Effect of ONO1714, a Specific Inducible Nitric Oxide Synthase Inhibitor, on Lung Lymph Filtration and Gas Exchange during Endotoxemia in Unanesthetized Sheep

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Background: The effect of nitric oxide synthase inhibitor on acute lung injury remains controversial. The current study was designed to examine effects of a newly synthesized and selective inducible nitric oxide synthase inhibitor, ONO1714, on endotoxin-induced lung injury in unanesthetized sheep.

Methods: Thirteen unanesthetized sheep chronically instrumented with a lung lymph fistula and vascular catheters for monitoring were prepared. Animals were randomly allocated into two experimental groups. In experiment 1, sheep (n = 6) were infused only with endotoxin (1 μg/kg) for 30 min. In experiment 2, sheep (n = 7) were pretreated with ONO1714 (0.1 mg/kg) before 30 min of endotoxin administration, and the endotoxin was infused in the same manner as in experiment 1. Mean pulmonary arterial pressure, left atrial pressure, systemic arterial pressure, and lung lymph flow were measured. Observation was continued over 5 h after endotoxin administration.

Results: ONO1714 did not cause any pulmonary hemodynamic changes at baseline or exert any influences on transient pulmonary hypertension and increased pulmonary vascular resistance during endotoxemia. However, inducible nitric oxide synthase inhibition with ONO1714 significantly reduced lung lymph filtration and improved abnormal oxygenation during endotoxemia. In addition, increased nitrate–nitrite in plasma and lung lymph in response to endotoxin was prevented by treatment with ONO1714.

Conclusions: These findings suggest that nitric oxide release by the inducible nitric oxide synthase pathway partially contributes to the increased permeability of pulmonary edema and decreased oxygenation during endotoxemia in sheep.

NITRIC oxide is an important mediator of various physiologic and pathologic conditions.[1] The formation of nitric oxide is synthesized by at least three distinct isoforms of the enzyme of nitric oxide synthase (NOS). In neuronal and endothelial cells, the NOSs are expressed constitutively. On the other hand, larger amounts of nitric oxide are released under inflammatory conditions by an inducible type of NOS.[1] Inducible NOS (iNOS) has been identified in many cell types in response to inflammatory cytokines and bacterial endotoxin.[1–3] Numerous cells, including endothelial cells, macrophage, neutrophils, and vascular smooth muscle cells in the lung, are capable of synthesizing and releasing nitric oxide.[4,5] The

overproduction of nitric oxide during sepsis has been considered to be an important mediator in both cardiopulmonary vascular dysfunctions during sepsis[6–9] and the pathogenesis of sepsis-associated lung injury, acute respiratory distress syndrome, or both.[10,11] Furthermore, a study using iNOS-deficient mice indicated that the extent of endotoxin-induced lung injury was significantly milder in iNOS-deficient mice when compared with wild-type mice.[12,13] Therefore, it has been speculated that pharmacologic inhibition of iNOS is beneficial in preventing the development of acute respiratory failure in sepsis. Aminoguanidine, a relatively selective iNOS inhibitor,[14] has been shown to attenuate endotoxin-induced acute lung injuries in several animal models.[15–17] However, in the sheep model of endotoxin-induced lung injury, aminoguanidine significantly increased pulmonary artery pressure and lung lymph flow (Qlym) during endotoxemia.[18] These findings obtained by aminoguanidine were similar to those of nonselective NOS inhibitors observed in sheep,[19,20] although the effects of NOS inhibitors on gas exchanges were different between aminoguanidine and nonselective iNOS inhibitor.[19–21] Therefore, the role of iNOS in endotoxin-induced acute lung injury, especially lung water balance and gas exchanges, remains unclear. These inconsistent findings might be due to the pharmacologic selectivity of the agents. More selective blocking effects for iNOS are essential to investigate the role of acute lung injury and to evaluate a pharmacologic benefit of an agent on the sepsis-associated lung water balances and gas exchanges during endotoxemia.

ONO1714, a cyclic amidine derivative, has been shown to be more selective for iNOS than aminoguanidine. The inhibitory activity for iNOS was stronger than that of aminoguanidine[22] *in vitro*. Recently, several studies using this agent revealed beneficial effects on sepsis-associated lung dysfunction in small animal models.[23,24] However, little information exists on the pharmacologic benefit of ONO1714 on endotoxin-induced lung injury in a large animal model. Therefore, we investigated the effects of ONO1714 on lung lymph filtration and gas exchange in unanesthetized sheep with vascular and lung lymph monitoring using a clinically relevant model of acute respiratory distress syndrome with sepsis.

