# Alterations in Gene Expressions Encoding PreproET-1 and NOS in Pulmonary Tissue in Endotoxemic Rats

Sohel Zaedi,* Subrina Jesmin,* Seiji Maeda,* Nobutake Shimojo,* Iwao Yamaguchi,* Katsutoshi Goto,† and Takashi Miyauchi*,[1]

*Department of Cardiovascular Medicine, Institute of Clinical Medicine; and †Department of Pharmacology, Institute of Basic Science, University of Tsukuba, Ibaraki 305-8575, Japan

**Septic shock is characterized by hypotension and a hyporeactive response to vasopressor agents. The pathogenesis is due to vascular leaks and an increased synthesis of cytokines and nitric oxide (NO). The present study examined the time-dependent alterations of endothelin-1 (ET-1) and the expression of NO synthase (NOS) in lung tissue in a septic rat model. Normal Sprague-Dawley (SD) rats aged 10 weeks received 15 mg/kg lipopolysaccharide (LPS) and then were sacrificed at different time points (1, 3, 6, and 10 hrs). Rats that did not receive LPS were considered to be controls. Both systolic and diastolic pressure decreased in SD rats after LPS administration. Time-dependent onset of features of acute lung injury, such as the infiltration of inflammatory cells and thickening of alveolar septa, were seen in rats that received LPS. A 2.8-fold increase in the expression of preproET-1 level was observed in lung tissue 6 hrs after LPS administration. The expression of endothelial NOS (eNOS) was also altered in lung tissue in a time-dependent fashion. After the administration of LPS, there was a 16-fold increase in the expression of eNOS mRNA. The peak expression of inducible NOS (iNOS) in lung tissue specimens obtained from rats that received LPS was 45-fold higher than that in control rats. ET-1 is a potent vasoconstrictor and thereby may play an important role in the pathogenesis of acute lung injury in a septic rat model. The increased expression of NOS may result in excess NO production and may also play a role in the pulmonary complications of endotoxemia.** Exp Biol Med 231:992–996, 2006

**Key words:** LPS; sepsis; lung; endothelin-1; NOS

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan (15390077 and 15650130) and by a grant from the Miyauchi project of Tsukuba Advanced Research Alliance at the University of Tsukuba.

[1] To whom correspondence should be addressed at Cardiovascular Division, Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan. E-mail: t-miyauc@md.tsukuba.ac.jp

Received October 6, 2005.
Accepted November 22, 2005.

1535-3702/06/2316-0992$15.00


Endotoxin shock is a major cause of death in patients with septicemia (1, 2). This condition is characterized by systemic hypotension, hyporeactiveness to vasoconstrictors, and generalized tissue damage (3, 4). Septic shock is caused by lipopolysaccharide (LPS) and other bacterial products. Accumulating evidence indicates a role for vasoactive substances and cytokines in this disease process. When endotoxic shock develops, exaggerated amounts of proinflammatory cytokines are released, resulting in refractory hypotension, tissue hypoperfusion, and organ dysfunction (2–4). Acute lung injury has long been observed in association with sepsis or endotoxin shock (5).

Endothelin-1 (ET-1) has been identified as the most potent vasoconstrictor peptide discovered so far (6, 7). The plasma ET-1 level has been shown to be significantly higher in septic animals than in healthy animals (8). During sepsis, endotoxin and other microbial products that are released into the bloodstream trigger endothelial cells to increase production of ET-1, which causes local vasoconstriction (9, 10). The possible involvement of the ET system in human septic shock is further supported by a clear correlation between ET plasma levels and morbidity and mortality in septic patients (11, 12). ET has also been suggested to contribute to the dysfunction of several vital organ systems during septic shock. Because endothelial damage is a major feature of acute lung injury, we examined whether the potent endothelial vasoconstrictor peptide ET-1 plays a pathophysiological role in sepsis or acute respiratory distress syndrome (ARDS). The effect of ET-1 is most prominent in the pulmonary circulation, where the $ET_A$ and $ET_B$ receptors are widely distributed (13, 14). Previous investigators have noticed that intravenously infused ET-1 results in increased pulmonary artery pressure and lung edema (15, 16). Moreover, in isolated rat lungs in which the vasculature has been paralyzed, ET-1 enhances microvascular permeability, but the mechanisms involved have not yet been conclusively determined (17).

