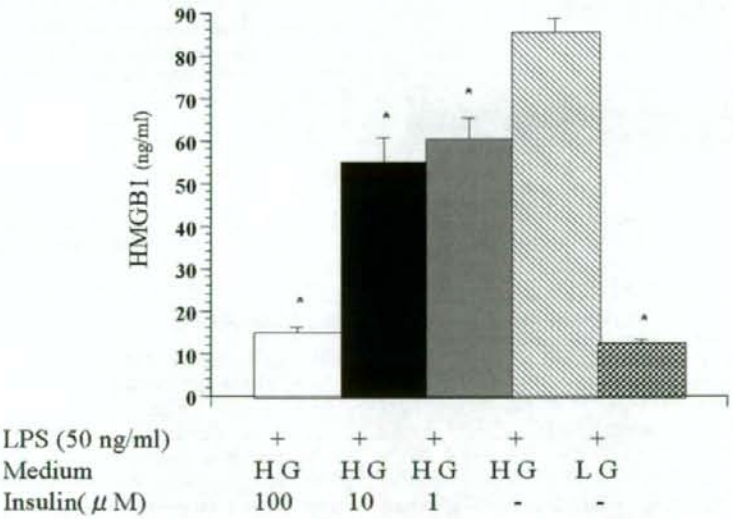


Figure 6. Effect of insulin in high glucose (*HG*) and low glucose (*LG*) medium on high mobility group box 1 (*HMGB1*) production by lipopolysaccharide (*LPS*)-stimulated murine macrophages. Murine macrophages treated with insulin were stimulated with LPS for 20 hrs. Conditioned media were collected and HMGB1 levels were determined by enzyme-linked immunosorbent assay. The *checkered bar* represents values measured for the LG medium, the *slashed bar* represents values measured for the HG medium without insulin, the *gray bar* represents values measured for the HG medium with 1 μM insulin, the *black bar* represents values measured for the HG medium with 10 μM insulin, and the *white bar* represents values measured for the HG medium with 100 μM insulin. All data are expressed as the means ± SE. *Denotes a significant difference compared with LPS in HG medium ($p < 0.05$).

tion, HMGB1 can have various effects (13). In the present study, we demonstrated that the expression of HMGB1 in the serum and lung tissue increased in hyperglycemic rats with LPS-induced lung injury. Moreover, hyperglycemia also increased histologic changes including interstitial and intra-alveolar inflammation, edema, congestion, and hemorrhage. These findings are consistent with previous studies, which showed that HMGB1 plays a critical role in the development of LPS-induced lung injury (13, 22).

LPS stimulation of macrophages activates several intracellular signaling pathways, including the IkappaB kinase (IKK)-NF-kappaB pathway (23). In addition, the proinflammatory effects of glucose metabolism include an increase in IKK-alpha, IKK-beta, and NF-kappaB binding with a decrease in IkapperB alpha (15). We found that insulin in high glucose medium suppressed the activation of NF-kappaB and prevented the phosphorylation of a molecule central to the IKK pathway, IkapperB. A previous study also demonstrated that insulin inhibits the activation of NF-kappaB (16, 24). Accordingly, the inhibition of IkapperB phosphorylation after insulin administration for hyperglycemia may lead to the inhibition of NF-kappaB activation. The *in vitro* effect of insulin on NF-kapperB/IkapperB suggests that there is a direct mechanism that is not necessarily dependent on a decrease in glucose concentrations. When IkapperB is phosphorylated after LPS stimulation, the activation of NF-kappaB may be related to the secretion of HMGB1 (25). We demonstrated that insulin in the high glucose medium inhibits LPS-induced NF-kappaB activation, which may participate in the inhibition of HMGB1 secretion in the LPS-induced lung injury model. However, in this study, we used two different species (rat *in vivo* study, and murine *in vitro* study) and acknowledge that there may be mechanistic differences between the two systems. Similar experiments using a mouse model of lung injury may clarify our results.

In the *in vitro* study, inhibiting HMGB1 secretion required a high concentration of insulin. Aljada et al. (24) has demonstrated the suppression of NF-kappaB by insulin in human aortic endothelial cells at more physiologically relevant concentrations. However, we used human insulin in our murine *in vitro* study and acknowledge that there may be differences. This might be the reason we needed a very high concentration of insulin to observe the inhibition of HMGB1 secretion and NF-kappaB activation.

We also demonstrated that insulin treatment significantly decreased the severity of lung injury and both serum and lung tissue HMGB1 levels in the LPS-induced septic rat model. Systemic in-

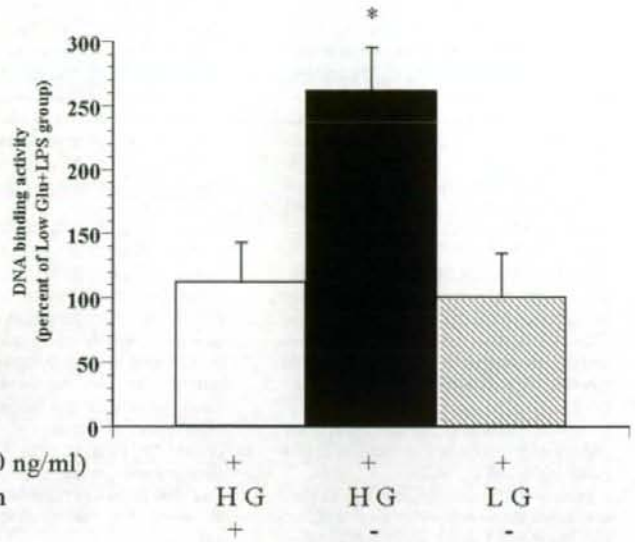


Figure 7. Effect of insulin on the lipopolysaccharide (*LPS*)-induced increase in the specific binding of p50/p65 to DNA. DNA binding activity of NF-kappaB p50/p65 is shown for nuclear fractions from LPS-stimulated RAW264.7 cells and low glucose (*Glu*) (*LG*) medium, high glucose (*HG*) medium, and HG medium with 100 μM insulin. The *dashed bar* represents values measured for the LG medium, the *black bar* represents values for the HG medium without insulin and the *white bar* represents values for the HG medium with insulin. All data are expressed as the mean ± SE. *Denotes a significant difference compared with LPS in HG medium at 1 hr ($p < 0.05$).

