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# Nafamostat mesilate inhibits the expression of HMGB1 in lipopolysaccharide-induced acute lung injury

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**Abstract**
*Purpose.* High mobility group box 1 (HMGB1) protein has recently been shown to be an important late mediator of acute lung injury and a promising therapeutic target. Nafamostat mesilate (NM) is a broad-range synthetic protease inhibitor with some anti-inflammatory action. The purpose of this study was to evaluate the effect of NM on HMGB1 in lipopolysaccharide (LPS)-induced lung injury in rats.
*Methods.* Male Wistar rats were given either saline (LPS group) or NM (NM+LPS group) 30 min before the intravenous injection of a bolus of LPS (5 mg·kg$^{-1}$). After the administration of LPS, injury to the lung and the expression of HMGB1, tumor necrosis factor-α (TNF-α), and plasminogen activator inhibitor-1 (PAI-1) were examined.
*Results.* Histological examination revealed that interstitial edema, leukocytic infiltration, and HMGB1 protein expression were markedly reduced in the NM+LPS group compared to the LPS group. Furthermore, the LPS-induced increases in PAI-1 activity and in plasma TNF-α concentrations were also lower in the rats given both NM and LPS than in the rats given LPS alone.
*Conclusions.* The anticoagulatory activity of NM may have inhibited PAI-1, while its anti-inflammatory activity blockaded TNF-α, thereby indirectly inhibiting HMGB1 and reducing tissue damage in the lung. These findings indicate that NM can inhibit the lung injury induced by LPS in rats. NM is an excellent candidate for use in new therapeutic strategies to prevent or minimize lung injury.

**Key words** LPS · Lung injury · Sepsis · Acute respiratory distress syndrome · Anticoagulant

## Introduction

Sepsis affects 18 million people every year, and with a mortality rate of almost 30% it is a leading cause of death worldwide. Sepsis remains a serious cause of morbidity and mortality worldwide [1–3]. Complications such as disseminated intravascular coagulation and acute respiratory distress syndrome remain refractory to therapy despite recent advances in therapeutic methods. Because sepsis is believed to be caused by an excessive inflammatory response, numerous clinical trials of anti-inflammatory strategies for the treatment of sepsis have been conducted over the past several decades. However, given that the activity of the immune system varies over the course of the disease, an anti-inflammatory strategy may not be helpful, and could even be harmful, to certain patients [4].

Recently, it has been demonstrated that the high mobility group box 1 (HMGB1) protein plays a key role as a late-phase mediator of lipopolysaccharide (LPS) lethality and systemic inflammation [5]. HMGB1 is an intranuclear protein that was originally identified as an important factor in the regulation of gene transcription [6]. In mice, serum concentrations of HMGB1 rise within 8 to 32 h after the administration of LPS [7], and the systemic administration of HMGB1 is lethal [7]. Once released from necrotic cells, or secreted by activated monocytes or macrophages, HMGB1 can bind to receptors for advanced glycation endproducts and signal through them. HMGB1 can stimulate the release of cytokines [7], and conversely, cytokines can control the further release of HMGB1 to the extracellular space [8]. Anti-HMGB1 antibodies are protective against LPS lethality in mice, even when they are administered after tumor necrosis factor-α (TNF-α) has passed its peak concentration [7]. Elevated concentrations of HMGB1 have been observed in the serum of patients who were septic, with the highest concentrations in nonsurvivors [7]. In recent studies, HMGB1 has been implicated in the activation of human monocytes [9], the induction of adhesion molecules in endothelial cells [10], and acute lung injury [11]. These observations suggest that HMGB1 is a key mediator of cell injury and that inhibiting it may improve clinical outcomes.

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The serine protease inhibitor nafamostat mesilate (6-amino-2-naphthyl *p*-guanidinobenzoate dimethansulfonate; NM) shows various modes of biological activity, and is capable of blocking a number of steps in the inflammatory process and the coagulation system [12–14]. Its primary activity is anticoagulatory, via the blockade of thrombin and active coagulation factors, but NM may also improve the disease profile during shock. Indeed, Uchiba et al. [15] showed that NM attenuated the pulmonary vascular injury and inhibited the rise in total serum complement hemolytic activity induced by endotoxin. Although these findings suggest that the protective effect of NM on lung injury is, in part, due to the inhibition of C5/C3 convertase activity, direct evidence is lacking. Therefore, to further investigate the mechanism by which NM reduces endotoxin-induced lung injury, we examined the effect of NM on HMGB1 production in the lungs of rats exposed to endotoxin.

