

LUNG, SPLEEN, AND KIDNEY ARE THE MAJOR PLACES FOR INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION IN ENDOTOXIC SHOCK: ROLE OF P38 MITOGEN-ACTIVATED PROTEIN KINASE IN SIGNAL TRANSDUCTION OF INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION

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ABSTRACT—Bacterial lipopolysaccharide (LPS) is known to induce endotoxic shock with inducible nitric oxide (NO) synthase (iNOS) expression and NO production. However, the major place for NO production in shock remains unclear. Although there is some literature about p38 mitogen-activated protein kinase (MAPK) in regulating LPS-induced iNOS expression, the results are contradictory. To interpret the precise cell mechanism and the role of p38 MAPK in the expression of iNOS during endotoxic shock, we carried out the following investigations. A severe endotoxic shock model was reproduced in mice 6 h after LPS injection. The plasma NO level was increased in a dose- and time-dependent manner after LPS stimulation and was suppressed by administration of SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl) imidazole], a highly specific inhibitor of p38 MAPK. The iNOS expression was increased in many organs, including heart, liver, spleen, lung, gut, and kidney in endotoxic shock. Among them, the highest expression of iNOS mRNA and protein was in the lung, moderate expression was in the spleen and kidney, and the lowest expression was in the heart, gut, and liver. The level of expression in lung was 5.5 times that of iNOS mRNA and was 3.1 times that of iNOS protein than in heart, and 1.6 and 1.8 times that of iNOS mRNA and 1.7 and 1.4 times that of iNOS protein than in spleen and kidney, respectively. The p38 MAPK activity increased after LPS injection, and SB203580 markedly reduced LPS-induced expressions of iNOS protein and mRNA in the lung. The results indicate that lung, spleen, and kidney are the major places for iNOS expression in endotoxic shock and are important therapeutic target organs for attenuating NO production in shock treatment.

KEYWORDS—Nitric oxide, inducible nitric oxide synthase, lipopolysaccharide, mitogen-activated protein kinase, lung

INTRODUCTION

Nitric oxide (NO) is a free gas radical produced from catalytic oxidation of L-arginine by NO synthases (NOSs). NO has been shown to have a number of important biological functions, including tumor cell killing, host defense mechanism against intracellular pathogens, vasodilatation, neurotransmission, inhibition of platelet aggregation, mediation of immune responses, and participation in both acute and chronic inflammation (1). NO produced from the constitutive forms of NOS, namely endothelial and neuronal NOS (eNOS or NOSIII, and nNOS or NOSI for short) (2), functions principally as a vasodilator and a neurotransmitter, respectively. Physiologically, only small amount of NO is produced by eNOS and nNOS, and is involved in the regulation of vascular tone and blood flow distribution (3). The inducible form, known as inducible NOS (iNOS or NOSII), is generally not present in resting cells but is induced in response to various stimuli, such as bacterial lipopolysaccharide (LPS), tumor necrosis factor (TNF), and inter-

leukins (IL) (4). In contrast to the constitutive forms of NOS, iNOS does not require Ca^{2+} /calmodulin for its activity. Increased expression of iNOS was associated with diverse disorders ranging from septic and hemorrhagic shock, rheumatoid arthritis, to chronic infections such as tuberculosis. For this reason, to treat septic and hemorrhagic shock, some investigators attempt to inhibit NO production by using pharmacological tools (3). Indeed, beneficial effects were recently reported in experimental models of septic shock that came from various inhibitors of iNOS, such as aminoguanidine (5), L-canavanine (6), S-substituted thiourea derivatives (7, 8), and L-NAME (9). However, it was reported that inhibition of iNOS with MEG did not improve survival in rat with cecal ligation and puncture, and administration of SNAP (a NO donor) could improve the vascular hyporeactivity and decrease the mortality rate of dog in hemorrhagic shock (10, 11). These contradictory results indicate that the role of NO production in the pathogenesis of shock should be further investigated, including the major tissue and signal transduction pathways for NO production in shock, to look for new approaches and targets for shock treatment.

There are at least three problems for further study. One is how to identify the major tissues for NO production in shock because NO is a gas-mediator with very short lifespan and is difficult to estimate. The second one is how to identify the signal transduction pathway for NO production in animal model because inhibitors for signal transduction research are quite expensive and usually not used in whole animals. The third one is how to reproduce a shock model in small animals,

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e.g., in mouse, because it should be studied in small animal models both to observe the tissue distribution of NO production and to afford the cost for signal transduction research in shock. In this study, we produced an endotoxic shock model in mouse, observed the changes of NO level in plasma and the tissue distribution of iNOS expression that implied NO production in shock, and we also investigated the role of p38 mitogen-activated kinase (MAPK) in the signal transduction of iNOS expression during endotoxic shock.

MATERIALS AND METHODS

Animals and materials

Pathogen-free male BALB/c mice, 10 weeks old, were obtained from Center of Laboratory Animal Science of the First Military Medical University.