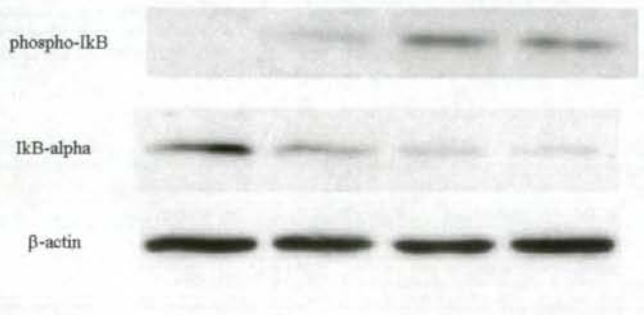


Figure 8. Effect of insulin on the lipopolysaccharide (*LPS*)-induced phosphorylation of IkapperB. Proteins were extracted from murine macrophages with or without insulin treatment 1 hr after stimulation with LPS (100 ng/mL). The cytoplasmic levels of phosphorylated IkapperB alpha, IkapperB alpha, and beta-actin were determined by Western blot analysis using antibodies specific for phosphorylated IkapperB alpha, IkapperB alpha, and beta-actin (1/1000 dilution). *LG*, low glucose; *HG*, high glucose.

flammation can occur during hyperglycemia and persist after blood glucose levels have retuned to normal. This can lead to an increase in the systemic inflammatory response to a subsequent moderate stress (14). An increase in systemic inflammation can lead to a greater risk of organ damage in septic shock patients. On the other hand, insulin treatment has been demonstrated to reduce the inflammation in various diseases (26). Therefore, insulin therapy may benefit patients with conditions that are often complicated by severe systemic inflammation including septicemia, meningitis, and pneumonia (27). This anti-inflammatory effect of insulin has also been shown in rats injected with endotoxin (28). In this study, we demonstrated that insulin treatment led to a decrease in the serum level of HMGB1 after LPS administration. These findings suggest that anti-inflammatory effect of insulin in the rat model of LPS-induced lung injury may be at least partially due to the ability of insulin to decrease HMGB1 levels in the serum and lung tissue.

This study only examined the short-term (up to 24 hrs) therapeutic effects of insulin treatment for hyperglycemia after the onset of lethal endotoxemia. However, recent clinical studies demonstrated that the anti-inflammatory effect of insulin was long-lived (17, 29). The results of this study cannot be extrapolated to chronic conditions in patients. Since long-term insulin treatment was effective in intensive care unit patients, we need further study of the long-term effects of insulin in a septic shock model.

In conclusion, hyperglycemia aggravated the pathophysiological findings in a rat model of LPS-induced lung injury and increased the serum level of HMGB1. The administration of insulin to treat hyperglycemia improved these findings. These observations indicated that insulin treatment for hyperglycemia could decrease the severity of lung injury by inhibiting the HMGB1 levels during the acute phase of LPS-induced lung injury in rats. As a caveat, in this study, we used small numbers of animals and the high levels of glucose might not be entirely relevant for clinical conditions in the intensive care unit.

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A Neutrophil Elastase Inhibitor, Sivelestat, Reduces Lung Injury Following Endotoxin-Induced Shock in Rats by Inhibiting HMGB1

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Abstract—Neutrophil elastase (NE) plays an important role in the progression of acute lung injury (ALI). Sivelestat sodium hydrate (Sivelestat) is a highly specific synthetic inhibitor of NE. High mobility group box 1 (HMGB1) is one of the key mediators in the development of sepsis. The aim of this study was to evaluate the effect of sivelestat and to determine whether it can reduce lipopolysaccharide (LPS)-induced acute lung injury in rats. Rats were randomly divided into a negative control group, an LPS-induced sepsis group, and a group treated with sivelestat prior to LPS administration. Animals in the sivelestat group received a bolus of 10 mg/kg of sivelestat injected into the intraperitoneal cavity before the LPS treatment. Furthermore, rats were administered sivelestat at 0, 1, 3, and 6 h following LPS treatment. We measured cytokine and HMGB1 levels in the serum after the induction of sepsis. In addition, we observed histopathology, wet/dry weight ratio, inducible nitric oxide synthase and HMGB1 expression in the lung tissue. Lung histopathology was significantly improved in the sivelestat group compared to the LPS group. Serum and pulmonary HMGB1 levels were lower over time among sivelestat-treated animals. Furthermore, inhibition of NF-κB activity was observed with the administration of sivelestat. These results suggest that sivelestat reduces LPS-induced lung injury at least partially by inhibiting inflammation and NF-κB activity.

KEY WORDS: inflammation; sepsis; high mobility group box 1; cytokine; NF-κB; nitric oxide.

INTRODUCTION

The high case fatality rate of sepsis is due to complications throughout the major organ systems that can occur by various mechanisms. In spite of advances in therapy, the mortality rate for severe sepsis remains high, reaching 80% to 90% for septic shock with multiple organ dysfunction [1]. Acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) are well defined clinical disorders that are a significant cause of morbidity and mortality in intensive care units (ICU). The most frequent cause of ARDS and ALI is sepsis. [2]. Despite recent increases in our understanding of the molecular underpinnings of sepsis, most of these complications remain refractory to treatment [3]. The respiratory system in particular is severely affected and difficult to treat [4].

Neutrophil elastase (NE, also called leukocyte elastase) is a serine protease found primarily in the azurophilic granules of neutrophils. Intracellular NE is a key molecule of the innate immune system and is required for effective killing of phagocytosed bacterial and fungal pathogens [5, 6]. In addition, Moraes TJ *et al.* [7] reviewed the potentially pathologic role of NE in ARDS and ALI, including the ability of extracellular NE

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to cause endothelial damage and increase vascular permeability.

Sivelestat is a specific neutrophil elastase inhibitor that has no other known antiprotease effects [8]. Previous studies have reported that sivelestat can reduce lung injury associated with systemic inflammatory response syndrome (SIRS) in humans [9] and also decrease endotoxin-induced lung injury in animal models [10, 11]. These results suggested that sivelestat might be useful for the treatment of SIRS and ARDS/ALI. However, the protective mechanisms of sivelestat are not well understood.

HMGB1 was recognized as a late-phase mediator in the pathogenesis of sepsis [12] and was thought to contribute to the pathology and mortality of sepsis as an inflammatory mediator [13]. Recent studies indicate that HMGB1 plays a role in the enhancement of various inflammatory processes. Indeed, HMGB1 mediates cell-to-cell signaling by binding to Toll-like receptors (TLRs) [14, 15]. HMGB1 also acts as a procoagulant, thereby enhancing the inflammatory response in septic shock [16]. In addition, HMGB1 is present in the serum of sepsis patients, with higher levels associated with increased mortality [17, 18].

The release of HMGB1 from macrophages was first reported in response to stimulation by endotoxin, and increased serum levels of HMGB1 have been observed in sepsis [12, 19]. Endotoxin, also called lipopolysaccharide (LPS), is found in the outer membrane of Gram-negative bacteria and induces immune responses by interacting with TLR4 [20]. The release of HMGB1 from macrophages is regulated by various inflammatory mediators and activates signal transduction pathways, including the NF-κB pathway. Specifically, nitric oxide and TNF-alpha induce HMGB1 release in response to LPS [21, 22].

We hypothesized that sivelestat prevents acute lung injury in a rat model by inhibiting serum and tissue HMGB1. To test this hypothesis, we investigated the impact of sivelestat administration on serum and lung levels of HMGB1, and lung histopathology in rats with LPS-induced lung injury. To further elucidate the mechanism of this effect, we assessed the effect of sivelestat by measuring the activity of NF-κB in the lung tissue.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250–300 g (Kyudou, Saga, Japan) were used in all experiments. All protocols conformed to the National Institute of Health (NIH) 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*.

