Effect of Inhaled \(N^G\)-Nitro-L-Arginine Methyl Ester on Candida-Induced Acute Lung Injury*

Kazuhiro Nagata, MD; Yoshinobu Iwasaki, MD, PhD; Yoshizumi Takemura, MD; Hidehiko Harada, MD; Ichiro Yokomura, MD, PhD; Shinji Fushiki, MD, PhD; and Masao Nakagawa, MD, PhD

**Study objectives:** Nitric oxide (NO) and peroxynitrite play a crucial role in acute lung injury (ALI). Whether NO synthase (NOS) inhibition is beneficial in the treatment of lung injury remains controversial. The objective of this study was to test the hypothesis that local inhibition of NOS in the lung reduces lung injury.

**Design:** We developed a model of Candida-induced ALI in the mouse by IV injection of *Candida albicans*. To evaluate the effect of NOS inhibitor, mice were pretreated by inhalation of saline solution or \(N^G\)-nitro-L-arginine methyl ester (L-NAME) before induction of Candida-induced ALI.

**Measurements and results:** After inhalation of 1 mM aerosolized L-NAME, nitrite-nitrate concentrations in BAL fluid (BALF) were significantly lower at 24 h and 48 h than those in mice treated with *C. albicans* alone. Tumor necrosis factor-\(\alpha\), interleukin-1\(\beta\), and macrophage inflammatory protein-2 concentrations in lung homogenates, measured by enzyme-linked immunosorbent assay, and neutrophil counts in BALF were decreased by inhalation of L-NAME (\(n = 6\) per group). Immunohistochemical analysis of inducible NOS (iNOS) and nitrotyrosine, a major product of protein nitration by peroxynitrite, revealed that alveolar macrophages and alveolar epithelial cells were positive for both substances in Candida-induced ALI. Inhalation of L-NAME markedly suppressed iNOS protein expression and nitrotyrosine production. Histologic evidence of lung injury decreased and survival improved after inhalation of L-NAME.

**Conclusions:** We conclude that NO might play a crucial role in the pathogenesis of Candida-induced ALI, and such injury might be reduced by local inhibition of NOS. Our findings suggest that inhalation of L-NAME is beneficial in the treatment of Candida-induced ALI.

(\*CHEST 2003; 124:2293–2301)

**Key words:** acute lung injury; Candida; \(N^G\)-nitro-L-arginine methyl ester; nitric oxide

**Abbreviations:** ALI = acute lung injury; BALF = BAL fluid; CA = aerosolized saline solution; IL = interleukin; iNOS = inducible nitric oxide synthase; L-NAME = \(N^G\)-nitro-L-arginine methyl ester; MIP = macrophage inflammatory protein; NO = nitric oxide; NOS = nitric oxide synthase; PBS = phosphate-buffered saline solution; TNF = tumor necrosis factor

*Candida albicans* is increasingly responsible for nosocomial sepsis in critically ill patients who receive intensive care procedures involving intravascular catheters, broad-spectrum antibiotics, extensive surgery, and immunosuppression for neoplastic disease or allograft preservation.\(^1\)–\(^3\) Candida-induced acute lung injury (ALI) is closely associated with candidiasis.\(^4\) In the presence of both conditions, mortality exceeds 60%. Experimentally, severe candidiasis increases the serum tumor necrosis factor (TNF) level and lung wet/dry ratio. Moderate ALI with capillary obstruction and interstitial hemorrhage is associated with yeast-mycelial transformation\(^5\); however, few reports have described the mechanism of Candida-induced ALI.