## Methods

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

Animals were randomly assigned to one of three groups: (1) LPS-group rats ($n = 15$) were injected intraperitoneally with 0.9% NaCl solution 30 min before the intravenous injection of a bolus of LPS (5 mg·kg$^{-1}$) into the tail vein; (2) NM+LPS-group rats ($n = 15$) received an intraperitoneal injection of NM (1 mg·kg$^{-1}$) 30 min before the intravenous injection of a bolus of LPS (5 mg·kg$^{-1}$) into the tail vein; and (3) control-group rats ($n = 15$) were given an intraperitoneal injection of 0.9% NaCl solution 30 min before the intravenous injection of a bolus of 0.9% NaCl solution into the tail vein. Animals were killed at 6, 12, and 24 h after the intravenous injection. After the rats had been anesthetized with sevoflurane, their vena cavae were clipped, their aortas transected, and normal saline was injected into the atria of their hearts. Lung tissue specimens were quickly removed and processed as indicated below.

### *Histological analysis*

Left lungs were obtained, with the rats under sevoflurane anesthesia, from animal's in the control, LPS, and NM+LPS groups 12 h after the administration of LPS. The lung tissue specimens were treated with 10% formalin. Samples were embedded in paraffin and cut into 4-μm sections. The sections were stained with hematoxylin and eosin.

### *Measurement of TNF-α secretion*

A silicone catheter was inserted into the animal's right jugular vein under sevoflurane anesthesia and samples of venous blood (0.2 ml) were obtained from the left external jugular vein at various time points (0 h, 1 h, 3 h, 6 h, 9 h, and 12 h) after the administration of LPS. Serum TNF-α production was assayed using an enzyme linked immunosorbent assay (ELISA) sandwich method (R&D Systems, Minneapolis, MN, USA). TNF-α was detected in the serum samples using the manufacturer's protocol. A450 values were determined using an ELISA reader.

### *Reverse transcription-polymerase chain reaction (RT-PCR)*

Total RNA was extracted from the lungs using the acid guanidinium thiocyanate–phenol–chloroform method. RNA concentrations were determined using a spectrophotometer (DU640; Beckman, Fullerton, CA, USA) at 260 nm. RT-PCR was used to quantitatively assess changes in plasminogen activator inhibitor-1 (PAI-1) RNA. First-strand cDNA synthesis was done using a commercial RT-PCR kit (Toyobo, Tokyo, Japan), in a 50-μl reaction mixture containing 1 μg of total cellular RNA. PCR was also carried out using the RT-PCR kit, and a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT, USA) under suitable conditions for PAI-1 amplification. The primers and conditions used for each mRNA were as follows. Primers for PAI-1 were 5′AT GAGATCAGTACTGCGGACGCCA and 5′GCAC GGAGATGGTGCTACCATCAGACTTGT, and PCR conditions were 35 cycles of 1-min denaturation at 94°C, 1-min annealing at 74°C, and 2-min extension at 72°C. Primers for β-actin were 5′GTTCCGATGCCCC GAGGATCT and 5′GCATTTGCGGTGCACGAT GGA; and PCR conditions were 30 cycles of 1-min denaturation at 94°C, 1-min annealing at 60°C, and 1-min extension at 72°C. The lengths of PCR products were determined by 1.5% agarose gel electrophoresis, followed by staining with ethidium bromide, and recorded using an FP-6000 system (Funakoshi, Tokyo, Japan). Quantification of mRNA bands was performed by densitometry, using the National Institutes of Health (NIH) Image program. The intensity of the PCR product of the target gene was normalized to the intensity of β-actin.