Anesthesia was induced by 4% sevofluran. Animals were randomly assigned to one of three groups: (1) LPS group, (2) sivelestat group, or (3) negative control group. In the LPS group, the rats received an infusion of 0.9% NaCl solution (1.0 ml/kg) into the intraperitoneal cavity 30 min prior to LPS administration. When LPS (7.5 mg/kg) was administered, the rats simultaneously received 0.9% NaCl solution (1.0 ml/kg) into the vein. The rats received an infusion of 0.9% NaCl solution (1.0 ml/kg) into the intraperitoneal cavity 1, 3 and 6 h following LPS administration. In the sivelestat group, the rats received an infusion of sivelestat (10 mg/kg) into the intraperitoneal cavity 30 min prior to LPS administration. When LPS (7.5 mg/kg) was administered, the rats simultaneously received sivelestat (10 mg/kg) into the vein. The rats received an infusion of sivelestat (10 mg/kg) into the intraperitoneal cavity 1, 3 and 6 h following LPS administration. Sivelestat was donated by Ono Pharma Co. Ltd. (Osaka, Japan). In the negative control group, the rats received an infusion of 0.9% NaCl solution (1.0 ml/kg) into the intraperitoneal cavity a total of five times to correspond with injections in the other groups. Before and after surgery, animals in all groups had unlimited access to food and water.

Wet-to-dry Weight (*W/D*) Ratio

Animals were euthanized with an overdose of sodium pentobarbital 12 h following LPS treatment. After the administration of sodium pentobarbital, the chest was opened and the right ventricle was injected with 10 ml of phosphate-buffered saline (PBS) to wash blood cells from the pulmonary circulation. The lungs were removed, weighed, and then dried in an oven at 80°C for 48 h. The dried lungs were weighed again to calculate pulmonary wet/dry ratios.

Histological Analysis

Lung sections were stained with hematoxylin and eosin. A pathologist blinded to group assignment analyzed the samples and determined levels of lung injury according to Murakami's technique [23]. Briefly, 24 areas in the 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,

infiltration of inflammatory cells, and hemorrhaging. The mean score for each of the parameters was then used for analysis purposes.

Measurements of Cytokine and HMGB1 Secretions

HMGB1, IL-6 and TNF-alpha levels were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (HMGB1: Shino-Test Corporation, Tokyo, Japan; IL-6 and TNF-alpha: R&D Systems Inc, Minneapolis, MN). The absorbance at 450 nm was determined using an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA).

Nitrite/Nitrate Determination

In biological fluids NO is rapidly deactivated by oxidation to nitrite and nitrate. After collecting the blood samples by cardiac puncture, we detected NO via nitrite/nitrate. Serum nitrite/nitrate concentration in plasma was determined using commercial kits according to the manufacturers' protocols (R&D Systems Inc., Minneapolis, MN, USA). This kit measured nitrite/nitrate concentration using a modification of the Griess assay. The absorbance was measured at 540 nm. nitrite/nitrate concentration was calculated using a nitrite/nitrate standard curve and expressed as micromoles per liter.

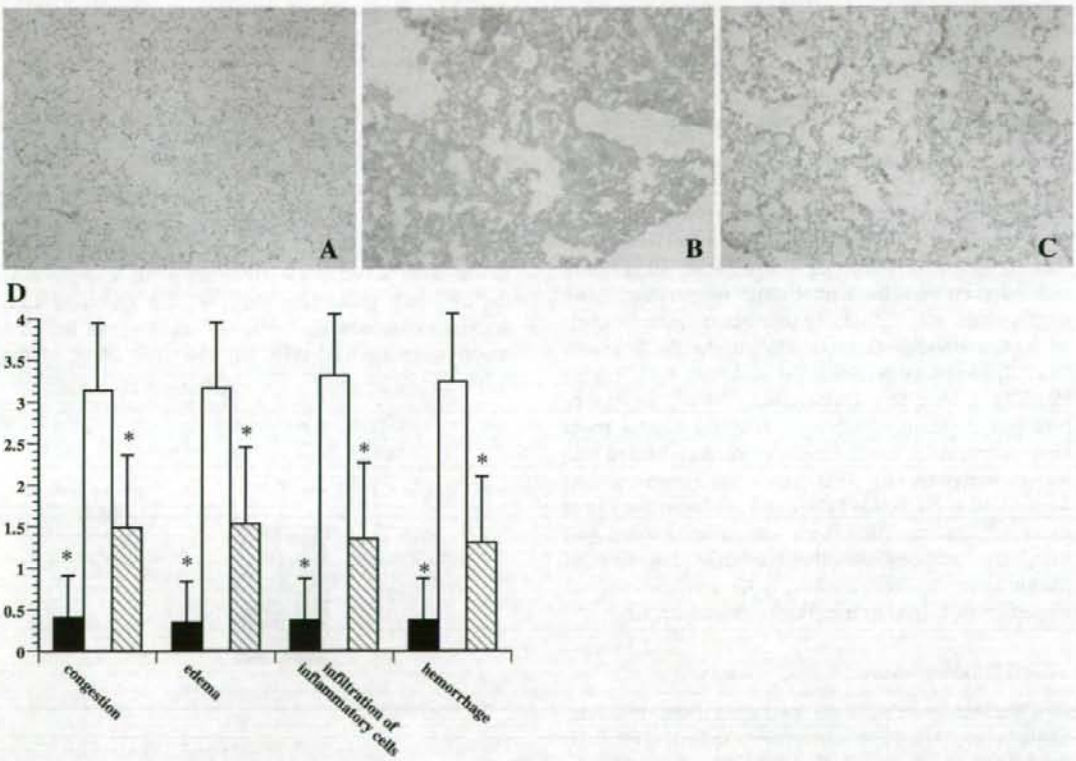

Fig. 1. Effects of sivelestat on lung injury in LPS-treated rats and changes in the lung histology score. Lung tissue was obtained from rats in the negative control group (**a**: magnification ×40), LPS group (**b**: magnification ×40), or sivelestat group (**c**: magnification ×40). Sections shown are stained with hematoxylin and eosin. **d** Histological changes were scored based on findings of congestion, edema, inflammation, and hemorrhage 12 h after treatment with LPS. *White bars* represent the negative control group, black bars represent animals administrated LPS only, and the *slashed bars* represent animals injected with sivelestat before LPS treatment. The data are expressed as the mean±SE. *Asterisk* denotes a significant difference compared with the LPS group ($p<0.05$).

Immunohistochemical Analysis

Tissue samples were fixed immediately in 4% paraformaldehyde and incubated at 4°C overnight, embedded in O.C.T. Compound (Sakura Finetechnical Co., Tokyo, Japan), and sectioned at 5 μm. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 and sodium azide (1 mg/ml) for 10 min followed by blocking of non-specific protein binding by 10% sheep serum for 10 min. Blocked sections were incubated with mouse anti-HMGB1 (Shino-Test Corporation, Tokyo, Japan) polyclonal antibody (1:1,000 dilution) overnight at 4°C. The sections were rinsed with PBS three times for 5 min and then were incubated with peroxidase-conjugated anti-mouse IgG. After three PBS rinses, the slides were stained using the biotin avidin peroxidase complex system from an LSAB2 kit (Dako, Via Real Carpinteria, CA, USA). Following development, slides were counterstained with Mayer's hematoxylin and mounted.