Nitric oxide (NO) is a highly reactive radical

---

*From the Department of Medicine (Drs. Nagata, Iwasaki, Takemura, Harada, Yokomura, and Nakagawa), Kyoto Prefectural University of Medicine; and Department of Pathology and Applied Neurobiology (Dr. Fushiki), Research Institute for Neurologic Diseases and Geriatrics, Kyoto Prefectural University of Medicine, Kyoto, Japan.
Manuscript received July 16, 2002; revision accepted June 18, 2003.
synthesized from L-arginine by the action of NO synthase (NOS). The levels of nitrate/nitrite in serum or BAL fluid (BALF) are elevated in patients with ARDS and correlate with disease severity. Many experimental studies of ALI have shown the presence of inducible NOS (iNOS) and nitrotyrosine, markers of peroxynitrite tissue damage, in alveolar spaces. Thus, NO generated by iNOS may play a critical role in the pathogenesis of ALI. However, whether a reduction in endogenous NO by NOS inhibitors is beneficial in ALI remains controversial because NO has a wide array of activity. For example, NO alters cytokine expression, causes vasodilatation, damages tissue, and kills microbes. In all studies examining the effects of NOS inhibition on ALI, NOS inhibitors were systemically administered by IV, intraperitoneal, or subcutaneous injection.

We hypothesized that NO generated by iNOS might play a crucial role in Candida-induced ALI by forming peroxynitrite, and that local reduction of NO in the lung might improve lung injury and outcome. We thus developed a model of ALI by administering C. albicans to mice. In addition, to evaluate the effect of locally reducing of NO in lung, the mice were pretreated by inhalation of N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) before C. albicans administration.

**Materials and Methods**

**Animals**

These experiments were approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University of Medicine. Specific pathogen-free BALB/c mice (5- to 6-week-old male mice) [Japan SLC; Kyoto, Japan] were used in all experiments. All mice were housed in the animal care facility at Kyoto Prefectural University of Medicine until the end of the experiments.

**C. albicans**

C. albicans (TIMN 1623; a gift from Teikyo University; Tokyo, Japan) was maintained at −85°C in Sabouraud broth supplemented with 5% dimethylsulfoxide, and was transferred to Sabouraud dextrose agar (Eiken Chemical; Tokyo, Japan) at 37°C before use. Yeast-phase blastospores for infusion were suspended in sterile saline solution, sedimented (400g, 10 min, 4°C), and resuspended in sterile saline solution to 5 × 10\textsuperscript{7}/mL as determined with a hemocytometer.

**Experimental Protocols**

Mice were infected IV with 10\textsuperscript{7} C. albicans cells via a caudal vein to develop a model of Candida-induced ALI. Via a nose-only aerosol chamber, mice received aerosolized saline solution (SA) [CA group] or various concentrations of L-NAME (L-NAME inhalation group) for 2 h before C. albicans administration with an ultranebulizer (NE-U17; Omron; Kyoto, Japan), driven at a rate of 0.75 mL/min. The ultranebulizer produces particles 1 to 8 μm in diameter. It is estimated that particles 2 to 5 μm in diameter reach and are deposited in small airways or alveoli. L-NAME (Sigma Chemical; St. Louis, MO) was dissolved in saline solution at concentrations of 0.01 mM, 0.1 mM, 1 mM, or 10 mM. The mice in the control group were exposed to SA for 2 h and then received an injection of 0.2 mL of saline solution.

**BAL**

After anesthetization (pentobarbital, 60 mg/kg intraperitoneal), the trachea was exposed and intubated with a 27-gauge needle. BAL was performed by administration of 0.5 mL of sterile saline solution three times, and the total cell counts in BALF were determined with a hemocytometer. Fluid recovery was routinely ≥90%. Differential cell counts were performed with the use of cytospin preparations (Sakura; Tokyo, Japan) stained with Giemsa-type stain (Giff-Quick; American Scientific; McGaw Park, IL). The BALF was immediately centrifuged at 500g for 10 min at 4°C, and the cell-free supernatant was stored at −80°C for nitrite/nitrate analysis.

**Nitrite and Nitrate Analysis**

The BALF supernatant was thawed and mixed with methanol (1:1, volume/volume) to precipitate protein and then centrifuged at 10,000g for 20 min at 4°C. The supernatant was applied to a nitrogen oxides analyzing system (ENO-11; EICOM; Kyoto, Japan). This system utilizes the high-performance liquid chromatography-Griess method, and nitrite/nitrate are measured separately (n = 6 per group).