*Western blots for plasminogen activator inhibitor-1 quantification*

Proteins were extracted from the lungs using tissue protein extraction reagent (T-PER, Pierce Biotechnology, Rockford, IL, USA). Lung tissue specimens were homogenized with T-PER. The homogenates were boiled for 5 min before dithiothreitol was added. Proteins were subjected to sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) in a 12% gel, and transferred onto polyvinylidine fluoride (PVDF) sheets (Millipore, Bedford, MA, USA). Membranes were washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T) and 5% skim milk for 1 h at room temperature. Following three washes with PBS-T, membranes were incubated with PAI-1 (Santa Cruz, CA, USA) at 1:1000 in PBS-T for 1 h at room temperature. After three further washes, peroxidase-labeled goat anti-rabbit IgG polyclonal antibody (Zymed, South San Francisco, CA, USA) was added, at 1:1000, to PBS-T, and incubated for 1 h at room temperature. After three final washes, the blots were developed using Enhanced Chemiluminescence (ECL) reagents (Amersham, Buckinghamshire, UK) and were exposed to Hyperfilm ECL (Amersham).

*Immunohistochemical analysis*

Left lungs were obtained from animals in the control, LPS, and NM+LPS groups under sevoflurane anesthesia at 12 h. The tissue samples were fixed immediately in 4% paraformaldehyde at 4ºC overnight, embedded in OCT Compound (Sakura Finetechnical, Tokyo Japan), and cut into 5-μm sections. Immunohistochemistry was performed after endogenous peroxidase activity was blocked with 0.3% $H_2O_2$ and sodium azide (1 mg·ml$^{-1}$) for 10 min, and nonspecific protein binding was blocked with 10% sheep serum for 10 min. The blocked sections were incubated with anti-HMGB1 polyclonal antibody (1:1000 dilution) overnight at 4ºC. The sections were then rinsed three times, for 5 min each time, with PBS and were then incubated with peroxidase-conjugated anti-mouse IgG. Following three rinses of 5 min each with PBS, the slides were stained using an LSAB2 kit (Dako, Carpinteria, CA, USA), including the biotin-avidin-peroxidase complex system. After development, the slides were counterstained with Mayer's hematoxylin and mounted.

*Statistical analysis*

All data values are presented as means ± SE of the means. The data were analyzed by analysis of variance and Scheffe's post-hoc test for multiple comparisons of paired groups, and by an unpaired *t*-test for single comparisons. A $P$ value of less than 0.05 was considered to be statistically significant.

## Results

*Effect of NM on lung tissues*

Microscopic observation of lung tissue samples taken 12 h after LPS administration showed interstitial edema and infiltration by neutrophils in both the LPS (Fig. 1 C,D) and the NM+LPS (Fig. 1 E,F) groups. No histological alterations were seen in the control group (Fig. 1 A,B). The NM+LPS group showed markedly reduced interstitial edema and inflammatory cell infiltration in comparison to the LPS group.

*Effect of NM on TNF-α concentration*

The serum level of TNF-α reached a maximum 3 h after the administration of LPS. At all assay times, the TNF-α level in the serum was significantly lower in the NM+LPS group than in the LPS group (Fig. 2).

*Effect of NM on PAI-1 levels*

PAI-1 mRNA expression in lung tissue increased at 6 h and 12 h after the administration of LPS. The increase was significantly larger in the LPS group than in the NM+LPS group at 12 h. PAI-1 protein levels in the lung tissue were also higher in the LPS group than in the NM+LPS group at 12 h (Figs. 3B and 4).

*Effects of NM on HMGB1 expression*

HMGB1 was slightly expressed in lung tissues in the control group (Fig. 5A), strongly expressed in lung tissues in the LPS group (Fig. 5B), and expressed at an intermediate level in the NM+LPS group (Fig. 5C).

## Discussion

The present study demonstrated that the systemic administration of LPS resulted in interstitial and intra-alveolar inflammation, edema, congestion, and hemorrhage, and in the increased expression of HMGB1 in the lung. These observations are in agreement with previous reports describing the critical role that HMGB1 plays in the inflammation and development of LPS-induced lung injury [11,16,17].

During the inflammatory reaction, HMGB1 is released by macrophages and monocytes into the extracellular environment. There, it causes acute inflammatory injury to the lungs, characterized by neutrophil accumu-