LPS from *Escherichia coli* serotype 055:B5 was obtained from Sigma (St. Louis, MO). M-MLV reverse transcriptase and Taq DNA polymerase were the products of TaKaRa Biotech (Otsu, Japan). Mouse anti-iNOS monoclonal antibody, rabbit anti-mouse IgG-FITC, anti-p38 polyclonal antibody, and enhanced chemiluminescence assay kits were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). SB203580 (specific inhibitor of p38 MAPK) was bought from Calbiochem (San Diego, CA) and Sigma. The inhibitor was reconstituted in dimethyl sulfoxide (DMSO) at a stock concentration of 1 or 10 mmol/L. Oligo (dT)₁₂₋₁₈ was obtained from Promega (Madison, WI). The primers of iNOS, β -actin, and GAPDH were synthesized by Genset (Singapore), and γ -³²P-ATP was bought from Beijing Ya Hui Biological Company, with recombinant GST-ATF2 and 6xHis-p38 fusion proteins routinely prepared. All other reagents were of analytical grade.

Mouse model of endotoxic shock

Age-matched (6-8 weeks old, mean weight of 25 g) male BALB/c mice were used in this study. The animal was anesthetized with sodium pentobarbital (60 mg/kg, i.p.). With the aid of SMZ-1B stereo dissecting microscope (Nikon, Melville, NY), a narrowed PE50 polyethylene catheter (Clay Adams, Piscataway, NJ) was inserted into the carotid artery. The arterial blood pressure was monitored and recorded with a four-channel dynograph. After mean arterial pressure (MAP) was recorded in normal condition, the animals received an intraperitoneal injection of 5 mg/kg LPS in 0.2 mL of PBS. The blood pressure was continuously monitored and was recorded every 15 or 30 min. Six hours later, blood samples were obtained from the arterial catheter. The samples were immediately centrifuged at 2000g at 4°C for 15 min, and the plasma was stored at -80°C for NO and other measurements. The mice were then sacrificed by CO₂ inhalation. Tissue samples including heart, liver, spleen, gut, lung, and kidney were carefully dissected and immediately placed in sterile tubes, frozen in liquid nitrogen for 12 to 24 h, and stored at -80°C for future measurements. SB203580 was given orally at the dosages of 12.5 or 25 mg/kg 30 min before LPS injection in some of the animals.

All animal experiments were approved by the Animal Care Committee of the First Military Medical University and were conducted according to the guidelines of National Institutes of Health.

Measurement of NO level and iNOS protein expression

The NO level in plasma of mice was determined by Greiss reaction. iNOS protein expression in animal tissues was assessed by Western blot. Frozen tissue sample homogenates were made at 4°C by grinding 30 mg of tissue sample in 500 μ L of ice-cold cell lysis buffer (25 mmol/L HEPES at pH 7.6, 137 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L EGTA, 3 mmol/L β -glycerophosphate, 1 mmol/L Na₃VO₄, 1% Triton X-100, 1 mmol/L PMSF, and 1 μ g/mL aprotinin). The lysates were scraped into microcentrifuge tubes and sonicated twice for 20 s on ice, and were spun for 10 min at 10,000g at 4°C. Concentration of total protein in the homogenates was quantified by Bradford protein assay (Bio-Rad, Hercules, CA). Aliquots of 100 μ g of total tissue protein were mixed with 2x SDS sample buffer (100 mmol/L Tris-HCl at pH 6.8, 200 mmol/L DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) and were denatured by boiling for 10 min. Protein samples were electrophoresed on 7% SDS-polyacrylamide gel with 50 μ g of total protein

and were transferred to nitrocellulose membranes (Whatman, Clifton, NJ). After blocking with TTBS (100 mmol/L Tris-HCl at pH 7.5, 0.9% NaCl, and 0.1% Tween-20) for 1 h, membranes were reacted with anti-iNOS mouse monoclonal antibody at a dilution of 1:600 for 2 h at room temperature, and were then washed with TTBS for 10 min three times, and incubated with anti-mouse IgG horseradish peroxidase-conjugated antibody at a dilution of 1:2000 for 1 h at room temperature. By using an enhanced chemiluminescence reagent, the membrane was exposed to Fuji x-ray film. The bands of film were analyzed by using the Molecular Analyst Software (Bio-Rad) after scanning on a densitometry (Vilber Lourmat, France GS-700 Imaging Densitometer; Bio-Rad).

Determination of iNOS mRNA by RT-PCR

Total RNA of mouse tissues was isolated with Tripure isolation reagent (Boehringer Mannheim, Indianapolis, IN) by following the manufacturer's guidelines. The amount of RNA was calculated from optical density measurements at $\lambda = 260$ nm. Reverse transcription of the mRNA was performed with 5 μ g of total cytoplasm RNA in a final volume of 20 μ L using M-MLV reverse transcriptase system according to manufacturer's instructions (TaKaRa). The mixture was incubated at 37°C for 60 min, and then at 95°C for 5 min to destroy the reverse transcriptase.

PCR was performed in a 25- μ L reaction system containing 2 μ L of transcript product, 10 pmol of the four primers (Table 1), 0.8 mmol/L dNTP, and 0.5 U Taq DNA polymerase and Taq buffer (TaKaRa). The amplification cycle (containing a denaturation step at 94°C for 60 s, an annealing step at 56°C for 45 s, and an extension step at 72°C for 90 s) was repeated 29 times and was terminated by a final extension at 72°C for 10 min. PCR products were identified by electrophoresis in a 1% agarose gel stained with ethidium bromide, visualized, scanned under UV illumination ($\lambda = 312$ nm), and semiquantified by a gel image analytic system.

