Role of Nitric Oxide in Lipopolysaccharide-Induced Acute Lung Injury and Lipid Peroxidation in Rats

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Abstract

Background: Nitric oxide (NO) may act as an oxygen radical scavenger or as an antioxidant, and inhibit neutrophil superoxide anion production. In contrast, NO combines with superoxide to form peroxynitrite, a very damaging material whose decomposition results in the generation of a hydroxyl radical. This study was designed to determine the role of NO in the development of acute lung injury and lipid peroxidation induced by lipopolysaccharide (LPS) in rats.

Methods: Male Sprague-Dawley rats (200–250 g) were given one of the following treatments: intraperitoneal normal saline 0.5 ml, intraperitoneal E. coli LPS (5 mg/kg) in 0.5 ml normal saline, 4 mg/kg L-N^2-(1-iminoethyl)lysine (L-NIL) + LPS, or L-arginine (80 mg/kg) + LPS. Four hours after treatment, the rats were killed by an intraperitoneal pentobarbital injection (100 mg/kg) and plasma nitrate/nitrite concentration (Griess reagents) and lipid peroxide (LPO) concentration of the lung (Yagi’s method) were measured (n = 8). In the other sets of experiments, myeloperoxidase activity of the lung (n = 5) and protein concentration of the bronchoalveolar lavage (BAL) fluid (BCA protein assay reagents, n = 4) were assayed.

Results: LPS treatment increased plasma nitrate/nitrite concentrations approximately 6 times (20.9 ± 1.8 μM, P < 0.01) compared with the control group (3.6 ± 0.7 μM), and L-NIL treatment prevented this increase. L-NIL plus LPS treatment resulted in greater increase of LPO concentrations of the lung compared with the control (P < 0.05). Myeloperoxidase activity and protein concentrations of BAL fluids were higher in LPS and L-NIL plus LPS treatment groups than the control group.

Conclusions: These results suggest that inhibition of the increase of NO by selective inducible NO synthase inhibitor L-NIL may increase lipid peroxidation in septic rats. (Korean J Anesthesiol 2001; 41: S 7–S 12)


INTRODUCTION

Gram-negative sepsis (GNS) is characterized by hypotension, multiorgan dysfunction, and poor tissue perfusion. The morbidity and mortality rate for GNS remains high despite intensive antibiotic therapy and patient management. GNS is also the most common cause
of acute respiratory distress syndrome. \(^2\) Bacterial endotoxin or lipopolysaccharide (LPS) is believed to mediate the tissue damage and shock observed in GNS by initiating a cascade of events, including activation of the coagulation, fibrinolytic, and complement systems, and release of proinflammatory cytokines. \(^5\) Lung cell injury caused by endotoxin is probably mediated at least in part by generation of free radicals. Inflammatory cells, especially neutrophils, are one source of these toxic oxygen species, but intracellular generation of free radicals within lung cells per se may also be stimulated by endotoxin and account for some of the lung injury. \(^7\)

Proinflammatory cytokines and endotoxin (bacterial LPS) induce the expression of a distinct inducible isoform of nitric oxide synthase (iNOS) in various cell types. \(^5.6\) The production of large amounts of NO by iNOS has been implicated in the pathologic vasodilatation and hypotension associated with the sepsis syndrome. \(^5.6\) NO inhibits platelet aggregation and adhesion, expression of adhesion molecules on the neutrophil surface, \(^7\) hypertensive glomerular damage, \(^8\) and bronchoconstriction. In the pulmonary tissues, NO opposes the pulmonary vasconstrictor response to hypoxia and thus causes an increased shunt fraction. \(^7\)

NO can act as an oxygen radical scavenger \(^9\) or as an antioxidant, \(^11\) and inhibit neutrophil superoxide anion production. \(^12\) However, NO combines with superoxide to form peroxynitrite, a very damaging material, whose decomposition results in the generation of a hydroxyl radical. \(^13\) Therefore, it remains to be established whether the interaction between NO and oxygen free radicals leads to an increased toxicity or reduced it. The present study was aimed to determine the role of NO in the acute lung injury and lipid peroxidation induced by LPS in rats.

