Effect of inducible nitric oxide synthase on apoptosis in Candida-induced acute lung injury

Shigekuni Hosogi¹, Yoshinobu Iwasaki¹, Takahiro Yamada¹, Nobuyo Komatani-Tamiya¹, Atsushi Hiramatsu¹, Yoshihito Kohno¹, Mikio Ueda¹, Taichiro Arimoto¹ and Yoshinori Marunaka¹,²
¹Department of Respiratory Medicine and ²Department of Molecular Cell Physiology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan

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ABSTRACT

Excessive nitric oxide (NO) generated by inducible nitric oxide synthase (iNOS) aggravates acute lung injury (ALI) by producing peroxinitrite. We previously showed that the expression of iNOS and lung injury were suppressed by inhalation of a novel iNOS inhibitor, ONO-1714, in mice with Candida-induced ALI, and that nitric oxide produced by iNOS and apoptosis of epithelial cells were found to have a crucial role in Candida-induced ALI. In the present study, we investigated the effect of NO on the apoptosis of alveolar epithelial cells in Candida-induced ALI. Mice were pretreated by inhalation of ONO-1714 or saline (vehicle control of ONO-1714), and were given an intravenous injection of Candida albicans to induce ALI. After 24 h from injection of Candida albicans, we performed bronchoalveolar lavage and removed lung tissues. We assessed apoptosis on the basis of TUNEL staining and caspase 3 activity. Our results showed that apoptosis was suppressed by inhibition of iNOS-derived NO production by ONO-1714 inhalation. The augmented production of NO increased FasL, TNF-α, and mRNA production of Bax of lung that induced apoptosis of alveolar epithelial cells. Inhibition of iNOS-derived NO production by ONO-1714 inhalation ameliorated Candida-induced ALI and improved survival by suppressing apoptosis of alveolar epithelial cells.
that epithelial cells underwent apoptosis in Candida-induced ALI and showed that such apoptosis was reduced by alveolar macrophage depletion (26). However, the involved mechanisms remain unclear.

We also reported that nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) played a crucial role in Candida-induced ALI and demonstrated that the iNOS inhibitor L-NAME had a beneficial effect on Candida-induced ALI (17). Furthermore, we showed that a novel iNOS inhibitor, ONO-1714, a novel selective iNOS inhibitor (1S,5S,6R,7R)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0]heptane hydrochloride), reduced Candida-induced ALI (20). Numerous clinical and case studies have indicated that inhaled NO decreases pulmonary hypertension and improves hypoxemia in ARDS (24). In sepsis associated with overproduction of endogenous NO, the effectiveness of inhaled NO decreases. Inhaled NO has been shown to prime lung macrophages to produce reactive oxygen and nitrogen intermediates (28). At present, inhaled NO therapy still remains experimental.

Overproduced NO interacts with oxygen free radicals, thereby forming a potent oxidant, peroxynitrite (8). Numerous experimental studies on ALI have demonstrated that peroxynitrite induces apoptosis by causing DNA oxidative injury, and the induced hydroxyl radicals impair the mitochondrial electron-transport chain. Apoptosis thus has a crucial role in Candida-induced ALI.

In the present study, we examined the role of NO, produced by iNOS, in apoptosis of alveolar epithelial cells in a mouse model of Candida-induced ALI. We also used ONO-1714 provided by ONO Pharmaceutical Co., Ltd. (Osaka, Japan), to study the effect of NO on apoptosis of alveolar epithelial cells in Candida-induced ALI.

MATERIALS AND METHODS

Animals. The experiments in the present study 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 males; Japan SL C 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 experiments.

Candida albicans. Candida albicans (C. albicans, TIMN 1623, a gift from Teikyo University, Tokyo, Japan) was maintained at −85°C in Sabouraud’s dextrose agar (Eiken Chemical, Tokyo, Japan) at 37°C before use. Yeast-phase blastospores for infusion were suspended in sterile saline, sedimented (400 g, 10 min, 4°C), and resuspended in sterile saline to 1.0 × 10^7 cells/0.2 mL/mouse as determined with a hemocytometer as reported in our previous study (17).