In the present study, we infused male Sprague-Dawley (SD) rats with LPS to create a model of endotoxemia. Lung tissue specimens were obtained from these rats and analyzed for time-dependent alterations in the expression of the gene

encoding preproET-1 and genes encoding different types of nitric oxide synthase (NOS). Before the animals were sacrificed, the systolic and diastolic blood pressure were measured, and after sacrifice the extent of lung injury by LPS was also assessed.

## Materials and Methods

**Animal Preparation.** Ten-week-old male SD rats (weight, 300–350 g) were obtained from Charles River Japan Inc. (Yokohama, Japan) and cared for according to the Guiding Principles for the Care and Use of Animals specified in the 1964 Helsinki Declaration. Endotoxemic shock was induced by ip administration of 15 mg/kg bacterial LPS (dissolved in sterile saline) from *Escherichia coli* 055:B5. The systolic and diastolic blood pressure were recorded before the animals were killed. Groups of animals (10 per group) were sacrificed by means of pentobarbital injection at different time points after LPS administration (1, 3, 6, and 10 hours). The control group received an equal volume of sterile saline (2 ml/kg) instead of LPS. At the indicated time, lung tissues were gently harvested, immediately frozen in liquid nitrogen, and stored at −80°C. Lung tissue was postfixed in 4% paraformaldehyde overnight and processed routinely for paraffin embedding. All animals received care that complied with guidelines of the University of Tsukuba, and the university's Graduate School of Medicine Animal Care and Use Committee approved the experimental procedure.

**Histopathological Analysis.** For histopathological analysis, tissue specimens (six from each group) were fixed in 4% buffered formalin solution, dehydrated, embedded in paraffin, and sliced into 5-μm–thick sections. After paraffin was removed, slides were stained with hematoxylin-eosin by use of standard methods.

**Hemodynamic Analysis.** On the day of the experiment, the rats were anesthetized with pentobarbital sodium (40 mg/kg ip), and a microtip pressure transducer catheter (SPC-320; Millar Instruments, Houston, TX) was inserted into the left carotid artery. Arterial blood pressure and heart rate were monitored and were recorded with a polygraph system (an AP-601G amplifier [Nihon Kohden, Tokyo, Japan], an AT-601G tachometer [Nihon Kohden], and a WT-687G thermal-pen recorder [Nihon Kohden]).

**Enzyme Immunoassay for Plasma ET-1.** The concentration of ET-1 in plasma specimens (8 from each group) was determined using an ET-1 Enzyme Immuno Assay Kit (Immuno-Biological Laboratories, Fujioka, Japan). This kit is a solid-phase sandwich enzyme-linked immunoabsorbent assay (ELISA) that uses two kinds of highly specific antibodies. The ELISA was done according to the manufacturer's instructions.

**Quantitative Real-Time Polymerase Chain Reaction (PCR).** Total RNA in lung tissue specimens (10 from each group) was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction with Isogen (Nippon Gene, Toyama, Japan). Briefly, the lung tissue was homogenized in Isogen (100 mg tissue/1 ml Isogen) with a Polytron tissue homogenizer (model PT10SK/35; Kinematica, Lucerne, Switzerland). The precipitated RNA was extracted with chloroform, precipitated with isopropanol, and washed with 75% vol/vol ethanol. The resulting RNA was resolved in diethyl pyrocarbonate–treated water, treated with DNase I (Takara, Shiga, Japan), and extracted again with Isogen to eliminate the genomic DNA. The RNA concentration was determined spectrophotometrically at 260 nm. Total tissue RNA was primed with 0.05 μg oligo d $(pT)_{12–18}$ and reverse transcribed by omniscript reverse transcriptase with a first-strand cDNA synthesis kit (Qiagen, Tokyo, Japan). The reaction was performed at 37°C for 60 mins.