GAPDH gene was also amplified in parallel and was regarded as the internal control of each sample. The reproducibility of iNOS in RT-PCR was confirmed by at least three samples in each group.

Analysis of p38 MAPK activity

p38 MAPK activity was measured by immune complex kinase assay using p38 MAPK antibody with ATF-2 as a substrate. Briefly, frozen tissue homogenates were made at 4°C by grinding 30 mg of tissue samples in 500 μ L of ice-cold cell lysis buffer and transferring them into microcentrifuge tubes and sonicating them for 20 s on ice. The lysates were spun for 10 min at 10,000g at 4°C, 300 μ L of which (containing approximately 300 μ g of protein) was mixed with p38 MAPK antibody-conjugated agarose, followed by incubation overnight at 4°C with gentle rocking. After the mixture was centrifuged for 30 s at 4°C, the precipitate was washed twice with 500 μ L of kinase buffer (20 mmol/L HEPES at pH 7.6, 20 mmol/L MgCl₂, 0.1 mmol/L Na₃VO₄, 25 mmol/L β -glycerophosphate, and 2 mmol/L DTT). The precipitate was dissolved in 250 μ mol/L ATP, 10 μ g GST-ATF2, and 10 μ Ci of γ -³²P-ATP in 20 μ L of kinase buffer. The kinase reaction was performed for 30 min at 37°C, and was terminated by boiling for 5 min. The samples were vortexed and centrifuged for 2 min at 10,000g, and the sample was loaded onto a 12% SDS-polyacrylamide gel. The gel was dried and subjected to autoradiography after electrophoresis.

Statistical analysis

Most quantitative data are shown as mean \pm SD from at least three independent experiments. The mean value was compared using one-way analysis of variance (ANOVA) and the Bonferroni multiple comparison test. Qualitative variable was expressed as a number with percentages. Statistical significance was assessed by Student's *t* test using SPSS software, where *P* values less than 0.05 were considered significant.

RESULTS

Changes of plasma NO level in endotoxic shock mouse

The changes in MAP before and after injection of LPS in the 400 min of observation period in mice are illustrated in Figure 1. The baseline value for MAP was 127.5 \pm 9.0 mmHg and was

TABLE 1. PCR primers

	Accession No. in GenBank	Primer Sequence (5'-3')	Positions	Expected Size (bp)
Mouse iNOS	AF065923	GAAACAACAGGAACCTACCA	655-674	1098
		TCCATGCAGACAACCTTGG	1753-1735	
Mouse GAPDH	NM008084	CCCATCACCATTCCAGGA	257-276	575
		TGCTTCACCACCTCTTGAT	831-812	

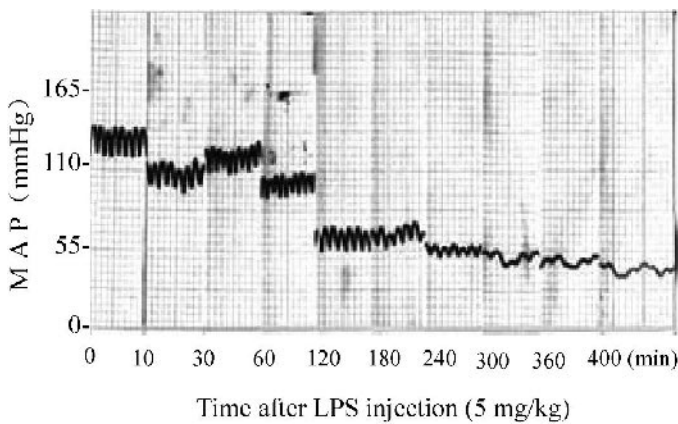


FIG. 1. Changes of mean arterial pressure (MAP) of mice after LPS injection. The data come from one of 10 animals.

stable during the course of the experiment in the control group. Administration of LPS (5 mg/kg, i.p.) caused a fall in MAP to 102.8 ± 6.8 mmHg at 10 min after LPS injection ($n = 3$, $P < 0.05$), and there was a slight but significant increase to 117 ± 9.6 mmHg at 30 min, then the MAP showed a progressive drop at 60 min and a severe drop to 42.0 ± 3.0 mmHg at 360 min. This indicated that animal had severe endotoxic shock 6 h after injection of LPS.