**Lung Tissue and Blood Sampling for Cytokine Analysis**

Before removal of the lungs, the pulmonary vasculature was perfused via the right ventricle with 1 mL of phosphate-buffered saline solution (PBS) containing 5 mM ethylenediamine tetraacetic acid. After removal, the whole lung was homogenized in 3 mL of lysis buffer containing 0.5% Triton-X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl\textsubscript{2}, and 1 mM MgCl\textsubscript{2} (pH 7.4), using a tissue homogenizer (Dremel; Racine, WI). The homogenate was incubated on ice for 30 min and then centrifuged at 3,000g for 10 min at 4°C. The supernatant was collected, passed through a 0.45-μm pore size filter (Gelman Science; Ann Arbor, MI) and then stored at −30°C until assessment of cytokine levels. Venous blood was drawn by cardiac puncture. The blood sample was immediately kept on ice and centrifuged at 3,000g for 10 min at 4°C. The serum was aspirated and stored at −30°C until analysis.

**Enzyme-Linked Immunosorbent Assay of Cytokines**

Mouse interleukin (IL)-1β, TNF-α, and macrophage inflammatory protein (MIP)-2 concentrations in the supernatant of the lung homogenate, and mouse IL-1β and TNF-α concentrations in serum were measured with the use of Quantikine M mouse IL-1β, TNF-α, and MIP-2 immunoassay kits (R&D Systems; Minneapolis, MN) [n = 6 per group].

**Determination of Colony-Forming Units of C. albicans in Lung Tissue**

Viable C. albicans within the lungs of infected mice was quantified by colony counting. Both lungs were removed aseptically and homogenized in 10 mL of sterile saline solution with a
tissue homogenizer. Twenty microliters of each lung homogenate was placed on Sabouraud dextrose agar and incubated for 24 h at 37°C, after which the colonies were counted (n = 6 per group).

**Histologic Examination of Lung**

After anesthetization, the trachea was exposed and intubated with a 27-gauge needle 24 h after infection. The lungs were inflated by administration of 0.5 mL of 4% paraformaldehyde. For hematoxylin-eosin staining, the lungs were removed en bloc and placed in freshly prepared 4% paraformaldehyde overnight. After fixation, the lungs were embedded in paraffin and cut to a thickness of 4 μm. They were then stained with hematoxylin-eosin (n = 3 per group).

**Immunofluorescence for iNOS**

The cells in BALF were resuspended in PBS cyt centrifuged onto glass slides, fixed in 4% paraformaldehyde for 30 min, and washed three times with PBS. The specimens were incubated for 24 h with polyclonal anti-mouse iNOS antibody (diluted 1/100). The glass slides were rinsed three times with PBS and incubated for 2 h with secondary fluorescein isothiocyanate-conjugated anti-mouse IgG (diluted 1/100; DAKO A/S; Glostrup, Denmark) for iNOS. After rinsing five times with PBS, the specimens were counterstained with propidium iodide and mounted in a shield (Vector; Vector Laboratories; Burlingame, CA) to prevent fluorescence decay (n = 3 per group).

**Immunohistochemistry for iNOS and Nitrotyrosine**

Paraffin-embedded lung tissue was processed for immunohistochemical analysis. The staining was performed as previously described, with minor modifications. The sections were dewaxed, dehydrated, and incubated with 10% normal goat serum to block nonspecific protein absorption. The sections were then incubated with polyclonal anti-mouse iNOS antibody (diluted 1/500; Affinity Bioreagents; Golden, CO), anti-nitrotyrosine polyclonal antibody (diluted 1/100; Upstate Biotechnology; Lake Placid, NY), or nonimmune rabbit IgG at 4°C overnight. A Vectastain ABC kit (Vector Laboratories) was used to visualize antibody binding. Bound antibodies were detected with biotinylated horse universal IgG secondary antibodies and the streptavidin-peroxidase complex, using diaminobenzidine tetrahydrochloride as substrate. Sections were counterstained with Mayer hematoxylin (n = 3 per group).