Western Blotting

Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel and transferred onto polyvinylidene difluoride sheets (Millipore, Bedford, MA). The membranes were washed with PBS containing 0.1% Tween 20 (PBS-T) and 5% skim milk for 1 hr at room temperature. After three washes with PBS-T, the membranes were incubated with antibodies (1:1000 dilution) to iNOS (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), HMGB1 (Shino-Test Corporation, Tokyo, Japan) or beta-actin (Abcam, Cambridge, UK) for 1 h at room temperature. After three washes, peroxidase-labeled secondary antibodies (ZYMED, South San Francisco, CA) were added at 1/1,000 in PBS-T and incubated for 1 hr at room temperature. Blots were subsequently developed using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK) and exposed on Hyperfilm ECL (Amersham, Buckinghamshire, UK).

NF-κB Binding Assay

Nuclear proteins were extracted from the lung tissue using NE-PER extraction reagents (PIERCE Biotechnology, Rockford, IL, USA). 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, USA). The absorbance at 450 nm was determined using an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical Analysis

All data were presented as the mean±standard error of the mean (SEM). Independent groups were compared using Mann–Whitney's *U*-test. Statistical analysis was performed using the Kruskal–Wallis test. A p value of <0.05 was considered to be statistically significant.

RESULTS

Effect of Sivelestat on the Lung in a Rat Model of LPS-Induced Septic Shock

In the negative control group, no pulmonary histological alterations were observed (Fig. 1a). Among the LPS affected rats, lung tissue specimens observed 12 h after the administration of LPS showed edema formation and interstitial infiltration by neutrophils and reduced the alveolar spaces (Fig. 1b). Sivelestat treatment significantly reduced the interstitial edema and inflammatory cell infiltration in comparison to the LPS group (Fig. 1c). Histology scores, based on the number of areas with congestion, edema, inflammation, and hemorrhaging, were all significantly higher in the LPS group than in the negative control group (Fig. 1d). All scores were lower in the sivelestat group compared to the LPS only group (Fig. 1d). At 12 h following LPS administration, the *W/D* ratios of the lungs in the LPS group were elevated (Fig. 2). The *W/D* ratios of the

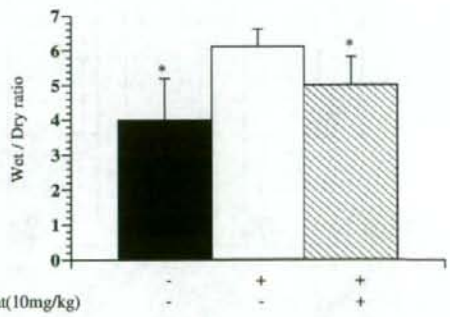

Fig. 2. Effects of sivelestat on lung wet-to-dry weight (*W/D*) ratio of LPS-treated rats. The lung *W/D* ratio 6 h following LPS administration is shown for each group ($n=6$ for each group). The *white bar* represents animals in the negative control group, the *black bar* represents animals administrated LPS only, and the *slashed bar* represents animals injected with sivelestat before LPS treatment. The data are expressed as the mean±SE. *Asterisk* denotes a significant difference compared with the LPS group ($p<0.05$).

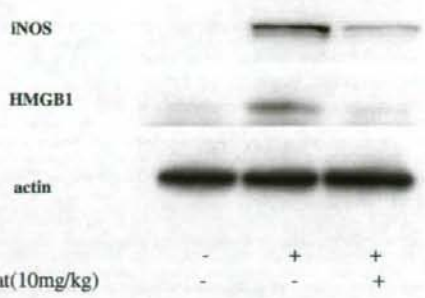

Fig. 3. Changes in iNOS and HMGB1 protein expression in rat lung tissue following LPS treatment. Rats were given either saline (LPS group) or sivelestat (sivelestat group) prior to the administration of LPS. The expression of iNOS or HMGB1 in lung tissue 12 h following LPS treatment in both groups was detected by Western blot.

lungs in the sivelestat group were significantly lower than those in the LPS group and were comparable to the values of the control animals (Fig. 2).

Measurement of Serum Nitrite/Nitrate

Nitrite/nitrate are metabolic products of NO and often used as markers to indicate NO formation. Nitrite/nitrate concentration was measured in plasma 0, 3, 6, 9, 12 and 24 h following LPS treatment (Fig. 4). After LPS

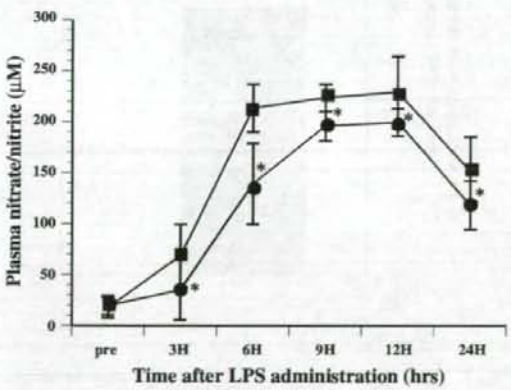

Fig. 4. Effect of LPS administration on serum levels of nitrite/nitrate. Serum concentration of nitrite/nitrate was measured 0, 3, 6, 9, 12, and 24 h following LPS administration (7.5 mg/kg, I.V., $n=6$ for each group). The LPS group is represented by squares and the sivelestat group is represented by circles. All data are expressed as the mean±SE. *Asterisk* statistically significant ($p<0.05$) with respect to the LPS group.

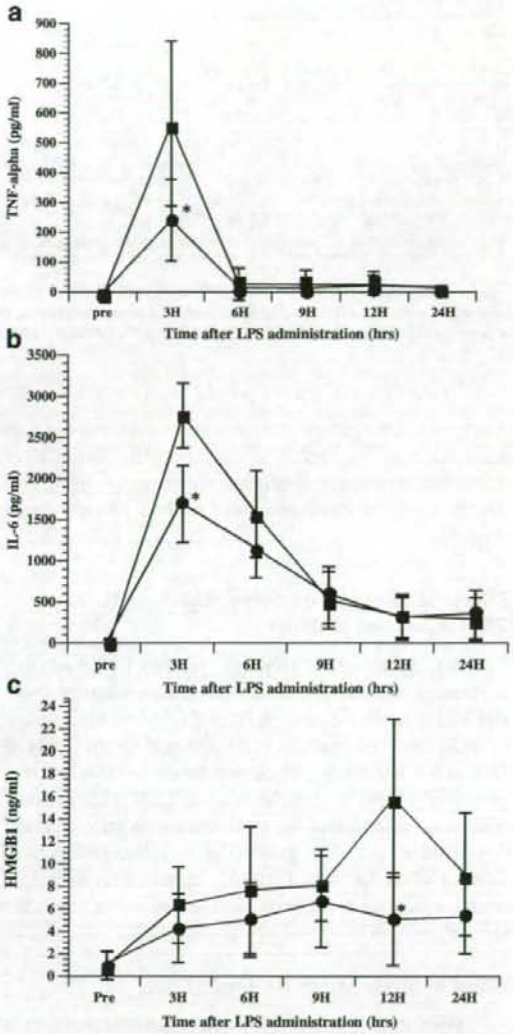

Fig. 5. Temporal changes in serum TNF-alpha, IL-6, and HMGB1 concentrations following LPS treatment in rats. Rats were given either saline (LPS group), or sivelestat (sivelestat group) prior to the administration of LPS. Serum concentrations pre-treatment and 3, 6, 9, 12, and 24 h following LPS treatment are shown for TNF-alpha (**a**), IL-6 (**b**), and HMGB1 (**c**). The LPS group is shown with *circle symbols* ($n=6$) and the sivelestat group is shown with *square symbols* ($n=6$). All data are expressed as the mean±SE. *Asterisk* denotes a significant difference compared with the LPS group ($p<0.05$).