**METHODS**

**Animals**

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Konyang University Medical School. Animals were housed with free access to standard rodent diet and water. Male Sprague-Dawley rats (Charles River Japan, Japan) weighing 200 to 250 g were divided into four groups.

**Experimental protocol**

Group 1 received 0.5 ml normal saline intraperitoneally. Group 2 received intraperitoneal Escherichia coli LPS (serotype 0127 : B8) 5 mg/kg in 0.5 ml normal saline. Group 3 was given 4 mg/kg L-NIL (Sigma Chemical, USA) dissolved in normal saline and LPS 5 mg/kg. Group 4 was given L-arginine (Sigma Chemical, USA) 80 mg/kg in normal saline and LPS 5 mg/kg. Four hours after treatments, the rats were killed by an intraperitoneal pentobarbital injection (100 mg/kg) and plasma nitrate/nitrite concentration and lipid peroxide (LPO) concentration of the lung were measured \((n = 8)\). In the other sets of experiments, myeloperoxidase activity of the lung \((n = 5)\) and protein concentration of the bronchoalveolar lavage (BAL) fluids \((n = 4)\) were assayed.

**Nitrate/nitrite assay**

Nitrate/nitrite concentration was determined by a spectrophotometric method based on the Griess reaction. \(^4\)

Briefly, 200 \(\mu\)l samples of plasma were mixed with nitrate reductase and enzyme cofactor mixture at room temperature for 3 h and added with 50 \(\mu\)l of 30% zinc sulfate and 740 \(\mu\)l of distilled water. After mixing using vortex, the samples were centrifuged at 14,000 rpm for 4 min. One half ml of supernatant was mixed with 500 \(\mu\)l of Griess solution, consisting of 1% sulfanilic acid, 0.1% naphthylethylendiamine dihydrochloride, and 2.5% phosphoric acid, and incubated for 20 min at room temperature. The absorbance of the reaction product reads 540 nm. The concentrations were determined from a linear standard curve between 1 and 50 \(\mu\)M of sodium nitrite.

**Measurement of LPO**

A portion of the excised lung was homogenized with a fivefold dilution of phosphate buffered saline using a high-speed homogenizer. The supernatant was used as a sample after centrifugation of the homogenate for 15 min at 1,000 g. LPO was measured by Yagi’s method \(^5\) using 1,1,3,3-tetramethoxypropane as a standard. One half ml of
diluted supernatant was mixed with 4 ml of 1/12 N H\textsubscript{2}SO\textsubscript{4} and 0.5 ml of 10% phosphotungstic acid and the mixture was centrifuged at 4,000 rpm for 10 min. The sediment was suspended in 4 ml of distilled water, and 1 ml of TBA reagent (0.67% thiobarbituric acid solution + glacial acetic acid, 1:1) was added. After the reaction mixture was heated at 95°C for 60 min, 5 ml of n-butanol were added and the mixture shaken vigorously. After centrifugation at 4,000 rpm for 15 min, the n-butanol layer was taken for fluorescence spectrophotometric measurement (Hitachi F-2000, Hitachi Ltd., Japan) which was made at 515 nm excitation and 553 nm emission.

**Myeloperoxidase activity of lung**

A portion of the excised lung was homogenized for 30 to 45 sec. Samples were sonicated for 30 sec and underwent centrifugation at 2,300 g for 30 min at 4°C. The spectrophotometric assay buffer (composed of 0.0005% hydrogen peroxide and 0.167 mg/ml of o-dianisidine dihydrochloride in 100 mM of phosphate buffer, pH 6.0) was prepared at room temperature. Fifty µl of each sample was mixed with 1.45 ml of assay buffer, and the change in absorbance at 460 nm was measured.\textsuperscript{16} Myeloperoxidase activity was defined as the δ optical density at 460 nm over 1 min.