The levels of mRNA expression of preproET-1, endothelial NOS (eNOS), and inducible NOS (iNOS) in the lung were analyzed by quantitative reverse-transcriptase PCR with TaqMan probes in an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA). The gene-specific primers and TaqMan probes were synthesized using Primer Express software, version 1.5 (Perkin-Elmer), according to the published cDNA sequences for preproET-1, eNOS, iNOS, and β-actin mRNA. The sequences of the oligonucleotides were as follows: preproET-1 forward: 5′-TCTACTTCTGCCACCTGGACAT-3′, preproET-1 reverse: 5′-GAAGGGCTTCCTAGTCCA TACG-3′, and preproET-1 probe: 5′-CATCTGGGTCAA-CACTCC-3′; eNOS forward: 5′-GATCCTAACTTGCCTTG CATCCT-3′, eNOS reverse: 5′-TGTAATCGGTCTTGCCA GAATCC-3′, and eNOS probe: 5′-CTGGTATTG-CACCCTTCC-3′; iNOS forward: 5′-GTGGGTGGCCTCGA GTTC-3′, iNOS reverse: 5′-CCAATCTCGGTGCCCATG TAC-3′, and iNOS probe: 5′- CTGCCCCTTCAATGGTT-3′; and β-actin forward: 5′-GGCCGGGACCTGACA-3′, β-actin reverse: 5′-GCTGTGGTGGTGAAGCTGTAG-3′, and β-actin probe: 5′-ACTACCTCATGAAGATCC-3′.

The expression of β-actin mRNA was used as an internal control. The PCR mixture (25 μl total volume) consisted of 450 n*M* of both forward and reverse primers for preproET-1, eNOS, iNOS, and β-actin (Perkin-Elmer); 200 n*M* of FAM-labeled primer probes (Perkin-Elmer); and TaqMan Universal PCR Master Mix (Perkin-Elmer). Each PCR amplification was performed in triplicate, using the following profile: 1 cycle of 95°C for 10 mins, and 40 cycles of 94°C for 15 secs and 60°C for 1 min. To obtain the standard curve in the real-time quantitative PCR, serial dilutions of rat lung cDNA were performed using a range of concentrations (1×, 2×, 4×, 8×, and 16×). A water-based reaction mixture was prepared as a negative control.

**Statistical Analysis.** Results are expressed as mean ± SD, and the sample number equals the number of animals in each group. Mean values were compared by means of one-factor analysis of variance, followed by the Scheffé's test to account for the problem of multiple comparisons. Differences were considered to be statistically significant at a $P$ value of $<0.05$.

## Results

Both the systolic and diastolic blood pressure after LPS administration were significantly lower than those in control rats (Fig. 1A and B). Lung tissue specimens from control rats showed no morphological abnormalities. However, in lung tissue specimens obtained from rats that received LPS, congestion, neutrophil infiltration, and thickening of alveolar septa were observed.

The expression of preproET-1 was increased after LPS administration in lung tissues in SD rats and this expression was peaked at 6 hrs (Fig. 2). Furthermore, the immunoreactive ET-1 level in plasma was significantly higher in plasma after LPS administration at all time points, and the peak level was observed 6 hrs after LPS administration (levels in the LPS group were 19-fold higher than those in the control group). In lung tissue specimens, expressions of the genes encoding iNOS and eNOS were remarkably increased after LPS administration in septic rats, compared with control rats (Fig. 3A and B).

## Discussion

The present study demonstrates that the expression of preproET-1 in lung tissue is increased in LPS-treated rats and peaks at 6 hrs of sepsis. The expressions of the genes encoding eNOS and iNOS are remarkably upregulated in the lung tissue of SD rats after LPS administration. It should be noted that the plasma level of ET-1 is also significantly increased after LPS administration. Moreover, the features of acute lung injury are evident in septic lung tissue. Both the systolic and diastolic blood pressure are significantly decreased in LPS-treated rats.