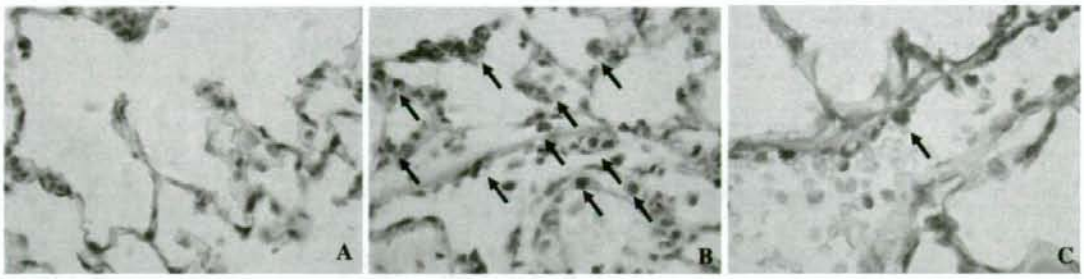

Fig. 6. Changes in HMGB1 protein expression in rat lung tissue following LPS treatment. Immunohistochemical analysis to detect HMGB1 in lungs from animals killed 12 h following intravenous saline administration; (**a**, magnification ×400), 12 h following LPS treatment (**b**, magnification ×400) or from animals treated with sivelestat and killed 12 h following LPS treatment (**c**, magnification ×400). *Arrows* point to positive cells.

administration, the levels of serum nitrite/nitrate were increased. Sivelestat treatment significantly decreased the nitrite/nitrate levels compared to the LPS only group (Fig. 4).

Effects of Sivelestat on Serum Levels of IL-6, TNF-alpha, and HMGB1

Administration of sivelestat prior to LPS treatment in rats significantly decreased the concentration of TNF-alpha at 3 h post-treatment (Fig. 5a). Similarly, groups treated with sivelestat showed decreased serum levels of IL-6 at 6 h later (Fig. 5b). Serum levels of HMGB1 also increased after the administration of LPS. This increase was less prominent in rats treated with sivelestat compared to the LPS group (Fig. 5c). Serum levels of IL-6, TNF-alpha and HMGB1 remained constant in negative control group rats at all assay times (data not shown).

Effect of Sivelestat on the Lung Tissue

Western blot analysis showed that the expression of HMGB1 in the lung tissue increased 12 hr following LPS administration. This increase was less pronounced among sivelestat treated rats compared with the LPS group (Fig. 3). At the same time point, immunohistochemical analysis revealed that cells expressing HMGB1 increased following LPS treatment (Fig. 6b). In contrast, the percentage of cells expressing HMGB1 did not significantly increase in the rats receiving sivelestat prior to LPS treatment (Fig. 6c). Similar to the expression of HMGB1, the expression of iNOS in the lung tissue increased as a result of LPS treatment. This increase was also less pronounced among sivelestat treated rats compared with the LPS group (Fig. 3). Administration of LPS led to a robust activation of the NF-κB transcription factor p50/p65. However, administration of sivelestat partially suppressed this increase (Fig. 7).

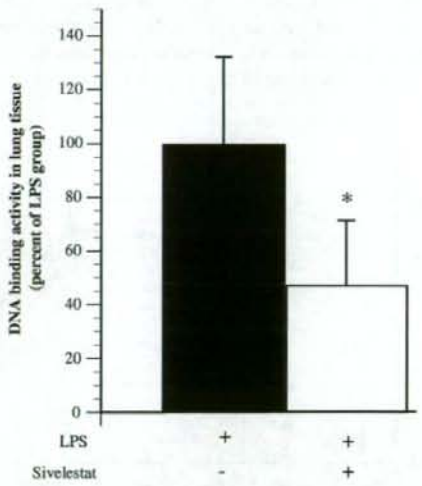

Fig. 7. Effect of sivelestat on the LPS-induced increase in specific binding of p50 and p65 to DNA. Nuclear fractions were harvested 6 h following LPS treatment from rats with or without sivelestat administration (n=6 for each group). The DNA binding activity assay showed a marked decrease in the p50/p65 binding activity in nuclear fractions from lung tissue cells in the sivelestat group. All data are expressed as the mean±SE. *Asterisk* denotes a significant difference compared with LPS group (p<0.05).

DISCUSSION

In this study, we demonstrated that the administration of sivelestat significantly reduced the lung injury and wet/dry ratio of lung tissue following LPS treatment (Figs. 1 and 2). Acute lung injury in acute respiratory distress syndrome (ARDS) is considered an acute inflammatory condition by many clinicians. Although the pathology of this syndrome is poorly defined, recent evidence suggests that neutrophil elastase (NE) plays a role in the development of acute lung injury, which is characterized by increased permeability and resulting pulmonary edema [5, 24].

Inducible NOS (iNOS) is typically activated only in systemic inflammatory conditions, including septic shock. The up-regulation of iNOS is mediated by specific proinflammatory agents, such as TNF-alpha [25]. Increased expression of iNOS induces nitric oxide (NO) release, leading to organ damage. NO is believed to play a key role in inflammation, and has been implicated in a wide range of disease processes [25]. In this study, we demonstrated that iNOS and nitrate/nitrate were significantly increased in the LPS-induced lung injury model. Treatment with sivelestat, however, decreased these mediators (Figs. 3 and 4). These results suggest that improvement of LPS-induced lung injury by treatment with sivelestat might be related to a decrease in NO.

Our findings showed that serum levels of cytokines and HMGB1 were significantly inhibited by the administration of sivelestat prior to LPS treatment (Fig. 5a–c). Several cytokines, including TNF-alpha and IL-6, can cause neutrophil-mediated lung tissue injury [26]. The significant decrease in lung injury among rats treated with sivelestat, therefore, may be partially attributable to decreased levels of these pro-inflammatory cytokines. Our findings suggested that sivelestat may inhibit not only serum cytokine levels, but also levels of HMGB1 in both serum and lung tissue (Figs. 3 and 5c). This is the first report of sivelestat administration inhibiting HMGB1 in a rat model of sepsis. These results build on the growing body of evidence suggesting the importance of HMGB1 in the initiation of acute lung injury [13].

Various pathways have been identified as potential mechanisms behind the inhibition of HMGB1 secretion. Stimulation of multiple cytokines induces the release of HMGB1 [23]. In particular, evidence supports a potential role for TNF-alpha and NO in the regulation of LPS-induced HMGB1 release [23, 27]. In the present study, the TNF-alpha and IL-6 levels in the serum were significantly lower in the sivelestat-treated group than in the LPS group. The decrease of TNF-alpha and NO in the sivelestat group suggests that the inhibition of cytokine and NO secretion by sivelestat in systemic inflammation mediates the inhibition of HMGB1.