**Analysis of Outcome**

Survival was assessed in 20 mice classified into two subgroups (CA group and L-NAME inhalation group; n = 10 per group) treated IV with 10⁷ C albicans cells. Survival was observed over the course of 5 days. Survival was estimated by the Kaplan-Meier method, and curves were compared by log-rank tests.

**Statistical Analysis**

All data, expressed as means ± SE, were analyzed by one-way analysis of variance. The statistical significance of differences between groups was assessed by the Fisher protected least-significant difference test; p < 0.05 was considered to indicate statistical significance.

**RESULTS**

**Nitrite and Nitrate Levels in BALF**

To determine the optimal concentration of aerosolized L-NAME, mice were exposed to CA or various concentrations of L-NAME for 2 h before C albicans administration. Mice were anesthetized and killed before BAL. BALF nitrite/nitrate levels significantly increased 24 h after administration of C albicans in the CA group. The nitrite/nitrate levels after candidemia were inhibited by L-NAME in a dose-dependent manner by concentrations from 0.01 to 1 mM. Nitrite/ nitrate levels were similar after treatment with aerosolized 1 mM and 10 mM L-NAME. After pretreatment with aerosolized 1 mM L-NAME, the nitrite/nitrate level was significantly lower than that in the CA group (Fig 1). To confirm the duration of the effect of aerosolized L-NAME, mice were exposed to saline solution or 1 mM L-NAME for 2 h before C albicans administration.
administration. BAL was performed 0 h, 24 h, and 48 h after *C. albicans* administration.³⁻⁵ After pretreatment with 1 mM aerosolized L-NAME for 2 h before administration of *C. albicans*, the increases in the BALF nitrite/nitrate levels induced by *C. albicans* were significantly inhibited at 24 h and 48 h (Fig 2). Aerosolized L-NAME significantly inhibited the production of NO in Candida-induced ALI for at least 48 h. We therefore selected 1 mM aerosolized L-NAME (L-NAME inhalation group) for subsequent treatment in our study.

### Cytokine Levels in Lung Tissue

To confirm the influence of inhaled L-NAME on cytokine production, cytokine analysis was performed 24 h after infection. The IL-1β, TNF-α, and MIP-2 levels in lung tissue from the CA group were significantly higher than the corresponding levels in lung tissue from the control group. The IL-1β, TNF-α, MIP-2 levels in lung tissue from the L-NAME inhalation group were significantly lower than those in lung tissue from the CA group (Fig 3).

### Neutrophils in BALF

To confirm the influence of inhaled L-NAME on neutrophil accumulation, BAL was performed 24 h after infection. The number of neutrophils in BALF from the CA group was significantly higher than that in BALF from the control group. The number of neutrophils in the L-NAME inhalation group was significantly lower than that in the CA group (Fig 4, left, a).

### Lung Colony-Forming Units

To confirm the influence of inhaled L-NAME on candidal infection in lung, candidal colony-forming units in lung were measured 24 h after infection. The

---

**Figure 3.** Effect of L-NAME inhalation on TNF-α (left, a), IL-1β (center, b), and MIP-2 (right, c) levels in lung tissue after IV injection of *C. albicans*. Data are given as means ± SEM of six animals. *p < 0.001 as compared with CA group.

**Figure 4.** Left, a: Effect of L-NAME inhalation on neutrophils in BALF after IV injection of *C. albicans*. Right, b: Effect of L-NAME inhalation on colony-forming units (CFU) in lung tissue after IV injection of *C. albicans*. Data are given as means ± SEM of six animals. *p < 0.001 as compared with CA group.
candidal level in lung tissue cultured after the infusion of $5 \times 10^6$ cfu of *C. albicans* did not differ significantly between the control group and L-NAME inhalation group (Fig 4, right, b).

**Immunofluorescence for iNOS**

The expression of iNOS was significantly elevated in alveolar macrophages in BALF from the CA group. The expression of iNOS in polymorphonuclear cells in BALF was generally faint or negligible. Faint expression of iNOS was found in the L-NAME inhalation group. No expression of iNOS was found in the control group (Fig 5).