Within a period of 24 h postinjection of LPS, the plasma NO level increased markedly in a dose-dependent manner (data not shown). The injection of LPS caused a time-dependent increase in the plasma level of NO concentration. Six hours after LPS injection (endotoxic shock), the NO level in plasma increased from the basal level 35.6 ± 5.6 $\mu\text{mol/L}$ to 78.6 ± 16.8 $\mu\text{mol/L}$. The increase level of plasma NO caused by endotoxic shock were markedly suppressed by pretreatment with SB203580, but still significantly higher than that in control (Fig. 2).

iNOS expression in different tissues of normal and endotoxic shock mouse

To compare iNOS protein expression among different tissues, 50 μg of total protein from each organ was loaded per lane in SDS-polyacrylamide gel electrophoresis.

iNOS protein and mRNA could not be detected in heart, liver, spleen, kidney, and intestine, but could be detected in lung of normal mice (Fig. 3). We measured the iNOS mRNA and protein expression of different tissues in the same mouse of endotoxic shock group ($n = 5$) 6 h after LPS injection. The level of iNOS expression in endotoxic shock could be divided into three grades: the highest in lung, the moderate in spleen and kidney, and the lowest in heart, gut, and liver. The highest expression level of iNOS protein in lung was 3.1 times more than the lowest expression in heart ($P < 0.01$) and was 1.7 and 1.4 times more than the moderate expression in spleen and kidney ($P < 0.05$), respectively, in endotoxic shock mice. iNOS mRNA had the same trend in the tissues in which the highest expression in lung was 5.5 times more than the lowest expression in heart ($P < 0.01$) and was 1.6 and 1.8 times more than those in spleen and kidney ($P < 0.05$), respectively, in endotoxic shock mice (Fig. 4).

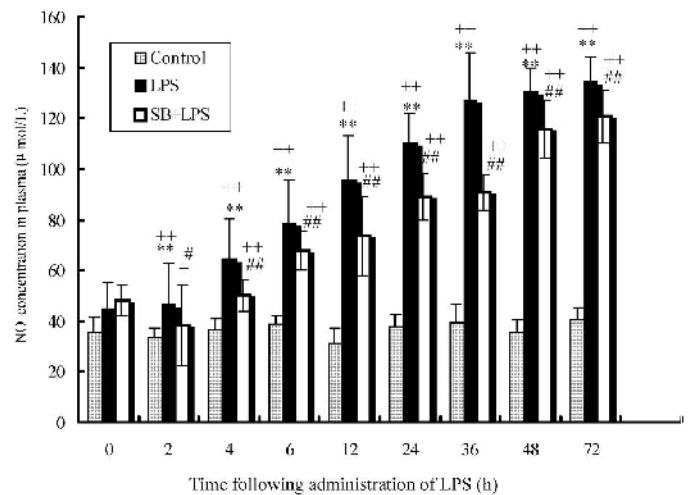


FIG. 2. Changes of NO level in plasma of mice during endotoxic shock. All data were expressed as the mean \pm SD of $n = 8$ animals studied at each time point. * $P < 0.05$ and ** $P < 0.01$ represent significant differences when versus those at 0 min; + $P < 0.05$ and ++ $P < 0.01$ represent significant differences when compared with control; # $P < 0.05$ and ## $P < 0.01$ represent significant differences between SB (15 $\mu\text{mol/L}$) + LPS and LPS-treated group at the same time point. The concentrations of NO in plasma increased significantly with time ($P < 0.05$) in LPS and SB + LPS groups. The NO concentration was significantly higher than at 0 h in LPS and SB + LPS groups ($P < 0.05$).

iNOS expression of lung tissue in mouse

In this study, an immunoblot of lung samples obtained after LPS injection is shown in Figure 5. We detected a weak 130-kD iNOS protein band in the lungs of saline-injected mice. Injection of LPS elicited a significant upregulation of iNOS protein expression in time-dependent manner.

The iNOS mRNA expression was also slightly detectable in lung from normal mice with the iNOS mRNA/GAPDH mRNA ratio of 0.05. Two hours after LPS injection, the ratio significantly increased to 0.78; at 4 and 12 h the ratio increased to 1.21 and 1.37, respectively, and decreased gradually at 36 h (Fig. 6).

During the replication of the endotoxic shock model, the rat was subjected to severe shock 6 h after injection of LPS (5 mg/kg), therefore, the subsequent experiments would select 6 h postinjection for detection of iNOS expression. The results of immunoblot and RT-PCR indicated that the expression of iNOS protein (Fig. 7) and mRNA (Fig. 8) showed a significant dose-effect relationship in the lung of mice.

Role of p38 MAPK in lung iNOS expression during endotoxic shock

Thirty minutes after LPS 5 mg/kg injection, p38 MAPK activity in lung tissue began to increase. The activity was maximum at 120 to 180 min, and began to descend after 240 min (Fig. 9). SB203580 could inhibit the increase of p38 MAPK in dose-dependent manner (Fig. 10).

The expression of iNOS protein and mRNA in lung tissues of LPS-treated mice was significantly higher than that in control mice. However, after pretreatment with p38 MAPK specific inhibitor SB 203580 at concentrations of 5, 12.5, and 25.0 mg/kg, the expression of iNOS protein (Fig. 11) in mice lung tissue was inhibited by $21\% \pm 2.8\%$, $48\% \pm 3.7\%$, and $75\% \pm 5.8\%$, and iNOS mRNA (Fig. 12) was inhibited by 37%