**Protein concentration of BAL fluids**

BAL (35 ml/kg) was performed 4 times with phosphate buffered saline (PBS, pH 7.4). BAL fluids were pooled from the four lavages and centrifuged at 400 g for 10 min. Protein concentration of the supernatant was measured by BCA protein assay kit (Pierce, USA). The absorbance of the reaction product reads 562 nm and the concentrations were determined from a linear standard curve between 20 to 2,000 µg/ml of bovine serum albumin.\textsuperscript{17}

**Statistical analysis**

Data are expressed as the mean ± SE. Statistical analysis was performed using one-way analysis of variance, followed by the Student-Newman-Keuls test for multiple comparison. Differences were considered to be statistically significant when P < 0.05.

**RESULTS**

**Nitrate/nitrite concentration of plasma**

LPS treatment increased plasma nitrate/nitrite concentrations approximately 6 times (20.9 ± 1.8 µM, P < 0.01) compared with the control group (3.6 ± 0.7 µM) and selective iNOS inhibitor L-NIL treatment prevented this increase. L-arginine plus LPS treatment also increased plasma nitrate/nitrite concentrations (15.4 ± 2.8 µM, P < 0.01), but these increases were comparable with those obtained in LPS only group (Fig. 1).

![Fig. 1. Plasma nitrate/nitrite concentrations (µM) at 4h after each treatment. Rats were treated with normal saline, LPS (5 mg/kg), L-NIL (4 mg/kg) plus LPS, or L-arginine (80 mg/kg) plus LPS. Values are presented as mean SE (n = 8). *P < 0.01 versus control.](image)

**LPO concentrations of lung (µM) at 4h after each treatment. Values are presented as mean ± SE (n = 8). †P < 0.05 versus control.**

![Fig. 2. LPO concentrations of lung (µM) at 4h after each treatment. Values are presented as mean ± SE (n = 8). †P < 0.05 versus control.](image)
LPO measurements

LPO concentrations of the lung were higher in L-NIL plus LPS treatment group (12.8 ± 0.26 μM, P < 0.05) than in saline treated control group (11.3 ± 0.26 μM) (Fig. 2).

Myeloperoxidase activity of lung

Lung myeloperoxidase activity was significantly increased in LPS (0.248 ± 0.004 d460 nm, P < 0.01) and L-NIL plus LPS treatment (0.238 ± 0.015 d460 nm, P < 0.01) groups compared with the control group (0.134 ± 0.011 d460 nm) (Fig. 3).

Protein concentration of BAL fluids

Protein concentrations of BAL fluid were increased more in LPS (391.8 ± 65.3 μg/ml, P < 0.05) and L-NIL plus LPS (372.7 ± 17.4 μg/ml, P < 0.05) groups compared with the control (191.5 ± 9.6 μg/ml) (Fig. 4).

DISCUSSION

Because NO contains an unpaired electron and is paramagnetic, it rapidly reacts with superoxide to form peroxynitrite in high yield. During physiologic conditions, superoxide is scavenged by endogenous superoxide scavengers (e.g., superoxide dismutase) and formation of peroxynitrite is minimal. During pathologic conditions, such as in the presence of increased concentrations of superoxide or after superoxide scavengers are exhausted, significant concentrations of peroxynitrite may be produced. Peroxynitrite directly causes oxidation, peroxidation, and nitration of biologically important molecules (e.g., lipids, proteins, DNA). Polysaturated fatty acids are particularly vulnerable to free radical attack by the process of hydrogen abstraction, causing membrane damage through lipid peroxidation, an irreversible reaction that occurs when unsaturated fatty acids react with oxygen radicals in the presence of iron.