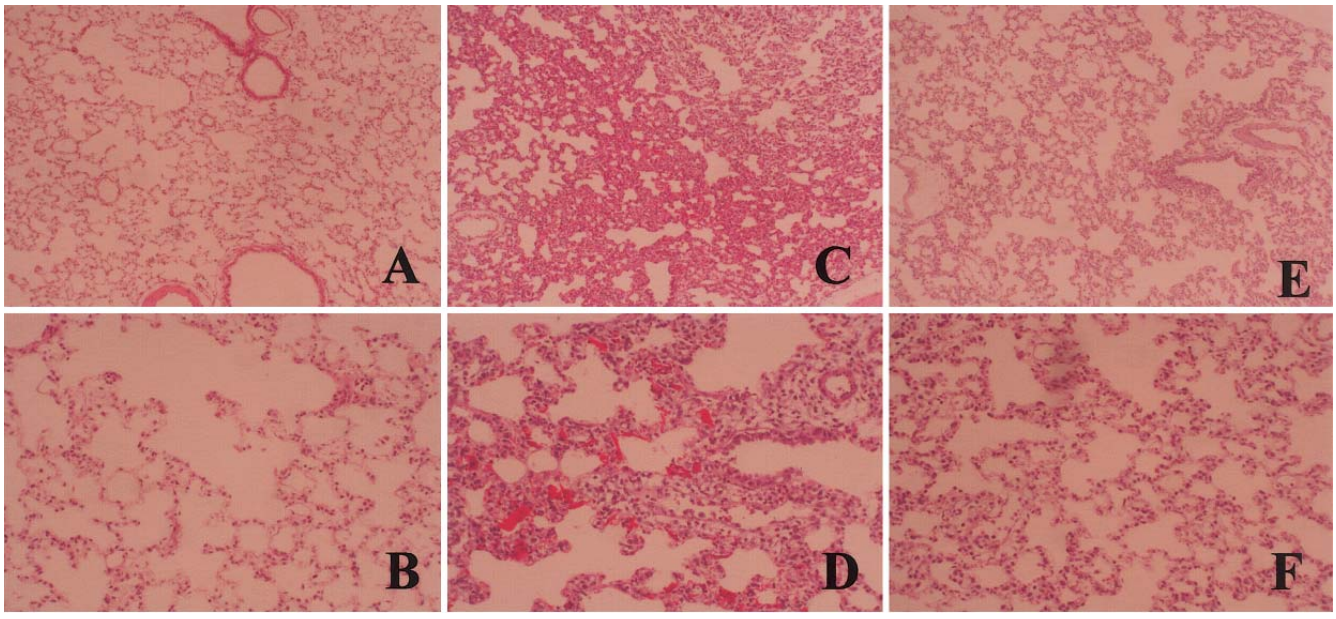



**Fig. 1A–F.** Effect of nafamostat mesilate (NM) on lung injury induced by lipopolysaccharide (LPS) in rats. Rats were given either: two doses of saline (i.p. followed by i.v., control group); saline (i.p.) and LPS (5 mg·kg$^{-1}$, i.v.; LPS group); or NM (1 mg·kg$^{-1}$, i.p.) and LPS (5 mg·kg$^{-1}$ i.v.; NM+LPS group). Lung injury was assessed 12 h after the second injection. Lungs shown are from the control (**A**,**B**), LPS (**C**,**D**), and NM+LPS (**E**,**F**) groups. **A**, **C**, **E**, H&E, ×40; **B**, **D**, **F** H&E, ×100

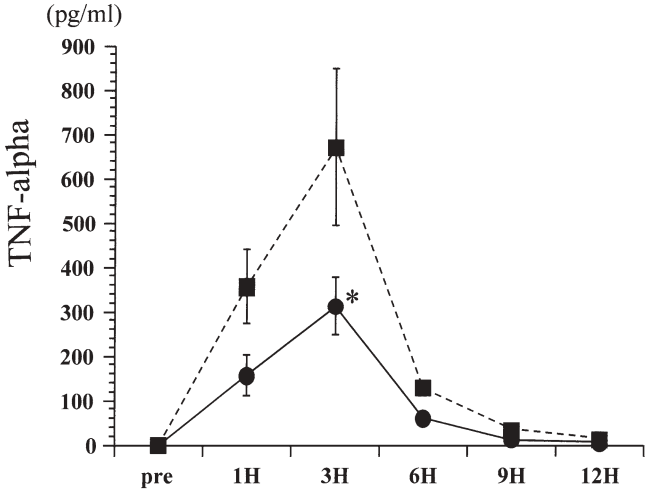
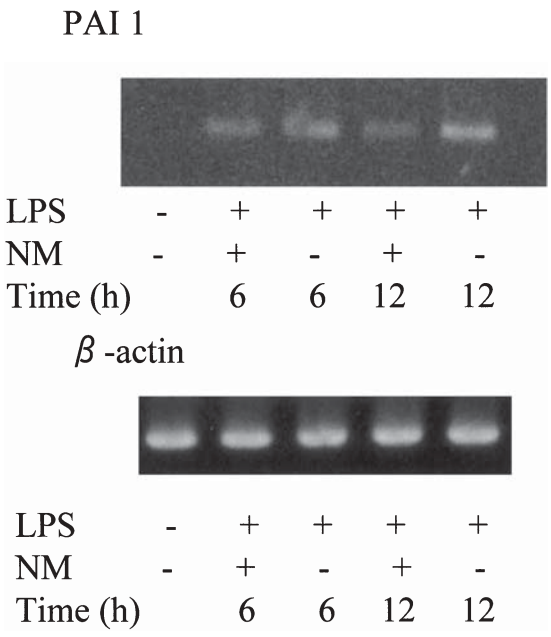



