An inhaled inducible nitric oxide synthase inhibitor reduces damage of *Candida*-induced acute lung injury

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ABSTRACT

Excessive nitric oxide (NO) generated by inducible nitric oxide synthase (iNOS) aggravates acute lung injury (ALI) by producing peroxynitrite. We previously showed by immunostaining that the expression of iNOS was suppressed by inhalation of N^G-nitro-L-arginine methyl ester in mice with *Candida*-induced ALI. This study tested the hypothesis that a novel iNOS inhibitor suppresses not only iNOS expression, but also iNOS messenger RNA (mRNA) production by interrupting a positive feedback loop at the time of NO production in *Candida*-induced ALI. Mice were pretreated by inhalation of saline or ONO-1714, a selective iNOS inhibitor, and were given an intravenous injection of *Candida albicans* to induce ALI. After inhalation of 1 mM aerosolized ONO-1714, the nitrite-nitrate concentration in bronchoalveolar lavage fluid (BALF) at 24 h was significantly lower than that after inhalation of saline. Tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) levels and neutrophils in BALF were decreased by inhalation of ONO-1714. Inhalation of ONO-1714 markedly suppressed nitrotyrosine production and inhibited the expression of iNOS mRNA as well as proteins in the lung. Survival was prolonged by inhalation of ONO-1714. We conclude that pretreatment with inhaled ONO-1714 suppresses the production of peroxynitrite and decreases oxidative stress associated with peroxynitrite in *Candida*-induced ALI.

In recent years, fungi have played an increasingly important role as pathogens in nosocomial infections, and nosocomial candidemia is associated with substantial mortality and long-term hospitalization (11, 22). Candidemia is the fourth most common bloodstream infection in the USA at present (2, 20). Increased prevalence of candidemia has been associ-

ated with severity of illness in hospitalized patients, the use of invasive devices or procedures, and the administration of broad-spectrum antibiotics (44). Mortality among patients with candidemia remains high, ranging between 50% and 80% (10, 43).

Nitric oxide (NO) plays an important role in a variety of physiological and pathophysiological processes. Three isoforms of NO synthase (NOS), neuronal NOS (nNOS, type 1), inducible NOS (iNOS, type 2), and endothelial NOS (eNOS, type 3), have been identified. These enzymes produce NO from L-arginine. After injection of lipopolysaccharide (LPS), mice lacking iNOS show not only decreased mortality, but also increase susceptibility to bacterial infection (17, 27, 29, 42). On the other

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hand, excessive production of NO may lead to the refractory hypotension, often observed in patients with septic shock (5, 7, 24, 41).

Previous studies (30, 45, 47) have reported that iNOS can be induced in a variety of cells, including macrophages, by various stimuli, including LPS, interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ). Excessive NO generated by iNOS aggravates acute lung injury (ALI) by producing peroxynitrite. Non-selective inhibition of NO formation could produce side effects by inhibiting the constitutive isoforms of NOS, which are responsible for the various physiological actions of NO.

Several studies (6, 31, 48) have shown that NOS expression is regulated by a feedback regulatory action of NO. However, it is still unclear whether in ALI the feedback mechanism functions. Therefore, we tried to confirm how iNOS expression including mRNA expression occurs in *Candida*-induced ALI; *i.e.*, we hypothesized that iNOS inhibitor suppresses not only iNOS expression, but also iNOS messenger RNA (mRNA) production by interrupting such a feedback mechanism at the time of NO production in *Candida*-induced ALI. We also studied how a specific iNOS inhibitor, ONO-1714, acts on *Candida*-induced ALI by measuring nitrotyrosine protein as an index of oxidative stress caused by peroxynitrite, as well as mRNA expression of iNOS, assessed by Western blot and reverse transcription-polymerase chain reaction (RT-PCR).

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 (4- to 6-week-old males; Japan SLC Co., 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 experiment.