Experimental protocol. Mice were infected intravenously with 1.0 × 10^7 C. albicans cells via a caudal vein to produce a model of Candida-induced ALI. Via a nose-only aerosol chamber, mice received aerosolized saline without (CA group) or with ONO-1714 (ONO-1714 + CA group) for 2 h before injection of C. albicans. Saline with or without ONO-1714 was administered by means of an ultranebulizer (NE-U17; Omron Co., Kyoto, Japan), driven at a rate of 0.75 mL/min and producing particles 1–8 μm in diameter. It was estimated that particles 2–5 μm in diameter reached and were deposited in small airways or alveoli. ONO-1714 (ONO Pharmaceutical Co.) was dissolved in saline at a concentration of 1 mM. Mice in the control group were exposed to aerosolized saline for 2 h and then given an injection of 0.2 mL saline.

Bronchoalveolar lavage (BAL). After anesthetization (pentobarbital 60 mg/kg i.p.), the trachea was exposed and intubated with a 27-gauge needle. BAL was performed by administration of 0.5-mL aliquots of sterile saline three times and the total cell number in the BAL fluid (BALF) was determined with a hemocytometer (n = 6 per group). The BALF was immediately centrifuged at 1 × 10^4 g 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, vol/vol) to precipitate protein and then centrifuged at 1 × 10^4 g for 10 min at 4°C. The supernatant was applied to a NOx analyzing system (ENO-11; EICOM Corp., Kyoto, Japan). This system utilizes the high-performance liquid chromatography-Griess method, and nitrite/nitrate were measured separately (n = 6 per group).

Western blot analysis. Western blot analysis for showing the presence of iNOS was performed on whole lung lysates (n = 6 per group). Protein (8 μg) was mixed with 5 × sample buffer heated to 95°C for 5 min, and fractionated on 7.5% SDS-polyacrylamide gel run at 100 V for 90 min. Cell proteins...
were transferred to nitrocellulose membrane at 100 V for 60 min. The membranes were blocked with Tris buffered saline (TBS) with 5% dry milk for 1 h, and incubated with rabbit polyclonal anti-mouse iNOS antibody (Santa-Cruz Biotech., Santa Cruz, CA, USA) in TBS/0.1% Tween20 and 5% dry milk overnight at 4°C. The blots washed 3 times with TBS/0.1% Tween20 and incubated horseradish peroxidase-conjugated anti-rabbit IgG antibody. Immunoreactive bands were developed using a chemiluminescent substrate (ECL Plus). Membranes were stripped by incubation in stripping buffer (Pierce Biotech., Rockford, IL, USA) for 15 min at 37°C. After washing, membranes were reprobed with anti-GAPDH antibody (Cell Signaling Tech.), and developed as described previously. An autoradiograph was obtained with exposure time of 30 s to 3 min. All the groups were harvested at the same time.

Histological examination of lung. The mice were anesthetized, and 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 10% paraformaldehyde. For hematoxylin-eosin (H&E) 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 H&E (n = 4 per group).

Immunohistochemical stain of iNOS and nitrotyrosine. Immunohistochemical staining for iNOS and nitrotyrosine was performed. The lungs were fixed overnight at 4°C in 10% buffered formalin and were embedded in paraffin and cut to a thickness of 4 μm. Slides were incubated in methanol with H2O2 for 30 min to block endogenous peroxidase activities. The slides were incubated with each properly diluted antibody for 12 h: diluted 1/1000, rabbit polyclonal anti-mouse iNOS antibody (Santa-Cruz biotech) and diluted 1/200, rabbit polyclonal anti-mouse nitrotyrosine antibody (Santa-Cruz biotech); second antibody, biotinylated anti-rabbit immunoglobulin G, and streptavidin-peroxidase conjugate contained in histofine SAB-PO Kit (Nichirei, Tokyo, Japan). Color was developed by diaminobenzidine (DAB), and sections were counterstained with methyl green (LAB VISION, Fremont, CA, USA) (n = 3 per group).

Terminal deoxynucleotidyl transferase-mediated dUTP Nick end-labeling (TUNEL) assay. Apoptosis in vivo was assessed by TUNEL. The lungs were fixed overnight at 4°C in 10% buffered formalin and were embedded in paraffin. An in situ Apoptosis Detection Kit (INTERGEN, NY, USA) was used to carry out TUNEL staining on sections of 4-μm thickness according to the manufacturer’s instructions. Color revelation was performed with DAB-H2O2. Nuclei were also stained with hematoxylin (Wako, Osaka, Japan). Positive staining was defined as a black-brown color, and background staining was blue. Ten fields at × 400 were randomly selected, and the numbers of TUNEL-positive and -negative epithelial cells were manually counted, and the apoptotic index was calculated ([TUNEL positive cells]/[TUNEL positive and negative cells] × 100 (%)).