**iNOS Immunoreactivity in the Lung**

Paraffin-embedded sections from the CA group exhibited marked immunostaining after treatment with polyclonal antibody to iNOS. Immunoreactivity for iNOS was primarily found in alveolar epithelial cells. In the L-NAME inhalation group, however, immunostaining of iNOS was markedly attenuated, and only light staining of alveolar epithelial cells was observed. No significant immunoreactivity was detected in the control group (Fig 6).

**Immunoreactivity of Nitrotyrosine**

Immunohistochemical images of lung specimens labeled with polyclonal antibody to nitrotyrosine are shown in Figure 7. In the CA group, immunohistochemical staining of protein nitrotyrosine residues was observed throughout the lung. The alveolar epithelial cells were darkly stained. After L-NAME inhalation, the alveolar epithelial cells were lightly stained. Scant staining was observed in lung tissue from the control group.

**Histology**

Twenty-four hours after *C. albicans* injection there was marked inflammatory cell infiltration in the interstitium and airspaces of the lung in the CA group. Alveolar hemorrhage, fibrin deposition, and extravasated erythrocytes in the perivascular interstitium of arterioles were also observed. L-NAME inhalation markedly attenuated the alveolar exudate.
and lung injury. No inflammatory change was observed in the control group (Fig 8).

**Serum Cytokine Levels**

The serum TNF-α and IL-1β levels in the CA group were significantly higher than those in the control group. The serum IL-1β and TNF-α levels in the L-NAME inhalation group were significantly lower than those in the CA group (Fig 9).

**Survival**

By day 2 after infection, mortality was 60% in the CA group. No mortality was noted by day 2 after infection in the L-NAME inhalation group. Median survival was 1.3 days in the CA group and 3.2 days in the L-NAME inhalation group. This difference was statistically significant \((p = 0.006\) by the log-rank test) [Fig 10]. We could not determine the causes of death in our study, but survival was improved by treatment with inhaled L-NAME in mice with lethal candidemia.

**Discussion**

On light microscopic examination, Candida-induced ALI is characterized by cellular aggregates surrounding yeast within blood vessels, extravasated erythrocytes in the perivascula interstitium of arterioles, alveolar hemorrhage, and fibrin deposition. Electron microscopic evaluation shows extensive vascular occlusion by leukocytes containing phagocytized yeast undergoing mycelial transformation to mature hyphae.5 Yokomura et al15 reported that intercellular adhesion molecule-1 plays a role in adherence of \(\text{C albicans}\) to pulmonary vascular endothelial cells, and that anti-intercellular adhesion molecule-1 antibody significantly reduces lung injury in candidemia. The adhesion of yeast to, and hyphal invasion of, the endothelium are considered essential features of Candida-induced ALI.16

Large amounts of NO produced by iNOS induction interact with oxygen free radicals derived from neutrophils and macrophages and form peroxynitrite...
by diffusion-limited reactions. Peroxynitrite causes extensive tyrosine nitration, resulting in the production of nitrotyrosine. Thus, peroxynitrite is considered a potent oxidant causing lung injury. Numerous clinical studies and case reports have demonstrated that inhaled NO decreases pulmonary hypertension and improves hypoxemia in ARDS. In sepsis, associated with overproduction of endogenous NO, the effectiveness of inhaled NO is decreased. Inhaled NO has been shown to prime lung macrophages to produce reactive oxygen and nitrogen intermediates. At present, inhaled NO therapy remains experimental.