Experimental design. *Candida 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 (400 g, 10 min, 4°C), and resuspended in sterile saline to 5×10^7 cells/mL. ONO-1714, (1S,5S,6R,7R)-7-chloro-3-imino-

no-5-methyl-2-azabicyclo [4.1.0] heptane hydrochloride, was provided by ONO Pharmaceutical Co., Ltd. (Osaka, Japan), and was solubilized with physiological saline immediately before use. We developed a model of *Candida*-induced ALI. Mice received aerosolized saline (non-treatment group) or various concentrations (10^{-3} , 10^{-2} , 10^{-1} , 1, or 10 mM) of ONO-1714 (ONO-1714 inhalation group) for 2 h, followed by an injection of 1×10^7 *C. albicans* cells. The control group was exposed to aerosolized saline for 2 h and then given an injection of 0.2 mL saline.

Analysis of bronchoalveolar lavage fluid (BALF) and immunohistochemistry. The trachea was exposed and intubated with a 27-gauge needle after anesthesia (pentobarbital, 60 mg/kg intraperitoneal). BAL was performed by administration of 0.5 mL sterile saline solution three times, and the count of total cell in BALF was determined with a hemocytometer. Fluid recovery was routinely 90%. Differential cell counts were performed with the use of cytopsin preparations (Sakura, Tokyo, Japan) stained with Giemsa-type stain (Giff-Quick; American Scientific, McGaw Park, IL). The BALF was immediately centrifuged at 500 g at 4°C for 10 min. The cell-free supernatant was stored at -80°C for nitrite/nitrate analysis. Nitrite/nitrate (NOx) were measured as followed. The BALF supernatant was thawed and mixed with methanol (1 : 1, volume/volume) to precipitate protein and then centrifuged at 10^4 g at 4°C for 20 min. 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. Cytokines (TNF- α and IL-1 β) were measured with the use of Quantikine M mouse IL-1 β , TNF-, and MIP-2 immunoassay kits (R&D Systems, Minneapolis, MN). The number of differential cells in the BALF was measured, and iNOS and nitrotyrosine were immunohistochemically examined as followed. Paraffin-embedded lung tissue was processed for immunohistochemical analysis. The section was dewaxed, dehydrated, and incubated with 10% normal goat serum to block nonspecific protein absorption. The section was 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 applied 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. The section was counterstained with Mayer hematoxylin.

RT-PCR. Total tissue RNA was isolated from snap-frozen lungs with the use of an RNeasy (R) Midi Kit (Qiagen Co., Tokyo, Japan) according to the manufacturer's protocol. Total RNA (10 µg) was reverse-transcribed in one step using RT-PCR *high-plus* (Toyobo Co., Osaka, Japan). The following primer sequences were used for iNOS: sense 5'-GGCTTGCCCCCTGGAAGTTTCTCTTCAAA GTC-3' and anti-sense 5'-AAGGAGCCATAATACT GGTGATG-3', with a final PCR product size of 441 base pairs (bp). The following primer sequences were used for β -actin: sense 5'-GAGAAGATGA CCCAGATCATG-3' and anti-sense 5'-GCCATCT CTTGCTCGAAGTC-3', with a final PCR product size of 350 bp (14). PCR was carried out on an iCycler (Bio-Rad Laboratories, Hercules, CA).

Western blot analysis. Aliquots of 30 µg of lung homogenate protein were diluted with sample buffer. Proteins were subsequently transferred to a nitrocellulose membrane (clear blot membrane-P, AE-6665; ATTO Co., Tokyo, Japan). After blocking with non-fat dry milk, the membrane was incubated with a 1 : 500 dilution of the primary antibody of anti-nitrotyrosine (MON 9029; SANBIO BV, Uden, Netherlands). The membrane was subsequently incubated with a 1 : 1000 dilution of anti-mouse IgG (SC-2005; Santa Cruz Biotechnology, Inc., CA) conjugated with peroxidase. The peroxidase reaction was carried out using ECL Western blotting detection reagents.

Pulmonary fluid measurement. The lung wet-to-dry (W/D) weight ratio was determined to assess the severity of pulmonary edema as described previously (25).