Analysis of caspase 3 activity. Caspase 3 activity was determined by measuring the proteolytic cleavage of the fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) (BioMol Research Laboratories, Plymouth Meeting, PA, USA). Briefly, crude lung lysate protein of 40–100 μg was incubated at 37°C for 30 min with 20 nM substrate in 500 μL lysis buffer (25 mM HEPES, pH 7.4, 5 mM EDTA, 2 mM DTT, and 0.2% Triton X-100) supplemented with protease inhibitors. The fluorescence of the cleaved reporter group was measured at excitation (380 nm) and emission (469 nm) in 30-min intervals to determine the rate of substrate hydrolysis. Values obtained were adjusted to a standard curve derived from AMC alone. Results were expressed as nanomoles per minute per milligram protein (n = 6 per group).

Lung tissue for cytokine analysis. Before removal of the lungs, the pulmonary vasculature was perfused via the right ventricle with 1 mM PBS containing 5 mM EDTA. After removal, the whole lung was homogenized in 3 mL lysis buffer containing 0.5% TritonX-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl2, and 1 mM MgCl2 (pH 7.4), using a tissue homogenizer (Dremel, Racine, WI, USA). The homogenate was incubated on ice for 30 min and was then centrifuged at 2,500 rpm for 10 min. The supernatant was collected, passed through a 0.45-μm-pore-size filter (Gelman Science, Ann Arbor, MI, USA), and stored at −30°C until assessment of cytokine level.

Enzyme-linked immunosorbent assay of cytokines. Mouse tumor necrosis factor α (TNF-α) and FasL
concentrations in the supernatant of the lung homogenate were measured with the use of Quantikine M mouse TNF-α and FasL immunoassay kits (R&D Systems, Minneapolis, MN, USA). The supernatants were studied and read at 490 nm (n = 6 per group).

**Quantification of RNA.** After isolation of total cellular RNA, Bax and β-actin mRNA levels were examined by real-time reverse transcription (RT) and polymerase chain reaction (PCR), done with a LightCycler (Roche Diagnostics, Basel, Switzerland). Total RNA samples from the lung tissue were isolated with an RNAeasy RNA extraction kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's protocol. First-strand cDNA was synthesized with 2 μg total RNA as a template, using a SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed using the LightCycler instrument (Roche Diagnostics) with LightCycler DNA-Master of 20 μL, SYBR Green I, 25 pmol oligonucleotide primers, cDNA solution of 2 μL, and 25 mmol/L MgCl₂ of 2.4 μL. After initial denaturation at 95°C for 10 min, reactions were cycled 40 times under the following conditions for Bax detection: 95°C for 5 s, primer annealing at 58°C for 10 s, and primer extension at 72°C for 20 s. Specific oligonucleotide primer sequences were as follows. For mouse Bax, forward: 5'-ACTA AAGTGCCCGAGCTGAT-3'; reverse: 5'-AGGACT CCAGCCACAAGAT-3' (amplifies a fragment of 164 bp). For mouse β-actin, forward: 5'-GGC CAGGTCATCACTATTG-3'; reverse: 5'-GAG GTCTTTACGGATGTCAAC-3' (amplifies a fragment of 164 bp). SYBR Green I fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during the cycle. At the end of each run, melting curve profiles were produced to confirm amplification of specific transcripts. Cycle-to-cycle fluorescence emission readings were monitored and quantified with the use of the second derivative maximum method and LightCycler Software (Roche Molecular Biochemicals, Indianapolis, IN, USA). Standard curves for Bax and β-actin were constructed using serial dilutions of total cDNA from Balb/c mouse lung tissue. Standard-curve samples were included in each run. Standards for both Bax and β-actin were defined to contain an arbitrary starting concentration, since no primary calibrators exist. Hence, all calculated concentrations are relative to the concentration of the standard (n = 6 per group).

**Survival.** Survival was assessed in 24 mice divided into two subgroups (CA group, ONO-1714 inhalation group, n = 12 per group) and treated intravenously with 1.0 × 10⁷ C. albicans cells. Survival was estimated by the Kaplan-Meier method over the course of 5 days. Survival curves were compared by log-rank tests.