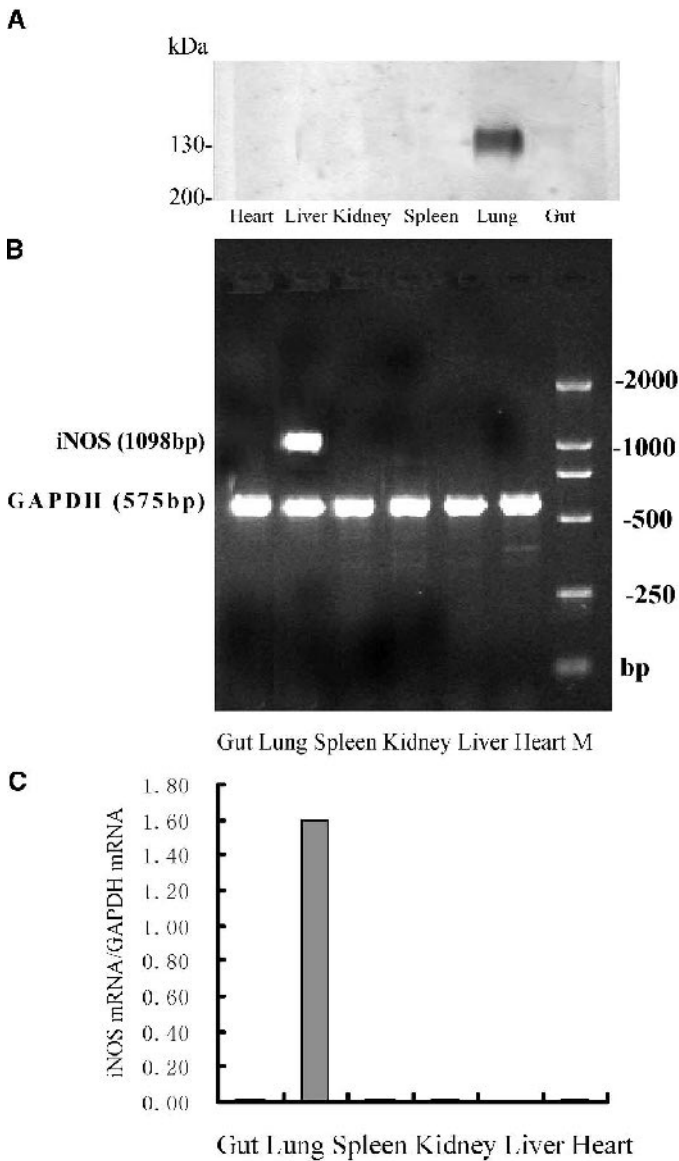


FIG. 3. The expression of iNOS protein (A) and mRNA (B and C) in different tissues of normal mice (A) Results of Western blot. (B) Results of agarose gel electrophoresis. (C) Semiquantification of iNOS mRNA/GAPDH mRNA ratio. One result of five independent experiments is shown.

$\pm 2.7\%$, $54\% \pm 2.9\%$, and $67\% \pm 3.8\%$, respectively, which were significantly lower than the control value ($P < 0.01$).

DISCUSSION

The major places for NO production in shock were investigated to look for new approaches and therapeutic targets in shock treatment. There were a few studies about the tissue distribution of iNOS expression in endotoxic shock, which focused on endothelium, macrophage, and vascular smooth muscle, but no one on viscera (10, 12–18). The role of p38 MAPK in iNOS expression was reported contradictorily. Some studies showed that p38 MAPK augmented iNOS expression after LPS stimulation, and the specific inhibitor of p38 MAPK, SB203580, suppressed iNOS expression in endotoxin-simulated primary glial cells (19, 20), whereas other reports (21, 22) seemed to argue that SB203580 had no effect on iNOS

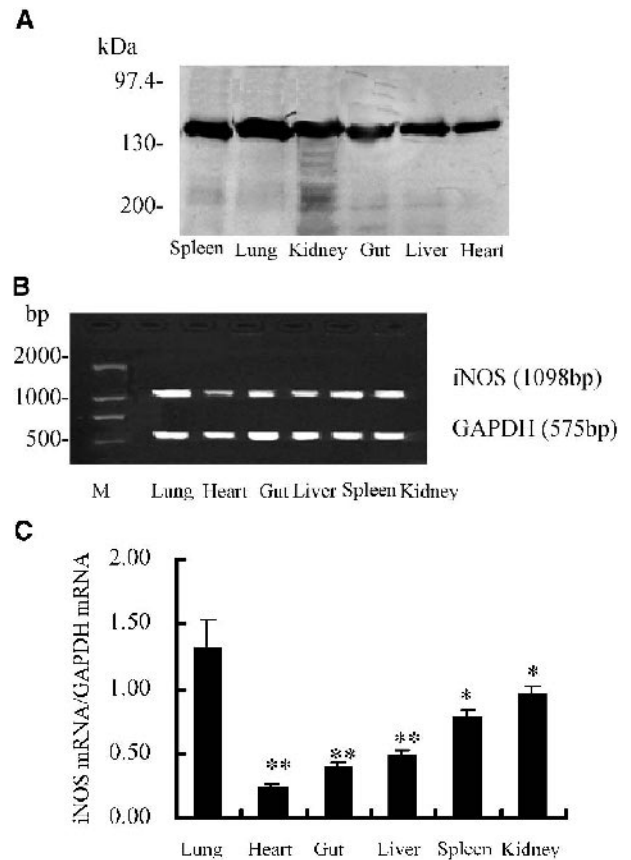


FIG. 4. The expression of iNOS protein (A) and mRNA (B and C) in different tissues of endotoxic shock mice (A) Results of Western blot. (B) Results of agarose gel electrophoresis. (C) Semiquantification of iNOS mRNA/GAPDH mRNA ratio. The results come from five independent experiments. * $P < 0.05$ and ** $P < 0.01$ represent significant differences when versus the ratios of lung.