**Fig. 2.** Temporal changes of tumor necrosis factor-alpha (*TNF-α*) serum concentration after LPS administration in rats. Rats were given either: saline (i.p.) and LPS (5 mg/kg, i.v.; LPS group), or NM (1 mg/kg, i.p.) and LPS (5 mg/kg i.v.; NM+LPS group). TNF-α serum concentration was measured hourly and is shown for both groups ($n = 6$ for each group). The *dashed line* represents values measured for the LPS group, while the *solid line* represents the values for the NM+LPS group. All data values are expressed as means ± SE. *Asterisk* denotes a significant difference in TNF-α levels ($P < 0.05$)

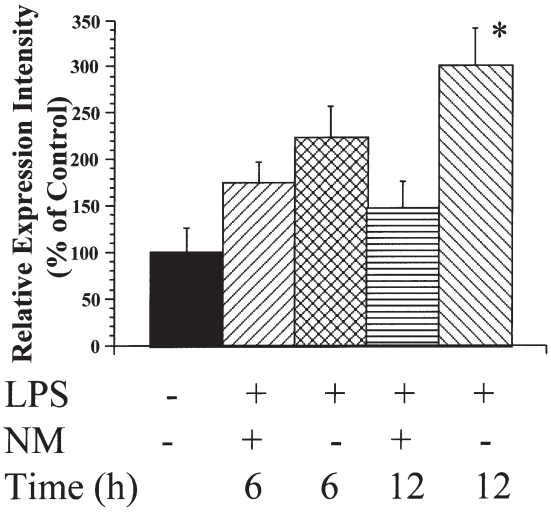
lation, edema, and increased pulmonary production of interleukin-1β, TNF-α, and macrophage inflammatory protein-2 [11]. HMGB1, which transmits signals into the intracellular environment via receptors for advanced glycation endproducts and toll-like receptors 4 and 2 [18,19], is also an important mediator of cell death. Previous studies have shown that HMGB1 is expressed at a relatively late stage following injury [5], a finding which concurs with our observation that the level of HMGB1 in the lung tissue increased 12 h after LPS administration.

Rats given NM before LPS injection had lower levels of HMGB1 in lung tissue and serum and more limited inflammatory changes in their lungs than rats injected with LPS alone. These findings suggest that NM inhibits the expression of HMGB1 and thereby reduces LPS-induced lung injury.

Sepsis is a complex condition with multiple etiological factors and diverse signs and symptoms in the human body [4]. Abnormal coagulation was reported to be important in the initial stage, causing both vascular injury in the lungs [20,21] and the formation of microthrombi. NM primarily acts as an anticoagulant, possibly through the proteolysis of proteins involved in coagulation. NM inhibits not only proteases such as thrombin, coagulation factors, kallikrein, plasmin, complement proteins, and trypsin but also the lipase phospholipase A2 [23,24]. Yoshikawa et al. [25] have suggested that NM inhibits microthrombus formation in