However, NO has contrasting effects on lipids, particularly on the oxidation of low-density lipoproteins in the pathogenesis of atherosclerotic lesions. NO can inhibit lipid peroxidation by inhibiting radical chain propagation reactions via radical-radical reaction with lipid peroxyl and alkoxyl groups. As a ligand to iron and other transition metals, NO modulates the prooxidant effects of iron and thereby limits the formation of hydroxyl radicals and iron-dependent electron-transfer reactions. NO inhibits cell and peroxynitrite-mediated lipoprotein oxidation in macrophage and endothelial cell systems.

In this study LPS increased plasma nitrate/nitrite concentrations, myeloperoxidase activity of lung, and protein concentration of BAL fluids. Selective iNOS inhibitor L-NIL prevented the increase of plasma nitrate/nitrite by LPS. However, L-NIL treatment results in increased LPO concentrations and myeloperoxidase activity of lung and produced protein concentrations of BAL fluids sig-

Fig. 3. Myeloperoxidase activity of lung (d460 nm) at 4 h after each treatment. Values are presented as mean ± SE (n = 5). *P < 0.01 versus control.

Fig. 4. Protein concentrations of BAL fluid (μg/ml) at 4 h after each treatment. Values are presented as mean ± SE (n = 4). †P < 0.05 versus control.
significantly above those seen in control rats. These results suggest that NO may act as an antioxidant and therefore inhibition of the increase of NO by selective iNOS inhibitor may increase lipid peroxidation in septic rats. However, Rubbo et al.\(^{25}\) have demonstrated that NO can both stimulate oxygen radical-induced lipid peroxidation and mediate oxidant-protective reactions in membranes at higher rates of NO production, with the prooxidant versus antioxidant outcome critically dependent on relative concentrations of individual reactive species. Thus additional studies may be required to determine whether increased NO acts as an antioxidant or not in various physiologic states of septic rats.

Systemic changes in septic syndrome are related with complex mechanisms including activation of the coagulation, fibrinolytic and complement systems, and release of proinflammatory cytokines. Activation and accumulation of polymorphonuclear cells are the initial events of tissue injury caused by release of oxygen free radicals, arachidonic acid metabolites, and lysosomal proteases.\(^{26}\) Extravasated polymorphonuclear cells become activated once in the inflammatory sites to secrete a variety of substances, such as growth factors, chemokines and cytokines, complement components, proteases, NO, and reactive oxygen metabolites, which are important mediators of tissue injury.\(^{27-29}\) Although L-NIL treatment elevated LPO concentrations, myeloperoxidase activity of lung, and protein concentrations of BAL fluids compared with control in our study, it did not increase those parameters over LPS group. Therefore further studies are required to determine whether the inhibition of the increase of NO by selective iNOS inhibitor affects lung injury or not in septic rats.

NO is synthesized via oxidation of the terminal guanidine-nitrogen atom of L-arginine by NOS in various cells, including vascular endothelium, platelets, macrophages, neutrophils, hepatocytes, and neural cells.\(^{20}\) Pre-treatment with L-arginine prevents the increase in LPO and the decrease in the synthesis and secretion of mucus induced by hypoxia-reoxygenation in gastric cells.\(^{31}\) However, L-arginine treatment did not increase the plasma nitrate/nitrite concentrations compared with LPS group in our study and McCall et al.\(^{22}\) presented that it does not affect the basal production of NO. This suggests that there are sufficient substrate for basal NO synthesis and L-arginine mobilization is not enhanced under conditions when the NOS is activated in vivo.

In order to measure plasma nitrate/nitrite concentrations, we used the Griess reagent.\(^ {11}\) However, as noted by others,\(^ {32,34}\) the Griess reaction gives unspecific reactions in protein-rich solutions such as plasma, serum, and especially in urine. Thus we assayed plasma nitrate/nitrite concentrations after using 30% zinc sulfate as a deproteinization step in this study.\(^ {35}\)

In summary, LPS-treated rats receiving L-NIL had higher LPO concentrations, myeloperoxidase activity of the lung, and protein concentrations of the BAL fluids than the control group. These results suggest that inhibition of the synthesis of nitric oxide by selective iNOS inhibitor L-NIL may increase lipid peroxidation in septic rats.

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