We demonstrated that sivelestat inhibited activation of nuclear factor κB (NF-κB) in rat lung tissue in the LPS-induced lung injury model. In acute lung injury, multiple signaling pathways lead to cytokine synthesis and release of HMGB1. Systemic inflammation, such as septic shock, also results in activation of various signaling pathways [29, 30]. Significantly, these signaling pathways induce activation of the transcription factor NF-κB, a central regulator of inflammation, cytokine synthesis and stress responses in many cell types, including macrophages [29, 31, 32]. In patients with septic lung injury, NF-κB activity in alveolar macrophages has been shown to be significantly increased [28]. Because NE activates NF-κB in LPS-induced lung injury [33], sivelestat might reduce NF-κB activation in lung injury through inhibition of NE (Figs. 6 and 7).

In a rat model of LPS-induced lung injury, sivelestat administration at 10 mg/kg can prevent acute lung injury. Our results suggest that sivelestat might therapeutically benefit septic patients not only through inhibition of HMGB1, but also through inhibition of inflammatory responses in systemic inflammatory conditions. Further research will be necessary to assess the effect of delayed sivelestat administration in LPS-induced septic shock models.

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High Mobility Group Box 1 Induces a Negative Inotropic Effect on the Left Ventricle in an Isolated Rat Heart Model of Septic Shock

— A Pilot Study —

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Tomoko Uchino, MD; Takayuki Noguchi, MD

Background Sepsis can be exacerbated by an inappropriate immune response and the severe impact of this disease on the cardiovascular system is well documented. High mobility group box 1 (HMGB1) protein is an important mediator in the pathogenesis of sepsis and its role in cardiovascular system dysfunction was investigated in an lipopolysaccharide (LPS)-induced rat model of sepsis.

Methods and Results Twelve hours after intravenous bolus injections of LPS (5 mg/kg), rats were killed and heart samples were harvested. Immunoblot analysis was performed to assess expression levels of HMGB1 in cardiac myocytes. Left ventricular developed pressure (LVDP) served as a measure of systolic function. LPS administration was associated with an increase in the expression of HMGB1 in cardiac myocytes and a decrease in cardiac function. Hearts from the LPS-treated rats were also perfused with recombinant HMGB1 and cardiac function measured. The dose-dependent effects observed with elevated HMGB1 included decreased LVDP, decreased left ventricular (LV) $+dP/dt_{max}$, decreased absolute value of LV $-dP/dt_{min}$, and increased LV end-diastolic pressure.

Conclusions HMGB1 stimulation produces a negative inotropic effect during septic shock, suggesting an important role for this molecule in cardiovascular system dysfunction during sepsis. (*Circ J* 2008; **72:** 1012–1017)

Key Words: Cardiac function; HMGB1; Negative inotropic; Sepsis

Mortality associated with septic shock is thought to be a consequence of severe derangements in the cardiovascular system,[1] with myocardial dysfunction playing a primary role.[2] Indeed, reduced cardiac contractile function has been observed both in septic patients[3,4] and experimental animal models of lipopolysaccharide (LPS)-induced sepsis.[5] The cellular and molecular mechanisms responsible for these effects, however, remain incompletely understood.

Recent studies in rat and mouse models of sepsis have partially elucidated these mechanisms. One key factor that has emerged is high mobility group box 1 (HMGB1) protein, which serves as a late-phase mediator in the pathogenesis of sepsis. HMGB1 is an intranuclear protein that was originally identified as an important gene regulator.[6] Following the activation of the inflammation cascade, HMGB1 is released from necrotic damaged cells and secreted by activated monocytes and macrophages. Its mechanism of action is presumably via its affinity for receptors for advanced glycation end-products, Toll-like receptor 2 and Toll-like receptor 4. HMGB1 binding to its receptors activates intracellular signaling pathways, such as the NF-kB pathway, which induce downstream cytokine release,[7] thereby enhancing the inflammatory response.[8,9]

LPS, a cell membrane component shed from Gram-negative bacteria, is a key mediator of septicemia and its pathogenic effects on the heart[10] but exactly how this endotoxin causes cardiac dysfunction remains largely unclear. We hypothesized that HMGB1 might be a key mediator in the pathogenesis of cardiac dysfunction and to test this, we exposed hearts from LPS-treated rats to recombinant HMGB1 protein.

Methods

Materials

Recombinant HMGB1 was purchased from Shino-Test Corporation (Tokyo, Japan) and LPS (O127:B8) was obtained from Sigma (St Louis, MO, USA). All reagents were of the highest available analytical grade.

Animals

Male Wistar rats weighing 250–300 g (Kyudou, Saga, Japan) were used in all experiments. All rats received humane care in compliance with the National Institute of Health (NIH) guidelines and the Principles of Laboratory Animal Care. All animals were housed with free access to food and water.

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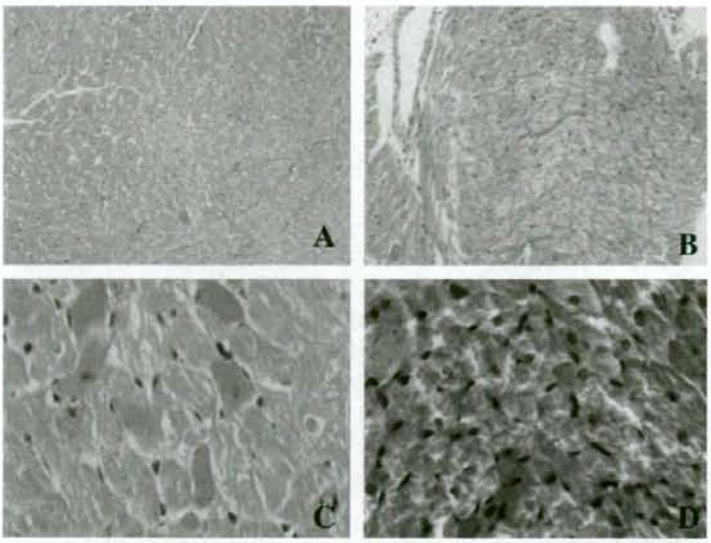

Fig 1. Effects of lipopolysaccharide (LPS) treatment on the gross histopathology of cardiac tissue. At 12 h after LPS injection, cardiac tissue from the control (A: ×100, C: ×400; H&E) and LPS groups (B: ×100, D: ×400; H&E) was analyzed and no gross changes were found in either group.

Experimental Protocols

The rats were randomly assigned to 1 of 2 treatment groups. The 6 animals in the control group received an intravenous bolus injection of 0.9% NaCl solution alone. The 6 animals in the LPS group received an intravenous bolus injection of LPS dissolved in 0.9% NaCl solution (5 mg/kg)[11] All rats were killed under general 2% sevofluren anesthesia 12 h following injection. Tissue specimens were immediately removed surgically and processed.

Histological Analysis

Heart tissue specimens were instilled with 10% formalin. The samples were embedded in paraffin and cut into 4-μm sections for staining with hematoxylin and eosin.