A




B

**Fig. 3A,B.** Changes in plasminogen activator inhibitor-1 (*PAI*-1) mRNA expression in lung tissue specimens after LPS administration in rats. **A** Rats were given either: two doses of saline (i.p. followed by i.v.; control group); saline (i.p.) and LPS (5 mg·kg$^{-1}$, i.v.; LPS group); or NM (1 mg·kg$^{-1}$, i.p.) and LPS (5 mg·kg$^{-1}$ i.v.; NM+LPS group). PAI-1 mRNA levels in the three groups were measured every hour by reverse transcription polymerase chain reaction (RT-PCR). **B** The signal intensities for the test mRNA, β-actin, and PAI-1 were quantified using an image analyzer. Expression intensities of test mRNA relative to that of β-actin were calculated, and expressed as percentages of values in the control group. All data values are expressed as means ± SE. *Asterisk* denotes significant difference vs the control group ($P < 0.05$)

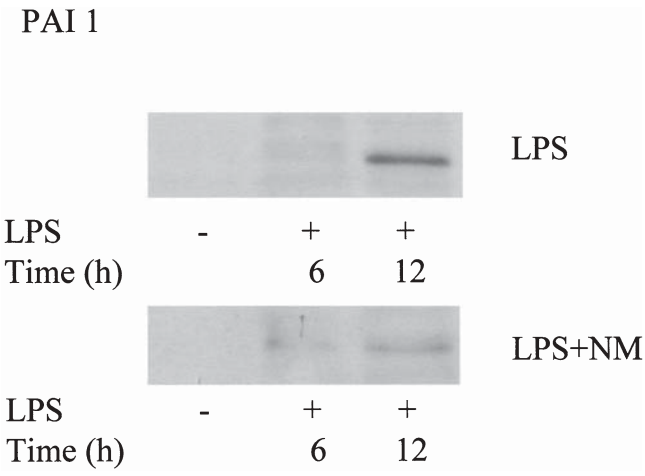



**Fig. 4.** Changes in PAI-1 protein expression in lung tissue specimens after LPS administration in rats. Rats were given either: saline (i.p.) and LPS (5 mg·kg$^{-1}$, i.v.; LPS group), or NM (1 mg·kg$^{-1}$, i.p.) and LPS (5 mg·kg$^{-1}$ i.v.; NM+LPS group). The expression of PAI-1 protein at 6 and 12 h after LPS administration was detected by Western blotting

rats with sepsis. Microthrombi are known to enhance the production of cytokines such as TNF-α [22,26], and these cytokines are thought to be the first mediators of the pathophysiologic events induced by LPS. Consequently, NM may reduce LPS-induced lung injury by reducing coagulation. Our results are consistent with this hypothesis. Our findings showed that the pre-administration of NM resulted in both lower serum levels of TNF-α and reduced lung injury. It follows that inhibiting the coagulation pathway may prevent lung tissue damage under inflammatory conditions.

Vascular endothelial injury plays a critical role in the onset of acute respiratory distress syndrome [27]. Stimulation by endotoxin or other molecules causes cytokines to be produced, and monocytes and macrophages to be activated, which can lead to the injury of vascular endothelial cells in the lungs and the accumulation of activated monocytes and macrophages in the lungs and other tissues [28]. NM appears to inhibit the ability of LPS-stimulated monocytes to produce cytokines [29] and to block the activation of the complement system, thereby reducing vascular endothelial and lung injury [16]. In the present study, pretreating rats with NM resulted in lower levels of TNF-α in the serum and HMGB1 in the lung, as well as reduced lung tissue damage. Our findings thus suggest that these features might be direct or indirect effects of NM.

A recent study demonstrated that HMGB1 interacted in a complex manner with the hemostatic system [10]. In particular, PAI-1, which inhibits plasminogen activation, is important for HMGB1 to exert its effect. Because NM inhibited the production of PAI-1, more