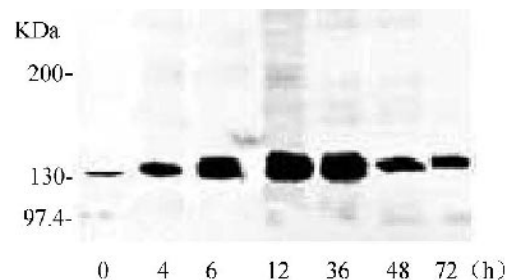


FIG. 5. Western blot analysis of the time course of iNOS protein expression in lung tissue of mice with endotoxic shock.

expression in LPS-induced mouse macrophages. Therefore, this study was designed to carry out a series of experiments by using molecular and cellular biology methods to investigate tissue distribution of iNOS expression and the role of p38 MAPK in the signal transduction of iNOS expression during endotoxic shock.

We successfully established the mice model of endotoxic shock. Mice showed a progressive biphasic decline in MAP after injection of LPS. MAP decreased from 127.5 ± 9.0 mmHg normal value to 42.0 ± 3.0 mmHg 6 h later, which indicated that these animals were in severe shock. It was shown that the increase of plasma NO level existed in a dose- and time-dependent manner and reached to 2.0 times of normal

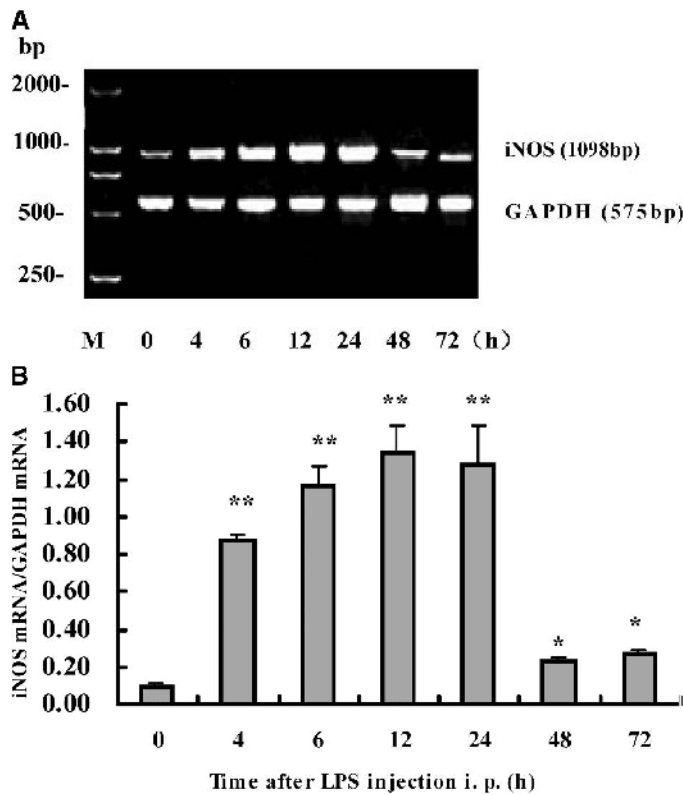


FIG. 6. The time course of iNOS mRNA expression in lung tissue of mice with endotoxic shock. (A) Results of agarose gel electrophoresis. (B) Semiquantification of iNOS mRNA/GAPDH mRNA ratio; data come from five independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. 0-h group.

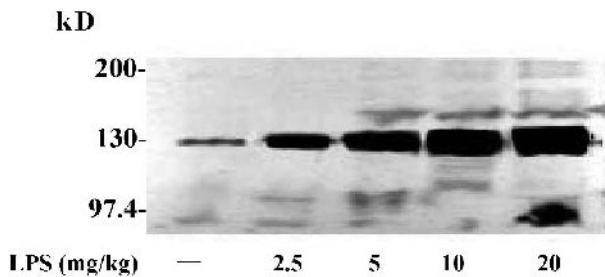


FIG. 7. Western blot analysis showing the relationship between the dosage of LPS injection and the expression of iNOS protein in lung tissues of BALB/c mice. Each group studied consists of three samples.

value 6 h post-LPS injection, which indicated that LPS really induced NO production in endotoxic shock mice and gave a bases for further study of the tissue distribution of iNOS expression. Administration of SB 203580 could partially and significantly suppress the increase of plasma NO level, which indicated that p38 MAPK was involved in the signal transduction of NO production and could serve as an auxiliary index for finding the major place of iNOS expression, in which the level of iNOS could be also suppressed by inhibition of p38 MAPK in coincidence with the alteration of plasma NO level.