**Statistical analysis.** All data, expressed as means ± SEM, were analyzed by one-way analysis of variance. Differences between groups were assessed by Fisher’s protected least-significant difference test; P < 0.05 was considered to indicate statistical significance.

**RESULTS**

**Nitrite and nitrate levels in BALF**

Mice were exposed to aerosolized saline with or without 1 mM ONO-1714 for 2 h before C. albicans administration. Twenty-four hours after administration of C. albicans, mice were anesthetized and killed before BAL. Pretreatment with aerosolized 1 mM ONO-1714 significantly suppressed nitrite/nitrate levels (Fig. 1A).

**The number of inflammatory cells in BALF**

To confirm the effect of iNOS-derived NO on inflammatory cell accumulation on Candida-induced ALI, we counted the number of inflammatory cells in BALF. The number of total cells in BALF was significantly higher in CA group than in control group, and the CA-induced elevation of the number of total cells was suppressed by ONO-1714 treatment (Fig. 1B). The population of neutrophils was significantly higher in CA group than in control group, and the CA-induced elevation of the number of neutrophils was suppressed by ONO-1714 treatment (Fig. 1C).

**Western blot analysis for iNOS in lung tissue**

Western blot analysis of protein of iNOS was performed to determine the effect of ONO-1714 on iNOS expression on whole lung lysates (Fig. 2A). The expression level of iNOS in lung tissues was significantly increased in the CA group compared with that in control group, and the increase was suppressed by ONO-1714 treatment (Fig. 2B).

**H&E staining**

Candida-induced ALI was histologically character-
NOS inhibitor reduces apoptosis in ALI

localized by a large number of inflammatory cells migrating into the alveolar spaces and the interstitium, in association with fibrin deposition, air-space edema, and alveolar hemorrhage. The alveolar walls showed destructive changes 24 h after administration of *C. albicans* (Fig. 3A). These findings were milder in ONO-1714 treated mice (Fig. 3B).

**Immunohistochemical staining of iNOS and nitrotyrosine**

Immunohistochemical staining of iNOS and nitrotyrosine was performed to confirm localization of the proteins and effect of ONO-1714 in three groups, since nitrotyrosine is considered as a marker for peroxynitrite formation and an indicator of nitrate stress. In the CA group, staining of iNOS was observed in alveolar epithelial cells (Fig. 4A). In the ONO-1714 + CA group, staining of iNOS (Fig. 4B) was attenuated as compared with that in the CA group (Fig. 4A). Nitrotyrosine in the CA group (Fig. 4C) was also attenuated by ONO-1714 treatment (Fig. 4D).

**TUNEL**

On TUNEL assay, no epithelial cell was positive in the lungs of control mice given an injection of 0.2 mL saline via a caudal vein. In the *Candida*-infected lung, a large number of alveolar epithelial cells were TUNEL positive (Fig. 5A-a), and the number of TUNEL positive cells was reduced by ONO-1714 treatment (Fig. 5A-b). Alveolar epithelial cells were examined in 10 randomly selected fields per slide per mouse. The apoptotic index was significantly decreased (*P* < 0.01) by ONO-1714 treatment (Fig. 5B).

**Analysis of caspase 3 activity**

Accumulating evidence indicates that activation of caspases is critical for many forms of apoptotic cell death. Caspase 3 is considered to play an essential role in triggering proteolytic pathways that lead to apoptosis. Caspase 3 activity was analyzed as an indicator of apoptosis in *Candida*-induced ALI and was compared among the 3 groups. Caspase 3 activity was increased in the CA group compared with that in control group (Fig. 6), and the increase was abolished in the ONO-1714 + CA group (Fig. 6). These observations indicate that caspase 3 activity was significantly suppressed (*P* < 0.01) by ONO-1714 treatment (Fig. 6).
Survival in mice decreased substantially after induction of *C. albicans* infection. All mice in the CA group died within 3 days after inoculation. Survival was significantly better in the ONO-1714 + CA group than in the CA group (*P* < 0.05 by the log-rank test), with delayed mortality during the 5 days of follow-up in the latter group (Fig. 9).