Immunoblotting Analysis

The hearts were harvested from all animals. After the blood was washed out using saline perfusion, the heart was homogenized with a T-PER (Tissue Protein Extraction Reagent: Pierce, Rockford, IL, USA) in a polytron homogenator (IKA Labortechnik, Staufen, Germany). The homogenates were then centrifuged at 10,000 g for 5 min at 4°C. The concentration of protein in the collected supernatant was measured by absorbance at 562 nm using the BCA Protein Assay Regent system (Pierce).

For gel electrophoresis, equal quantities of protein (100μg) were suspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) buffer. Protein samples were boiled for 1 min and separated using a 10% SDS-polyacrylamide gel. Protein runs using SDS-PAGE were immediately electrotransferred to polyvinylidene difluoride membrane (PVDF) (Millipore, Bedford, MA, USA) at 60 V for 3 h in a wet transfer system containing 20 mmol/L Tris-HCl/0.2 mol/L glycine in 20% methanol as the transfer buffer. The membrane was blocked with 5% nonfat dry milk in Tris/Tween buffer [25 mmol/L Tris-HCl; 2% Tween 20 (Bio-Rad Lab, Hercules, CA, USA); 0.14 mol/L NaCl] overnight at 4°C.

Antibodies to HMGB1 (Shino-Test Corporation) or β-actin (Abcam, Cambridge, UK) were used as the primary antibody. The immune serum was diluted 500-fold with 1% nonfat dry milk and incubated with gentle shaking for 1 h. The PVDF membrane was then rinsed 3 times with TBS/Tween buffer for 10 min. The secondary antibody was diluted 1,000-fold with 1% nonfat dry milk and incubated with the membrane for 1 h. The blot was then washed by rinsing with TBS/Tween 3 times for 10 min. The membrane was treated with enhanced chemiluminescence reagent (Amersham, Buckinghamshire, UK) and then exposed to X-ray film. After scanning the X-ray film, the band concentration was calculated by quantification of the integrated optical density using NIH Image J software package (NIH, Bethesda, MD, USA).

Measurement of HMGB1 Secretion

Serum samples were assayed using the ELISA sandwich method and 96-well plates that were precoated with monoclonal antibodies specific to rat HMGB1 (Shino-Test Corporation). The samples, negative controls, and diluted HMGB1 standard markers were added to each well. Detection of HMGB1 in the samples was performed according to the manufacturer's protocols. The A450 values were determined using an ELISA reader.

Isolated Heart Perfusion and Assessment of Cardiac Function

Cardiac function was determined by a modified isovolumetric Langendorff technique as described elsewhere[12–14] and expressed as left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), left ventricular (LV)+dP/dt$_{max}$, and LV−dP/dt$_{max}$. At the termination of the experiments, beating hearts were rapidly excised into oxygenated Krebs-Henseleit solution containing 11 mmol/L glucose, 2.0 mmol/L $CaCl_2$, 4.3 mmol/L KCl, 25 mmol/L $NaHCO_3$, 118 mmol/L NaCl, 1.2 mmol/L $MgSO_4$, and 1.2 mmol/L KH_2PO_4. Normothermic retrograde perfusion was performed with this solution in an isovolumetric and nonrecirculating mode. The perfusion buffer was saturated with a gas mixture of 95% O_2–5% CO_2 at a pH of 7.4. Perfusion pressure was maintained at 75 mmHg.

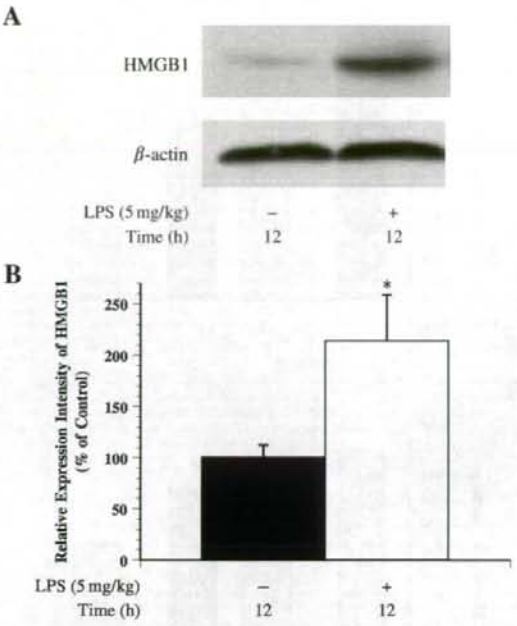

Fig 2. Effects of LPS treatment on high mobility group box 1 (HMGB1) expression in cardiac tissue. (A) Immunoblots showing HMGB1 and β-actin protein expression in the heart tissue from a representative control or LPS animal. (B) Signal intensities for the density of HMGB1 protein immunoblot bands (n=4 for each group). HMGB1 protein levels were quantified using an image analyzer and normalized to β-actin levels. Combined expression intensities of HMGB1 are shown as percentages of the control. Data are mean± standard error of the mean (SEM). *p<0.05 relative to the control group. See Fig 1 for other abbreviation.

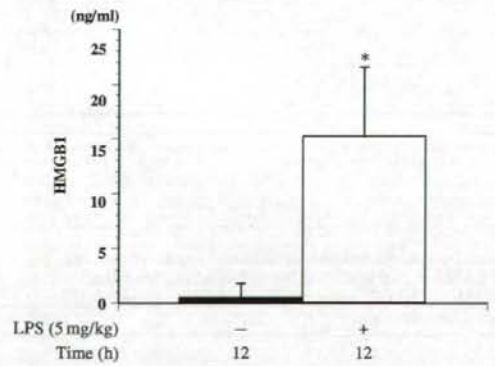

Fig 3. Differences in HMGB1 serum concentration between control (white bar; n=6) and LPS rats (black; n=6). All data are mean± SEM. An asterisk denotes a difference vs administration of LPS (p<0.05). See Figs 1,2 for abbreviations.

A latex balloon was inserted through the left atrium into the left ventricle, and filled with water (0.18–0.28 ml). LVDP, LVEDP, +dP/dtmax, and –dP/dtmax were continuously recorded with a computerized pressure amplifier-digitizer (BIOPAC Systems, Inc, Goleta, CA, USA). After 20 min of perfusion (to reach equilibrium), the inotropic effects were measured in the presence of varying concentrations of recombinant HMGB1 added to the perfusion solution. Myocardial temperature was maintained at 37°C through the circulation of warm water.

Statistical Analysis

All descriptive data are presented as the means ± standard error of the mean. The data were analyzed by repeated measurement ANOVA for multiple comparisons, and by unpaired t-test for single comparisons. A p-value <0.05 was considered to be statistically significant.

Results

Cardiac Tissue Pathology

We investigated whether LPS-induced sepsis would affect cardiac histopathology. No histological alterations were observed, however, in either the control or the LPS-treated group (Figs 1A–D).

HMGB1 Expression in Cardiac Tissue and Serum

We next examined whether LPS treatment would affect the levels of HMGB1 in cardiac tissue and serum. At 12 h after LPS treatment, heart tissue was isolated and analyzed by immunoblotting. Levels of HMGB1 protein in tissue from LPS-treated animals were at least 2-fold higher than those in the control group (Figs 2A,B). Prior to LPS administration, HMGB1 was barely detectable in the sera of rats from either group. Following LPS injection, HMGB1 levels increased markedly, so that by 12 h post-injection the expression was higher than in the control group (Fig 3).