It was demonstrated that iNOS expression, including the expression of iNOS mRNA and protein, was slight in normal lung tissue, and it was undetectable in other organs under normal conditions. Some investigators reported that many types of cells (including nerve fiber, endothelial cell, airway epithelial cell, and cultured epithelial cell line) could express

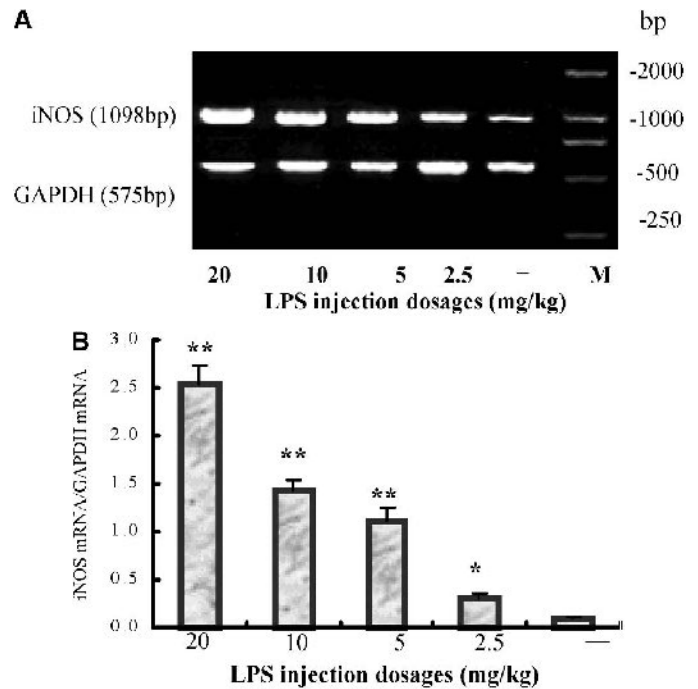


FIG. 8. The relationship between the dose of LPS injection and expression of iNOS mRNA in lung tissue of BALB/c mice by RT-PCR analysis. (A) Results of agarose gel electrophoresis. (B) Semiquantification of iNOS mRNA/GAPDH mRNA ratio. * $P < 0.05$ and ** $P < 0.01$ represent significant differences when versus the ratio of control. The statistical results came from five independent experiments.

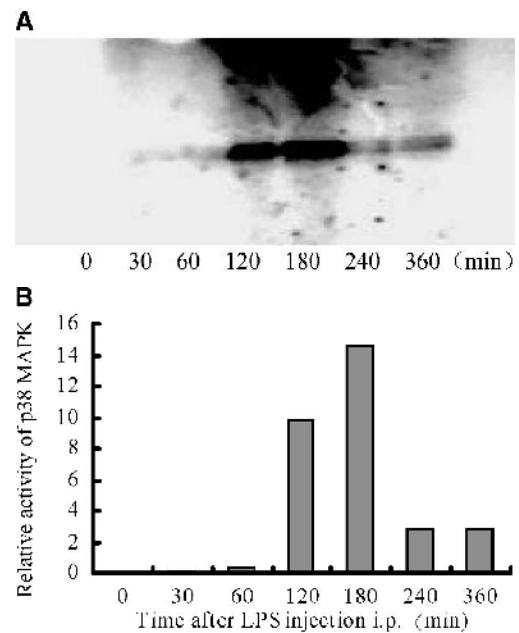


FIG. 9. Effect of LPS on p38 MAPK activity in lungs of BALB/c mice. Mice were pretreated with 15 $\mu\text{mol/L}$ SB203580 1 h after stimulation by LPS (5 mg/kg). Lung tissue extracts were subjected to immunokinase assay. p38 MAPK activity bands were detected by exposure to film (A) and the relative activity of p38 was quantified by densitometry (B). The result was one of three separate experiments.

nNOS and eNOS under unstimulated conditions (17, 23, 24). Just as in our experiment, some reporters indicated that iNOS expression existed also in normal human airway epithelia and rat lung macrophages (17, 25, 26). We consider that iNOS is not a constitutive or intrinsic synthase of normal lung tissue.

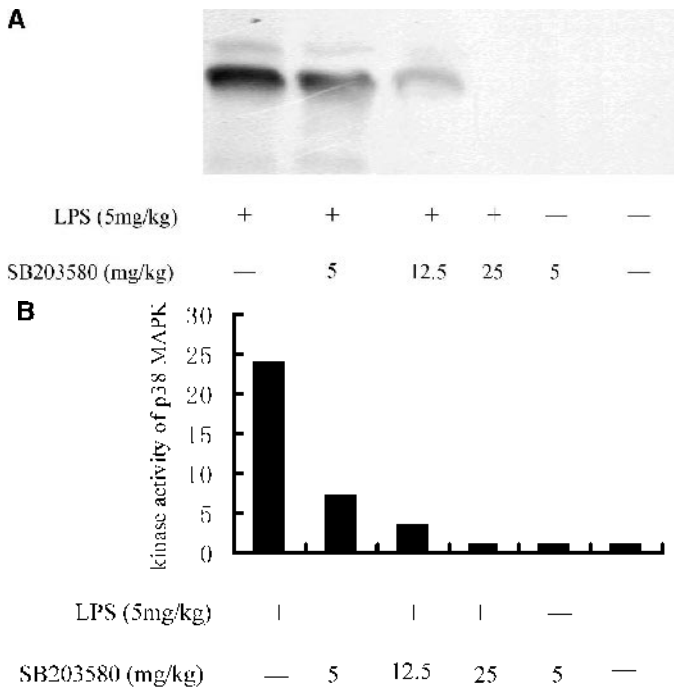


FIG. 10. Effect of different dosages of SB203580 on p38 MAPK activity in lungs of BALB/c mice treated by LPS. Mice were pretreated with various concentrations of SB203580 1 h after stimulation by LPS (5 mg/kg). Lung tissue extracts were subjected to immunokinase assay. p38 MAPK activity bands were detected by exposure to film (A) and the relative activity of p38 was quantified by densitometry (B). The result was one of three separate experiments.