**DISCUSSION**

Some studies (11, 12, 16, 27 29) have demonstrated that: 1) pulmonary production of NO is increased in response to severe sepsis or administration of LPS, 2) iNOS expression in the lung is increased during ARDS, and 3) in consistency with our present study iNOS inhibition attenuates acute lung injury (ALI) and improves survival. Rudkowski *et al.* (25) have reported that mice with deficient expression of iNOS (iNOS-knockout mice) attenuate ALI in response to LPS administration. These findings strongly support the idea that iNOS contributes to the development of sepsis-induced ALI. However, another study has

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**Quantification of Bax mRNAs**

We determined the mRNA level of the pro-apoptotic protein, Bax, by RT-PCR to examine its role in apoptosis associated with *Candida*-induced ALI. Bax mRNA was increased in the CA group and suppressed (*P* < 0.01) by ONO-1714 treatment (Fig. 7).

**Enzyme-linked immunosorbent assays for TNF-α and FasL**

To confirm the effects of inhaled ONO-1714 on cytokine production, cytokine analysis was performed 24 h after infection. The concentrations of TNF-α and FasL in the CA group were clearly higher than those in the control group (Fig. 8A, B). The increases in concentrations of TNF-α and FasL in the lung tissue were suppressed by ONO-1714 treatment (*P* < 0.01; Fig. 8A, B).
NOS inhibitor reduces apoptosis in ALI

**Fig. 4** Immunohistochemistry of iNOS (A, B) and nitrotyrosine (C, D) in lung tissues in *Candida*-induced ALI. A) iNOS in CA; B) iNOS in ONO1714 + CA; C) nitrotyrosine in CA; D) nitrotyrosine in ONO-1714 + CA. The bar indicates 10 μm.

**Fig. 5** TUNEL staining (A) and apoptotic index (B) in lung tissues in *Candida*-induced ALI. A-a) TUNEL staining in CA; A-b) TUNEL staining in ONO-1714 + CA. B) Apoptotic index. Data are represented as means ± SEM of 6 animals. **P < 0.01 compared with CA group. The bar indicates 10 μm.**
found that such apoptosis was reduced by depletion of alveolar macrophages. However, the underlying mechanisms still remain poorly understood.

Inhalation of ONO-1714 suppressed inflammatory cells in BALF in our model of *Candida*-induced ALI (Fig. 1). Razavi and colleagues have examined the effect of iNOS-generated NO on several steps of neutrophil trafficking in the lungs during sepsis including neutrophil sequestration and neutrophil migration into airspaces, reporting that neutrophils in BALF release free radicals and proteases and induce lung injury in sepsis (23). However, Pheng et al. (21) have indicated that inhaled NO inhibits neutrophil migration and cytokine production, suggesting that NOS inhibition can worsen lung injury. On the other hand, the findings shown in our present study suggest that depletion of inflammatory cells by inhibition of iNOS-generated NO may induce reduction of superoxide anions in lung tissues, as well as reduction of apoptosis of alveolar epithelial cells. To resolve the apparent discrepancy, further studies are required.

Pulmonary apoptosis has been reported to be associated with FasL, TNF-α, and Bax both in vitro and in vivo (3, 13). Other studies have shown that inhibition of excessive iNOS may also cause apoptosis of alveolar cells (9, 27). In the present study, alveolar cell apoptosis was observed at increased NO levels. We also studied the effect of ONO-1714 on the apoptosis. Although ONO-1714 inhibited iNOS, the ONO-1714-induced level of iNOS was not excessively lower than control, but was identical to control one. We also assessed apoptosis in *Candida*-induced ALI on the basis of TUNEL staining and caspase 3 activity in the present study. Our results showed that inhibition of iNOS-derived NO by ONO-1714 inhalation attenuated pulmonary apoptosis and improved survival in mice with candidemia. Nitrotyrosine is considered as a marker for peroxynitrite formation and an indicator of nitrate stress, suggesting that peroxynitrite would be produced by NO, and that the produced peroxynitrite would induce pulmonary apoptosis. Take-mura et al. (26) reported that epithelial cells underwent apoptosis in *Candida*-induced ALI and provided evidence that inhaled NO inhibits cytokine production, suggesting that NOS inhibition can worsen lung injury (21). Augmented production of NO in septic lungs may help to maintain lung function during the course of sepsis. We previously reported the role of NO in *Candida*-induced ALI and proposed that NO has a detrimental role in this model of ALI (26). In the present study, we demonstrated that inhibition of iNOS-derived NO by ONO-1714 inhalation attenuated *Candida*-induced ALI.