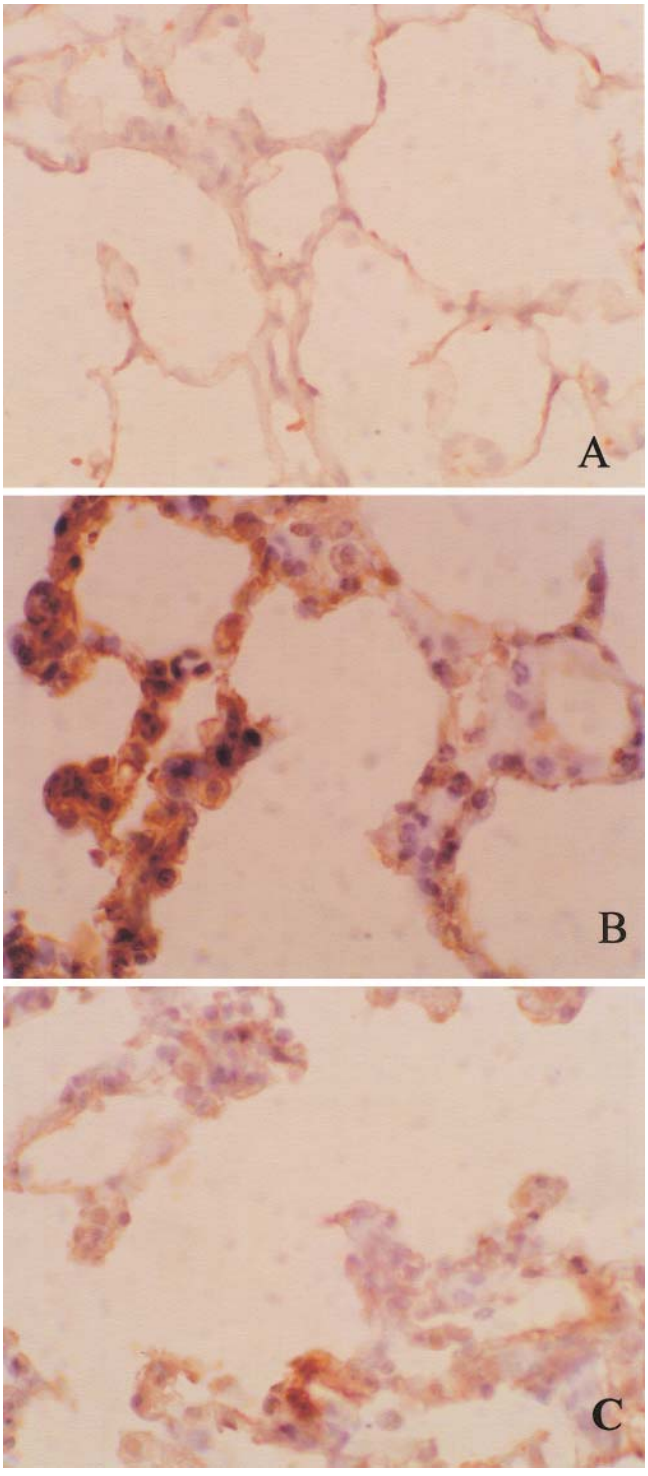



**Fig. 5A–C.** Changes in high mobility group box 1 (HMGB1) protein expression in lung tissue specimens after LPS administration in rats. Rats were given either: two doses of saline (i.p. followed by i.v.; control group); saline (i.p.) and LPS (5 mg·kg$^{-1}$, i.v.; LPS group); or NM (1 mg·kg$^{-1}$, i.p.) and LPS (5 mg·kg$^{-1}$ i.v.; NM+LPS group). Immunohistochemical analysis was used to determine HMGB1 expression 12 h after the second injection. Lungs from the control (**A**), LPS (**B**), and NM+LPS (**C**) groups are shown. **A–C**, at ×400

plasmin would have been produced in NM-treated rats. Plasmin can degrade HMGB1 proteolytically [10]. Previous studies have shown increased levels of PAI-1 in bronchoalveolar lavage fluid obtained from patients with acute lung injury [30], and overexpression of PAI-1 has been conclusively linked to the pathogenesis of acute lung injury. Mellgren et al. [31] showed that NM inactivated PAI-1 activity during experimental extracorporeal life support. Here we have shown that PAI-1 levels increase in LPS-induced lung injury and that the pre-administration of NM suppresses the increase. It has been shown that monocytes and macrophages stimulated by TNF-α secrete HMGB1 [7,8]. In our study, serum TNF-α levels were reduced by the administration of NM. These findings intimate that NM might have reduced the HMGB1 levels by reducing both PAI-1 and TNF-α levels.

In the present study, NM pre-administration markedly attenuated LPS-induced lung injury and reduced TNF-α, PAI-1, and HMGB1 levels in lung tissue. We propose that NM suppresses the activation of the cytokine network and coagulation pathway, thereby inhibiting the production of HMGB1 and reducing LPS-induced lung injury in rats. Furthermore, NM may also be able to reduce the lung injury characteristic of the systemic inflammatory response syndrome in humans. Further research is needed to better explore this possibility.

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