We found that the plasma ET-1 concentration increased in the LPS-treated SD rats. This observation is in accordance with previous findings in endotoxemic and septic animals and humans (18). The increase in plasma ET-1 levels in septic animals has been attributed to both upregulated ET-1 synthesis and impaired renal and pulmonary clearance of ETs.

The role of endogenous ETs in hypotension of sepsis and endotoxemia remains controversial. In the present study, systolic and diastolic blood pressure were significantly decreased after LPS administration. Conflicting results exist regarding the effectiveness of various ET receptor antagonists in reversing LPS-induced hypotension. Pretreatment of septic rats with the nonselective ET receptor antagonist SB209670 resulted in worsening of hypotension (19). By comparison, the nonselective ET-receptor antagonist bosentan improves survival but had no influence on LPS-induced hypotension in pigs. The reasons behind these contradictory results are not clear. We speculate, however, that many factors are involved in determining the influence of ET receptor antagonists on LPS-induced hypotension, such as the hemodynamic profiles of various septic shock models, the degrees to which endogenous ET levels are

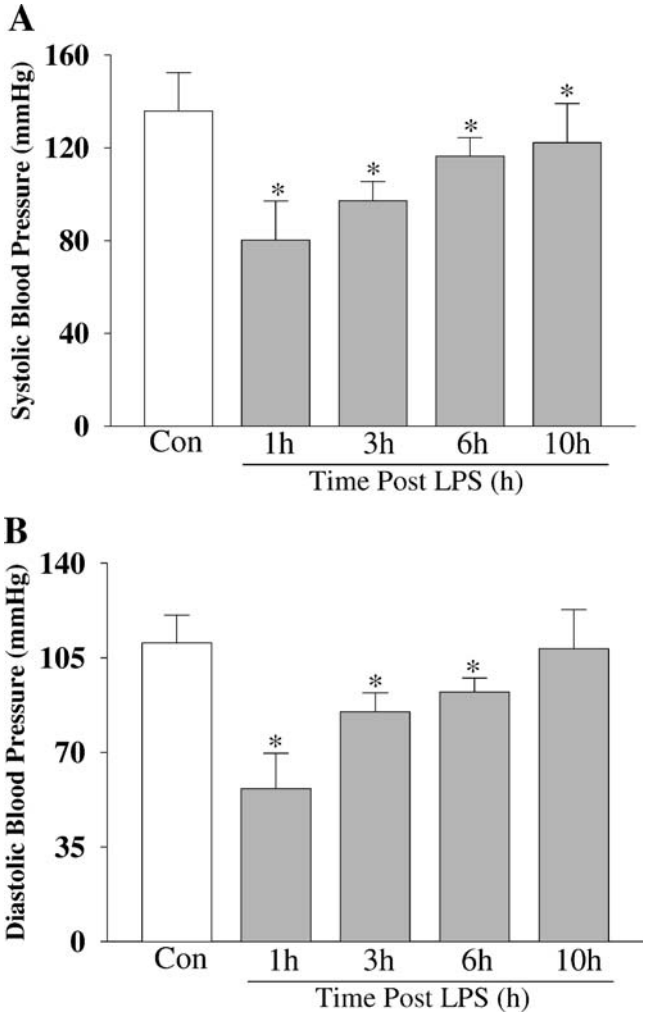


**Figure 1.** Changes in systolic (A) and diastolic (B) blood pressure in SD rats after LPS administration and in control rats ($n = 10$). Results are mean ± SD. $^*P < 0.01$, with respect to control.

elevated in these models, and the *in vivo* effectiveness and selectivity of various ET receptor antagonists.