We hypothesized that peroxynitrite derived from NO might play a crucial role in the pathogenesis of Candida-induced ALI, and that the inhibition of NOS might reduce lung injury by decreasing peroxynitrite production. However, some studies have shown that inhibition of endogenous NO by NOS inhibitors increases ALI and reduces survival. Walley et al demonstrated that intraperitoneal pretreatment with L-NAME increases proinflammatory cytokine levels and messenger RNA expression in endotoxin-induced ALI. Okamoto et al demonstrated that the subcutaneous administration of ONO-1714, a novel and selective iNOS inhibitor, reduces lung injury and improves survival at intermediate doses, but does not improve survival at low or high doses in rats with septic lung injury. In our preliminary study, pretreatment with IV L-NAME increased proinflammatory cytokine protein levels and reduced survival in mice with Candida-induced ALI (data not shown). These results suggested that excessive inhibition of NO production might diminish potentially beneficial effects (ie, enhancing organ circulation by vasodilatation, scavenging superoxide, and defense against infectious pathogens) and lead to worsening of lung injury and survival. In the present study, mice were pretreated with inhaled L-NAME to inhibit the production of NO and peroxynitrite locally in lung, while maintaining the beneficial systemic effects of these substances. We successfully reduced nitrite/nitrate levels in BALF by inhalation of L-NAME. Immunohistochemically, we demonstrated that inhalation of L-NAME significantly reduced the expression of iNOS and nitrotyrosine residue, used as markers of peroxynitrite tissue damage, in alveolar macrophages and alveolar epithelial cells. Consequently, lung injury was reduced. Wang et al showed that pretreatment with L-NAME inhibits iNOS expression and NO production after endotoxin administration in rats, while L-NAME competes for NO substrate (L-arginine). When NO was reduced or blocked at its origin, less hydroxyl radical and peroxynitrite were

---

**Figure 9.** Effect of L-NAME inhalation on serum TNF-α (left, a) and IL-1β (right, b) levels after IV injection of *C. albicans*. Data are given as means ± SEM of six animals. *p < 0.001 as compared with CA group.

**Figure 10.** Effect of L-NAME inhalation on survival in candidemia. All mice in the two groups were infected IV with 5 × 10⁶ *C. albicans* cells (n = 10 per group).
produced. Consequently, the reduction in oxidant stress by L-NAME induced the down-regulation of iNOS expression.

Proinflammatory cytokines, such as IL-1β and TNF-α, have been implicated in endotoxin shock. Proinflammatory cytokines stimulate the expression of iNOS and promote inflammatory processes. Neutrophil migration in the airway is increased by chemokines, such as MIP-2, derived from alveolar macrophages. TNF-α and IL-1β stimulate chemokine-induced neutrophil chemotaxis. Neutrophils in BALF release free radicals and proteases and induce lung injury in sepsis. We successfully reduced both cytokines in lung and BALF neutrophils by inhaled L-NAME. Our study demonstrated that inhibition of NO in alveolar space decreased proinflammatory cytokines and neutrophil migration to the lung. This might mitigate Candida-induced ALI. The inhalation of L-NAME reduced cytokine levels in serum as well as those in lung in Candida-induced ALI, although we believe that inhalation of L-NAME locally inhibited NO production in lung. Cytokine production was inhibited more strongly in lung than in serum as shown Figure 3 and Figure 9. This result suggests that the increase in serum cytokines was partially attributed to lung cytokines. Conversely, the reduction in lung cytokines might have induced the reduction in serum cytokines. We also demonstrated that inhalation of L-NAME did not affect candidal colony-forming units in lung, although the production of peroxynitrite in airspaces was reduced. This finding suggests that inhalation of L-NAME locally inhibits NOS activity without worsening candidal infection in lung.

We treated mice with inhaled L-NAME before infection in this study. The effect of inhaled L-NAME after the onset of infection should be evaluated in the future studies to confirm the clinical effectiveness of this treatment. In conclusion, our study demonstrated that NO derived from iNOS in alveolar epithelial cells and alveolar macrophages caused tissue injury in a model of Candida-induced ALI by overproduction of peroxynitrite and proinflammatory cytokines in lung and neutrophils accumulation in lung. The inhalation of L-NAME, which locally inhibits NO production, might reduce the production of peroxynitrite and other inflammatory components in lung. These effects may improve outcome in Candida-induced ALI. We also anticipate that inhaled L-NAME might have a beneficial effect on ALI resulting from other causes associated with the overproduction of NO and peroxynitrite.

References

20 Weinberger B, Fakhruzadeh L, Heck DE, et al. Inhaled nitric oxide primes lung macrophages to produce reactive oxygen...