Statistical analysis. All data, which are expressed as means $\pm$ SE, were analyzed by one-way analysis of variance. Differences between groups were compared by Fisher's protected least-significant difference test; $p < 0.05$ was considered to indicate statistical significance. Survival of two subgroups (non-treatment group, ONO-1714 inhalation group; $n = 12$ per group) was estimated by the Kaplan-Meier method, and curves were compared by log-rank tests.

RESULTS

Nitrite and nitrate levels in BALF

To determine the optimal concentration of aerosolized ONO-1714, the mice were exposed to saline or various concentrations of ONO-1714 for 2 h before *C. albicans* administration. The mice were anesthetized and euthanized before BAL. BALF NOx levels significantly increased 24 h after administration of *C. albicans* in the non-treatment group. The NOx levels after the induction of candidemia were inhibited by ONO-1714 in a dose-dependent manner by concentrations from 10^{-3} mM to 1 mM. NOx levels were similar after treatment with aerosolized 1 mM and 10 mM ONO-1714. After pretreatment with aerosolized 1 mM ONO-1714, the nitrite/nitrate level was significantly lower than that in the non-treatment group (Fig. 1A). We therefore selected 1 mM aerosolized ONO-1714 (ONO-1714 inhalation group) for subsequent treatment in our study to clearly study the effect of ONO-1714 possibly without other side effects.

Western blot analysis of nitrotyrosine

As shown in Fig. 1B, the amount of nitrotyrosine protein in the lung homogenates from the non-treatment group with *Candida* administration was significantly higher than that in the lung homogenates from the control group. The amount of nitrotyrosine protein in the ONO-1714 inhalation group was significantly lower than that in the non-treatment group.

Immunoreactivity of nitrotyrosine

Immunohistochemical images of lung specimens labeled with polyclonal antibody to nitrotyrosine are shown in Fig. 2. In the non-treatment group, immunohistochemical staining of protein nitrotyrosine residues was observed throughout the lung. The alveolar epithelial cells were darkly stained. After ONO-1714 inhalation, the alveolar epithelial cells were lightly stained. Scant staining was observed in the lung tissue of the control group (not shown).

Cytokine levels in BALF

To examine the influence of inhaled ONO-1714 on cytokine productions, cytokine analysis was performed 24 h after inoculation. The IL-1 β and TNF- α levels in BALF from the non-treatment group were significantly higher than the corresponding levels in the control group. The IL-1 β and TNF- α levels in BALF from the ONO-1714 inhalation group were significantly lower than those in the non-treatment

Fig. 1 A) Effect of inhaled ONO-1714 on nitrite and nitrate levels in BALF after intravenous injection of *C. albicans*. Data are means $\pm$ SEM of 6 animals. *, $p < 0.05$ as compared with non-treatment group. B) Nitrotyrosine protein expression in lung tissue examined by Western blotting. Data are means $\pm$ SEM of 3 animals. *, $p < 0.05$ as compared with non-treatment group.

group (Fig. 3A).

Total cell counts and neutrophils in BALF

To confirm the effect of inhaled ONO-1714 on neutrophil accumulation, we collected BALF 24 h after inoculation. The numbers of total cells and neutrophils in BALF from the non-treatment group were significantly higher than those in BALF from the control group. The numbers of total cells and neutrophils in the ONO-1714 inhalation group were significantly lower than those in the non-treatment group (Fig. 3B).

RT-PCR for iNOS

Total RNA was extracted from the lungs of mice

and was then electrophoresed and hybridized with cDNA probe for iNOS. As shown in Fig. 4, the expression of mRNA in lungs was significantly lower in the ONO-1714 inhalation group than in the non-treatment group.

iNOS immunoreactivity in the lung

Paraffin-embedded sections from the non-treatment group exhibited marked immunostaining with polyclonal antibody to iNOS. Immunoreactivity for iNOS was primarily found in alveolar epithelial cells, possibly both type I and II cells but not macrophages (Fig. 5A). In the ONO-1714 inhalation group, however, immunostaining of iNOS was markedly attenuated, and only light staining of alveolar epithelial cells was observed (Fig. 5B). No significant immunoreactivity was detected in the control group (data not shown).