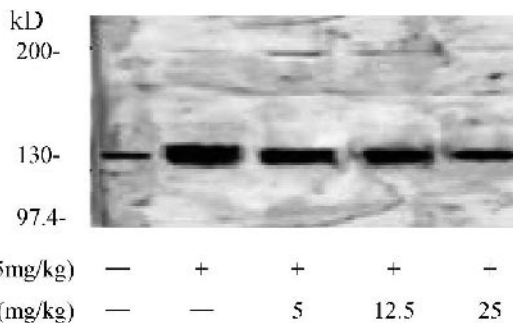


FIG. 11. Effect of SB203580 on iNOS protein expression in lung of mice with endotoxic shock. The result was one of five independent experiments.

The small quantity of iNOS results from trace LPS stimulation because lung is an open organ in communication with the outer environment. However, it reflects the fact that the lung tissue is highly sensitive to LPS. The iNOS gene transcription and translation were markedly increased among various organs, including heart, liver, spleen, gut, lung, and kidney in endotoxic shock mice. Of all studied tissues, the highest iNOS expression was in lung of mice in endotoxic shock. The writer also obtained a similar result during the study of ICAM-1 in shock (27). ICAM-1 could also be detectable in normal lung tissue of mice, and its expression was increased significantly after LPS treatment. All the evidence indicates that lung is the most susceptible organ to bacterial toxin and is a therapeutic target in shock treatment.

According to our previous and current studies, there are at least two therapeutic targets for NO in severe shock. One is to

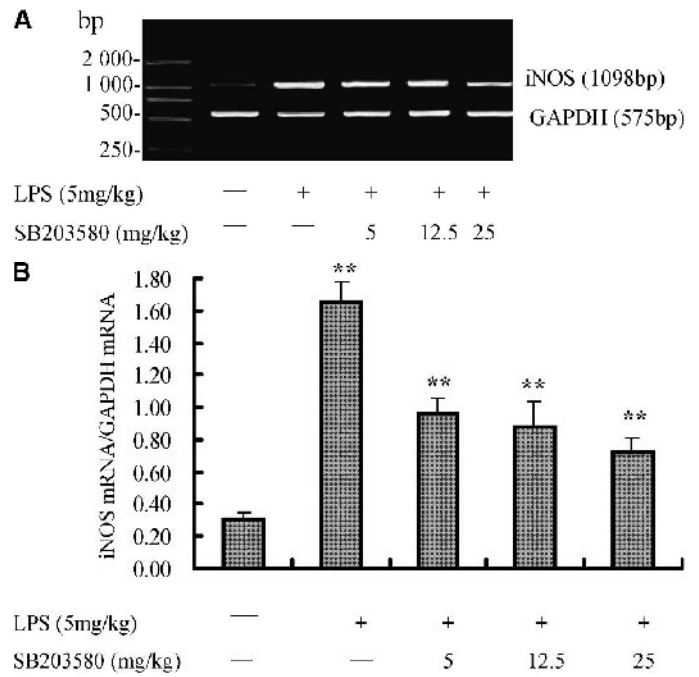


FIG. 12. Effect of SB203580 on iNOS mRNA expression in lung of mice with endotoxic shock. (A) Results of agarose gel electrophoresis. (B) Semiquantification of iNOS mRNA/GAPDH mRNA ratio. ***P* < 0.01 represents significant differences when versus the ratios of control. The statistical results came from five independent experiments.

reduce the production of peroxynitrite (ONOO⁻), a toxic oxidant formed by the reaction of NO and superoxide anion. It was shown by us that peroxynitrite might activate ATP-sensitive potassium channel (K_{ATP}) with arteriolar smooth muscle cell (ASMC) membrane hyperpolarization and low vasoreactivity in severe shock (28). ASMC hyperpolarization resulted in inhibition of potential operated calcium channel (POC) and reduction of Ca²⁺ influx, which in turn led to reduction of increased [Ca²⁺]_i of ASMC stimulated by norepinephrine (NE) and reduction of contractile response (29). Administration of tiron, a scavenger of oxygen free radicals, could decrease the formation of ONOO⁻ with the recovery of ASMC membrane potential. In our previous experiment, treatment by glybenclamide, a K_{ATP} blocker, and tiron which served as two restituting vasoreactivity agents, followed by injection of dopamine, got very good therapeutic efficacy in hemorrhagic shock (30). The second one is to reduce the production of NO because only large amounts of NO can react with O₂⁻ to form ONOO⁻ and produce a hyperpolarizing effect of ASMC (28, 31). In this study, it has been demonstrated that lung, spleen, and kidney may be the major target organ for attenuating NO production in shock treatment. However, how to inhibit the NO production in major places of body directly remains to be studied in the future.

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