The mechanism underlying the effect of NO may involve the modulation of pulmonary apoptosis. Several studies have shown that NO regulates apoptosis and inhibits the activity of many caspases in vitro as well as in vivo (3, 13). Other studies have shown that inhibition of excessive iNOS may also cause apoptosis of alveolar cells (9, 27). In the present study, alveolar cell apoptosis was observed at increased NO levels. We also studied the effect of ONO-1714 on the apoptosis. Although ONO-1714 inhibited iNOS, the ONO-1714-induced level of iNOS was not excessively lower than control, but was identical to control one. We also assessed apoptosis in *Candida*-induced ALI on the basis of TUNEL staining and caspase 3 activity in the present study. Our results showed that inhibition of iNOS-derived NO by ONO-1714 inhalation attenuated pulmonary apoptosis and improved survival in mice with candidemia. Nitrotyrosine is considered as a marker for peroxynitrite formation and an indicator of nitrate stress, suggesting that peroxynitrite would be produced by NO, and that the produced peroxynitrite would induce pulmonary apoptosis. Take-mura et al. (26) reported that epithelial cells underwent apoptosis in *Candida*-induced ALI and found that such apoptosis was reduced by depletion of alveolar macrophages. However, the underlying mechanisms still remain poorly understood.

Inhalation of ONO-1714 suppressed inflammatory cells in BALF in our model of *Candida*-induced ALI (Fig. 1). Razavi and colleagues have examined the effect of iNOS-generated NO on several steps of neutrophil trafficking in the lungs during sepsis including neutrophil sequestration and neutrophil migration into airspaces, reporting that neutrophils in BALF release free radicals and proteases and induce lung injury in sepsis (23). However, Pheng et al. (21) have indicated that inhaled NO inhibits neutrophil migration and cytokine production, suggesting that NOS inhibition can worsen lung injury. On the other hand, the findings shown in our present study suggest that depletion of inflammatory cells by inhibition of iNOS-generated NO may induce reduction of superoxide anions in lung tissues, as well as reduction of apoptosis of alveolar epithelial cells. To resolve the apparent discrepancy, further studies are required.

Pulmonary apoptosis has been reported to be associated with FasL, TNF-α, and Bax both in vivo and in vitro, and Fas ligand and TNF-α are central signals of apoptosis in LPS-induced ALI (10, 15). Bachofen and Weibel (1) have noted that type I pneumocytes exhibit decreased size and chromatin condensation as early morphologic changes in human ARDS. The DNA of alveolar epithelia of patients who died due to lung injury is fragmented, and Bax in alveolar epithelia of humans with diffuse alveolar damage is up-regulated (2, 7). The present study suggests that inhibition of iNOS-derived NO
NOS inhibitor reduces apoptosis in ALI

would be inflammatory cells. Therefore, our results indicate a possibility that iNOS-derived NO plays a key role in apoptosis by promoting over expression of these cytokines.

Our findings suggest that inhalation of ONO-1714 may have a beneficial effect on Candida-induced ALI by suppressing apoptosis of alveolar epithelial cells. We treated mice with inhaled ONO-1714 before induction of infection, although the effect of inhaled ONO-1714 after the onset of infection should be evaluated in future studies to confirm the effectiveness of this treatment.

In summary, our study showed that augmented production of NO increased the proapoptotic cytokines FasL, TNF-α, and Bax and induced apoptosis of alveolar epithelial cells in a mouse model of Candida-induced ALI. Inhibition of iNOS-derived NO ameliorated Candida-induced ALI by suppressing apoptosis of alveolar epithelial cells.

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Fig. 8 Concentrations of TNF-α (A) and FasL (B) in lung homogenates in control group, CA group, and ONO-1714 + CA group. Data are represented as means ± SEM of 6 animals. *P < 0.01 compared with control group. **P < 0.01 compared with CA group.

suppressed apoptosis of alveolar epithelium associated with the decrease in proapoptotic cytokines FasL, TNF-α, and Bax. We previously investigated the apoptosis in Candida-induced lung injury (26). In the study (26), we performed immunohistochemistry of Bax, indicating that Bax-positive cells were mainly epithelial cells. So, these observations lead us to a conclusion that the caspase activity is mainly elevated in epithelial cells expressing Bax mRNA. Therefore, TUNEL-positive cells would be mainly epithelial cells, although we could not neglect that a small portion of TUNEL-positive cells

Fig. 9 Effect of ONO-1714 on survival in candidemia. All mice in the 2 groups were infected intravenously with C. albicans (n = 12 per group).
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