Lung wet-dry weight ratio

We studied the effect of ONO-1714 inhalation on lung wet-dry ratio in ALI lungs. The lung wet-dry ratio was increased by *Candida* injection. ONO-1714 inhalation diminished the lung wet-dry ratio from the increased level to the normal one (Fig. 6).

Survival

By day 2 after inoculation, mortality was 40% in the non-treatment group. There was no mortality up to day 3 in the ONO-1714 inhalation group. Median survival was 1.6 days in the non-treatment group and 4.7 days in the ONO-1714 inhalation group. This difference was statistically significant ($p < 0.001$ by the log-rank test; $n = 10$). We could not determine the causes of death in our study, but survival was improved by treatment with inhaled ONO-1714 in mice with lethal candidemia.

DISCUSSION

Induction of iNOS is a much greater stimulus of NO production than activation of nNOS and eNOS. In physiologic states, NO may scavenge superoxide to protect lung tissues, but excessive production of NO may produce tissue damage caused by NO reacting with superoxide to form peroxynitrite, a strong oxidant (3, 19, 21). Peroxynitrite is considered an important oxidant in various diseases (35). In the present study, pretreatment with 1 μ M to 1 mM aerosolized ONO-1714, dose-dependently diminished nitrite/nitrate levels in BALF.

We examined the expression of nitrotyrosine in

Fig. 2 Immunostaining of lung tissue for anti-nitrotyrosine 24 h after inoculation (n = 3 per group). A) Non-treatment group: marked immunostaining of iNOS in alveolar epithelial cells. B) ONO-1714 inhalation group: weak staining of alveolar epithelial cells. Original magnification: $\times 400$.

Fig. 5 Immunostaining of lung tissue for iNOS 24 h after inoculation (n = 3 per group). A) Non-treatment group: marked immunostaining of iNOS in alveolar epithelial cells. B) ONO-1714 inhalation group: weak staining of alveolar epithelial cells. Original magnification: $\times 400$.

the lung by means of immunohistochemical staining and Western blotting to evaluate the oxidative stress caused by peroxynitrite derived from superfluous nitric oxide and superoxide in the lung. Our results showed that the production of nitrotyrosine in the lung was significantly suppressed by the inhalation of ONO-1714. Inhalation of ONO-1714 also significantly reduced the expression of iNOS and nitrotyrosine considered markers of peroxynitrite tissue damage, in alveolar epithelial cells. Among the many organs previously studied, the lungs express the highest iNOS activity in response to endotoxin injection in the rat (40). This finding suggests that the local administration of an iNOS inhibitor to the lung may be effective for the treatment of ALI.

Proinflammatory cytokines, such as IL-1 β and TNF- α , have been implicated in endotoxin shock (4, 8, 16, 33). Intravenous administration of these cytokines induces pulmonary edema and death, similar

to the septic shock syndrome (8, 38). iNOS can be expressed after exposure to endotoxin and cytokines in macrophages, neutrophils, mast cells, endothelial cells, and vascular smooth muscle cells (12, 17, 39). As for the random migration of neutrophils to the lung, macrophage inflammatory protein (MIP-2) has been shown to be a powerful neutrophil migration factor in rodents (15). Greenberger *et al.* (13) have reported that administration of anti-MIP-2 antibody prevents neutrophils from integration to the lung in a model of *Klebsiella* pneumonia. This effect was accompanied by decreased capability to remove *Klebsiella* from the lung (13). In the lungs, alveolar macrophages and airway epithelial cells are the main cells in which cytokines induce iNOS expression and NO formation (18). Kubota *et al.* (25) demonstrated that survival was prolonged despite decreased clearance of *Candida* from the lungs in alveolar macrophage-depleted mice as compared

Fig. 3 A) Effect of inhaled ONO-1714 on BALF IL-1 β (a) and TNF- α (b) levels after intravenous injection of *C. albicans*. B) Effect of inhaled ONO-1714 on total cell counts (TTC) (a) and neutrophils (b) in BALF after intravenous injection of *C. albicans*. Data are means $\pm$ SEM of 6 animals. *, $p < 0.05$ as compared with non-treatment group.