The pulmonary dysfunction during sepsis includes pulmonary hypertension, hypoxemia, and low lung compliance and may progress to acute respiratory distress syndrome (ARDS) (20). In this state, which is associated with a mortality rate of approximately 50% (21), the lung may become a net ET-1 producer and contribute to increased plasma ET-1 (22, 23). In healthy lungs, ETs are synthesized by bronchial epithelial cells, endothelial cells, macrophages, vascular smooth-muscle cells, and pulmonary neuroendocrine cells (24). ETs modulate pulmonary vascular tone through potent stimulation of pulmonary vascular smooth muscles (24). In the present study, we found that the expression of preproET-1 was increased in lung tissue after LPS administration. ET-1 is produced from prohormones known as preproETs. Previous studies have documented that the ET-1 concentration is elevated in the lungs of endotoxemic animals (25). The concept that ET-1 is

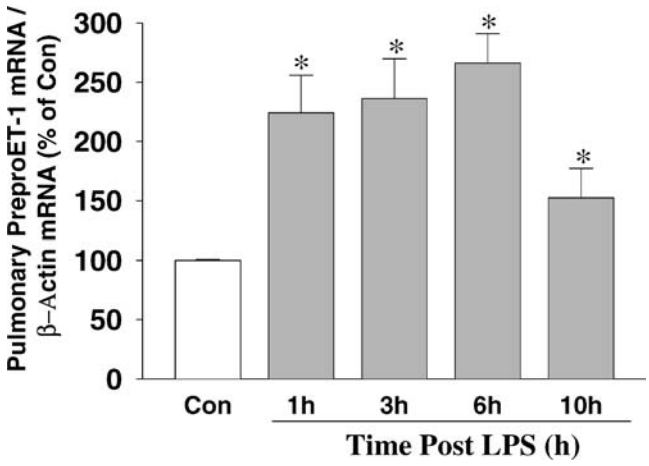


**Figure 2.** Changes in mRNA expression of preproeET-1 in lung tissue in SD rats after LPS administration and in control rats. Pulmonary tissue expression of the gene encoding preproET-1 gene was determined by real-time PCR ($n = 10$ for each group). Results are expressed as relative values (% of control). Results are mean ± SD. $^*P < 0.01$, with respect to control.

involved in the pathogenesis of acute lung injury during sepsis has come from the observation that ET-1 increases capillary fluid leakage and potentiates leukotoxin-induced lung edema (26). In humans with ARDS, not only is plasma ET-1 elevated but pulmonary ET-1 clearance is also reduced (27). Increased ET-1 level in septic lung may increase pulmonary vascular pressure. Because ET-1 may act as an immunomodulator, an increase in ET-1 may contribute to lung injury by inducing expression of cytokines, including tumor necrosis factor, IL-6, and IL-8. The lung is, however, a highly important organ under septic conditions, and more-detailed studies should address the gene- and protein-based regulation of different components of the ET system in a time-dependent manner after LPS administration.

Many investigators attributed the increase in pulmonary NO synthesis in animals with sepsis to the induction of iNOS expression, particularly in airway epithelial cells and macrophages. Significant attenuation of various indices of acute lung injury and prevention of microvascular leakage by selective iNOS inhibitors has strengthened the role of iNOS in LPS-induced acute lung injury (28). In the present study, we also found an approximately 50-fold increase in iNOS gene expression in lung tissue after LPS administration. This elevated iNOS expression might have a role in the cases of acute lung injury seen in the present study. ET-1 may also contribute to the development of acute lung injury in animals with sepsis through enhanced NO synthesis. ET-1 may act in autocrine, paracrine, and endocrine fashions to stimulate pulmonary NO synthesis through the induction of iNOS expression. Enhanced ET production by pulmonary endothelial cells and airway epithelial cells in response to LPS injection can activate $ET_A$ receptors and lead to an increase in intracellular cAMP synthesis and activation of the protein kinase C pathway,