Isolated Heart Perfusion and General Effects of LPS Treatment

Heart rate (HR) did not differ between the control and LPS groups (Fig 4A), but LPS group animals demonstrated a higher LVEDP at 12 h after exposure to LPS compared with the control groups (Fig 4B). As shown in Fig 4C, hearts subjected to LPS exhibited a significant impairment of LVDP. LV+dP/dtmax, the rate of pressure rise during contraction (Fig 4D), was lower in the LPS group than in the control group ($p<0.05$). LV–dP/dtmin, the maximum rate of relaxation (Fig 4E), was also lower in the LPS group ($p<0.05$). Twelve hours after exposure to LPS, the isolated hearts demonstrated significantly lower LVDPs.

Effect of HMGB1 on Myocardial Function in Control Animals

Perfusion with 0.1 μg/ml HMGB1 had no apparent effect on HR, LVEDP, LVDP, LV+dP/dtmax, or LV–dP/dtmin (data not shown). We then investigated the effect of 1 and 10 μg/ml HMGB1 on cardiac performance in the control animals. Perfusion with 10 μg/ml HMGB1 had no effect on HR (Fig 4A), LVEDP (Fig 4B) or LV–dP/dtmin (Fig 4E). It did, however lead to small decreases in LVDP (Fig 4C) and LV+dP/dtmax (Fig 4D), although these changes were not significantly different.

Effect of Combined LPS Treatment With HMGB1 on Myocardial Function

After LPS treatment, hearts were removed and perfused with HMGB1 for a period of 10 min and cardiac function was measured. Perfusion with 1 or 10 μg/ml HMGB1 resulted in no changes in HR (Fig 4A). Perfusion of 0.1 μg/ml HMGB1 had no effect on HR, LVDP, LVEDP, LV+dP/

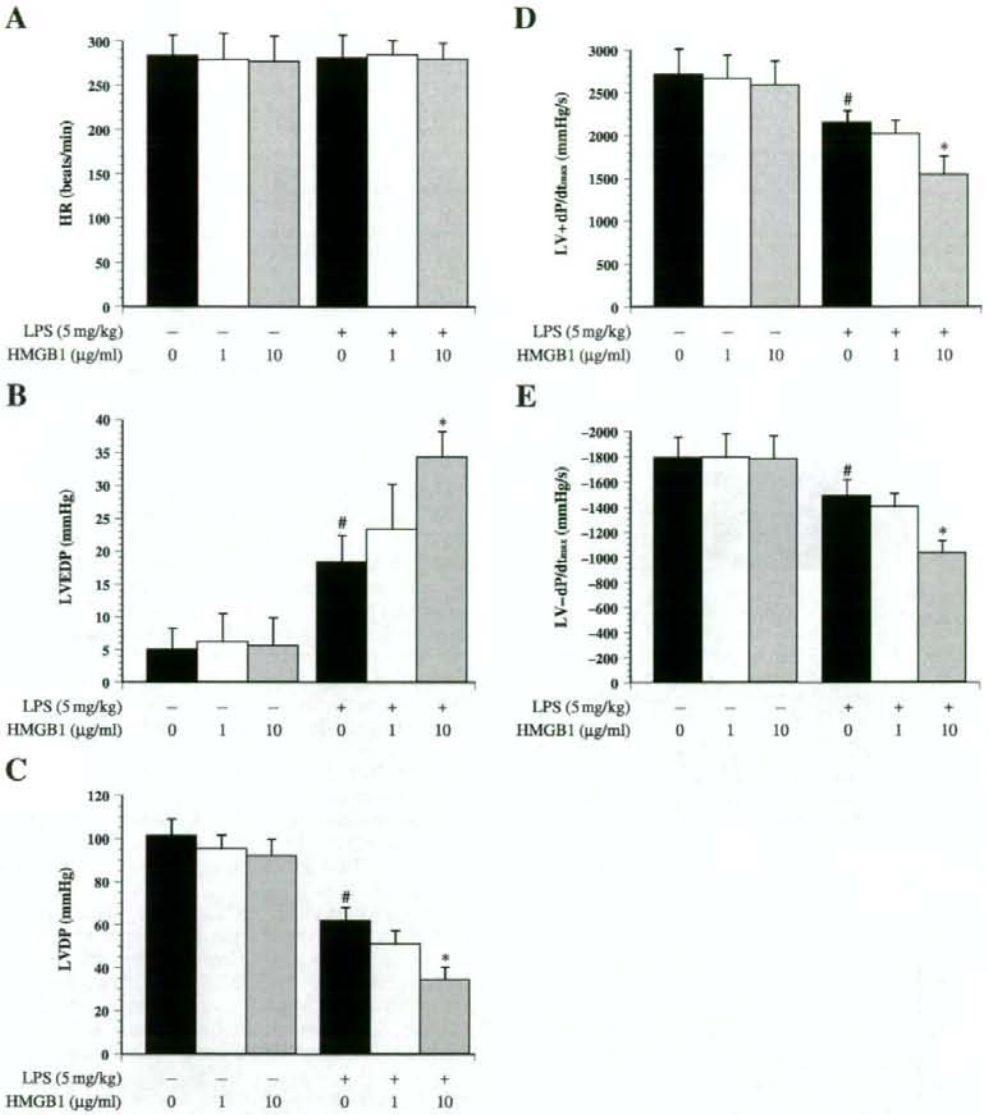

Fig 4. Effects of LPS and HMGB1 treatment on heart function in rats from a LPS-induced sepsis model. (A) Heart rate, (B) LVEDP, (C) LVDP, (D) +dP/dtmax, (E) −dP/dtmin. Data are mean ± SEM from 6 animals in the control and LPS-treated groups. *p<0.05 vs LPS (+) HMGB1 (0). #p<0.05 vs LPS (−) HMGB1 (0). LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure. See Figs 1,2 for other abbreviations.

dt_{max} or LV–dP/dt_{min} (data not shown). Hearts perfused with HMGB1 at concentrations of 1 or 10μg/ml, however, showed significantly increased LVEDP in the LPS group, compared with the control group (Fig 4B). In contrast, hearts perfused with HMGB1 at concentrations of 1 or 10μg/ml showed markedly decreased LVDP (Fig 4C), LV+dP/dt_{max} (Fig 4D), and LV–dP/dt_{min} (Fig 4E), relative to the control group. These observed effects on cardiac function occurred in an HMGB1 dose-dependent manner. We next examined the effect of 10μg/ml HMGB1 on hemodynamic parameters over time. All of the previously described effects were apparent within 1 min after exposure and remained relatively stable throughout the 10-min period of exposure (Figs 5A–D). After 10 min, HMGB1 was washed from the perfused hearts and all parameters immediately returned to baseline.

Discussion

Our results suggest that HMGB1 played an important role in causing decreased myocardial activity in a rat model of septic shock. Within 12 h of administration, LPS caused a significant increase in the level of HMGB1 in serum and cardiac tissue. In addition, recombinant HMGB1 repro-