Fig. 4 iNOS mRNA expression in the lung tissue as assessed by reverse transcription polymerase chain reaction (RT-PCR). Data are means $\pm$ SEM of 3 animals. *, $p < 0.05$ as compared with non-treatment group.

with alveolar macrophage-sufficient mice. In this study, we demonstrated that total cell and neutrophil counts in BALF were significantly decreased by in-

Fig. 6 Effect of inhaled ONO-1714 on lung W/D weight ratios after intravenous injection of *C. albicans*. Data are means $\pm$ SEM of 6 animals. *, $p < 0.05$ as compared with non-treatment group.

halation of ONO-1714. The random migration of inflammatory cells (mainly neutrophils to the lung) was also suppressed.

To our knowledge, the present study reports here for the first time to show that inhalation of ONO-1714 as a pretreatment suppresses the expression of iNOS in the lung not only at the protein lev-

el, but also at the mRNA level in *Candida*-induced ALI. Consequently, the lung W/D weight ratio was significantly lower and survival was significantly longer in the ONO-1714 inhalation group than in the non-treatment group. The present study demonstrated that inhalation of ONO-1714 reduced the tissue injury caused by peroxynitrite and suppressed the production of iNOS locally in the lung. Our results for the W/D ratio conflict with the finding of Okamoto *et al.* (37) who have reported that ONO-1714 did not reduce the W/D ratio. However, other studies have shown a significant increase in the lung W/D ratio when yeast-form *Candida* of 10^7 was injected into mice intravenously (1, 26, 32). Our results agree with those of a study by Kristof *et al.* (23), using mice lacking the iNOS gene.

Nuclear factor- κ B (NF- κ B) is a member of a family of transcriptional factors first described in B lymphocytes that regulate transcription of iNOS. Two nitric oxide-mediated effects on the NF- κ B activation pathway have been reported, a stimulatory effect and an inhibitory effect. Murine iNOS promoter contains 24 transcriptional factor binding sites, including those for NF- κ B and activator protein-1 (28, 47). Proteins of the NF- κ B family appear to be essential for the enhanced iNOS gene expression seen in macrophages exposed to LPS (46), and p65 NF- κ B is considered responsible for iNOS induction in astrocytes (9). Marcinkiewicz *et al.* (31) have reported that excessive production of NO could activate a positive feedback loop, leading to increased release of a variety of macrophage inflammatory mediators, such as IL-1 α and TNF- α . Their findings suggest an important self-amplifying mechanism, since iNOS is under NF- κ B control. Our findings that ONO-1714 significantly attenuated the expression of iNOS mRNA in the lung indicated that ONO-1714 may interrupt such a positive feedback.

ONO-1714 is 451-fold more potent than NG-monomethyl-L-arginine monoacetate (L-NMMA) in terms of inhibiting human iNOS, and ONO-1714 is 10-fold more selective for human iNOS than for human nNOS or eNOS. In fact, ONO-1714 is about 34-fold more selective for human iNOS than L-NMMA (36). One study has reported that treatment with ONO-1714 12 h after cecal ligation and puncture significantly reduces NOx levels in plasma and improves survival (37). Another study has shown that two types of treatments, pre-treatment and early post-treatment with ONO-1714 (within 2 h after the infusion of endotoxin) improve ALI (34), although the precise mechanism involved remains unknown.

ONO-1714 is unable to directly inhibit inflammatory mediators (*e.g.*, O₂⁻) other than NO. Thus, the prophylactic or therapeutic effectiveness of using an iNOS inhibitor in *Candida*-induced ALI may be limited. A better response may be achieved by combining ONO-1714 with drugs that suppress other toxic inflammatory mediators. Although inhaled ONO-1714 may be useful for the treatment of *Candida* ALI, whether the drug is effective after onset of infection or when given in multiple doses remains unclear.

In conclusion, pretreatment with inhaled ONO-1714, a new inducible nitric oxide synthase inhibitor, suppressed the production of peroxynitrite and decreased the oxidative stress caused by peroxynitrite in *Candida*-induced ALI. The iNOS inhibitory effect of ONO-1714 was seen at both protein and mRNA levels.

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