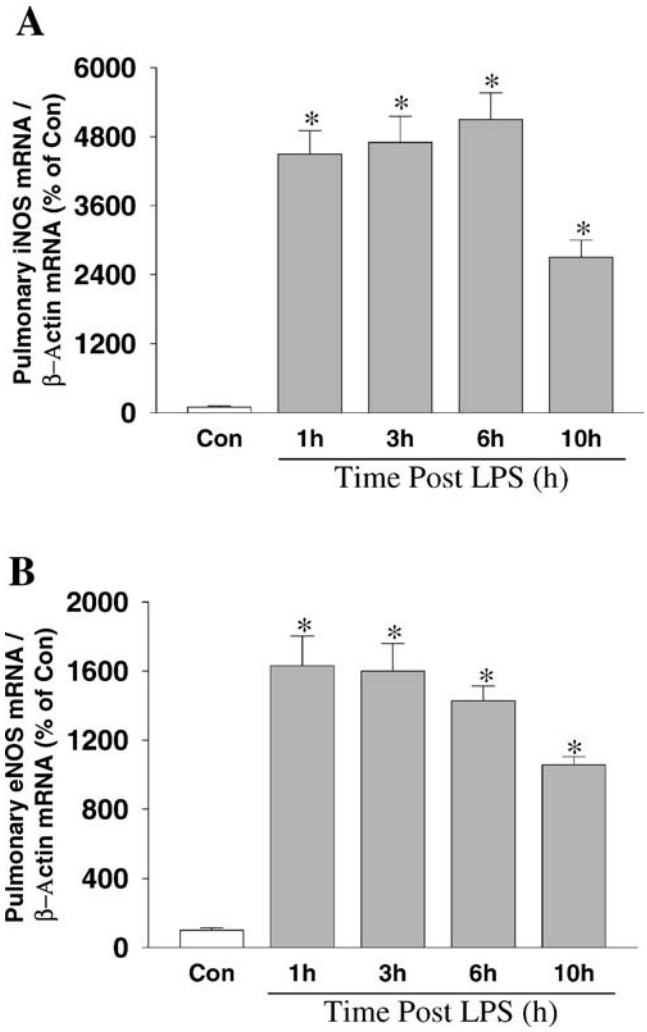


**Figure 3.** Changes in mRNA expression of iNOS (A) and eNOS (B) in lung tissue in SD rats after LPS administration and in control rats. Pulmonary tissue expression of the genes encoding iNOS and eNOS was determined by real-time PCR ($n = 10$ for each group). Results are expressed as relative values (% of control). Results are mean ± SD. $^*P < 0.01$, with respect to control.

both of which are known to cause transcriptional upregulation of iNOS expression (29).

The low concentration of NO produced by eNOS under normal physiological conditions is one of the major regulators of arterial blood pressure and regional blood flow. Some investigations have proposed that the maintenance of basal NO synthesis is critical for organ perfusion and survival in endotoxin shock and that the deleterious effects of NOS inhibitors in endotoxin shock might be related to the blockade of eNOS (30). Little is known about the potential roles of eNOS in endotoxin shock. Long-term eNOS overexpression prevented LPS-induced death by attenuating lung injury in eNOS transgenic mice and by the secondary prevention of organ damage due to the maintenance of blood pressure and organ perfusion after LPS injection (31). However, conflicting results also exist. eNOS knockout mice were as susceptible to LPS-induced

death as wild-type mice (32). But in the present study, we found that expression of the gene encoding eNOS in lung tissue was 16-fold higher in SD rats after LPS administration. We think that eNOS gene upregulation may be a compensatory change after LPS administration. In the late hours after LPS administration, eNOS expression began to be downregulated in the present study. Thus, the pathogenesis of acute lung injury after LPS administration in the rat model with normal eNOS expression and in the mouse model with eNOS overexpression or eNOS knockout may be different. Future studies should evaluate eNOS expression in the rat model after longer LPS exposure. The present study cannot rule out that overexpression of eNOS gene plays a role in the increased pulmonary NO production in SD rats after LPS administration.

In the present study, preproET-1 was upregulated in lung tissue after LPS administration in SD rats. In addition, the expression of the genes encoding iNOS and eNOS was also increased in lung tissue after LPS administration. Again, LPS caused the decrease in both systolic and diastolic blood pressure. Thus, there might be a loss of balance in the complex and tight interaction and regulation of ET-1 and NOS in lung tissue, as well as in systemic circulation, in rats after LPS administration.


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