Materials and Methods

This study protocol was approved by the institutional review board for the care of animals in Shinshu Univer-

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sity (Asahi Matsumoto, Japan). Care and handling of animals were in accordance with the guidelines of the National Institutes of Health.

Animal Preparation

Thirteen sheep weighing 30–39 kg were used. We prepared chronic lung lymph fistulae and placed catheters for hemodynamic monitoring. Sheep were anesthetized with 12.5 mg/kg intravenous pentobarbital sodium and then ventilated with 0.5–1.0% halothane using positive-pressure ventilation. Through two right thoracotomies, the efferent lymphatic channel from the caudal mediastinal node was cannulated with a thin silicon tube. The caudal mediastinal node tail was then ligated at the free margin of the inferior pulmonary ligament to block contamination by nonpulmonary lymph. Through a left thoracotomy, we directly implanted silicon catheters into the main pulmonary artery and left atrium. A silicon tube was inserted into the thoracic aorta *via* the carotid artery. An 8-French catheter sheath introducer (Cordis Laboratories, Miami, FL) was placed in the superior vena cava *via* the right jugular vein. On the day before the experiment, a 7-French thermodilution Swan-Ganz catheter (Becton Dickinson, Tokyo, Japan) was passed into the pulmonary artery through the Cordis introducer. The animals were allowed to recover for at least 7 days with free intake of food and water after the surgical procedures.

Measurements

All measurements were made under an awake and standing condition. Systemic arterial, pulmonary arterial, and left atrial pressures were continuously measured and recorded using calibrated pressure transducers (RMP-6008; Nihon Koden, Tokyo, Japan) and a recorder (WT-685G; Nihon Koden), respectively. Cardiac output was determined by the thermodilution method using a cardiac output computer (model 9520; Edwards, Santa Ana, CA). We collected lung lymph in heparinized tubes and measured Qlym every 30 min. The protein concentration in plasma and pooled lung lymph was determined by biuret method every 30 min, and the lymph–to–plasma protein concentration ratio (L/P) was calculated. Lymph protein clearance (Clym) was calculated by multiplying L/P by Qlym. Blood samples for blood gas analysis were drawn before endotoxin and 0.5, 1, 2, 3, 4, and 5 h after endotoxin from systemic and pulmonary arterial lines, respectively. Blood gas analysis (partial pressure of oxygen [P_{O_2}], partial pressure of carbon dioxide [P_{CO_2}], pH, oxygen saturation, and hemoglobin) were performed with use of a blood gas analyzer (ABL-2; Radiometer, Copenhagen, Denmark). Alveolar–arterial oxygen pressure difference (A-aD_{O_2}) and venous admixture (Q_S/Q_T) were calculated using standard equations. The plasma and lung lymph concentrations of nitrate–nitrite (NOx) were also measured using the Cayman Chemical Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Com-

pany, Ann arbor, MI). The first step is the conversion of nitrate to nitrite using nitrate reductase. The second step is the addition of Griess reagent,[25] which converts nitrite into a deep-purple azo compound. The plasma sample was taken from the systemic artery catheter. NOx concentrations in plasma and lung lymph were measured before endotoxin and 1, 3, and 5 h after endotoxin administration in sheep treated with and without ONO1714.

Experimental Protocols

Sheep were allocated to two experimental groups. In experiment 1 (endotoxin alone; n = 6), after stable baseline measurements over 2 h, *Escherichia coli* endotoxin (1 μg/kg, diluted in 30 ml sterile normal saline, *E. coli* 0127; B8; Difco Laboratories, Franklin Lakes, NJ) was infused. In experiment 2 (endotoxin combined with ONO1714 treatment; n = 6), ONO1714 ((1S,5S,6R,7R)-7-chloro-30imino-5-methyl-2-azabicyclo[4.1.0] heptane hydrochloride) was supplied by Ono Pharmaceutical Company Ltd. (Osaka, Japan). ONO1714 (0.1 mg/kg) was dissolved with 20 ml normal saline and intravenously administered for 5 min. The treatment of ONO1714 was performed 30 min before endotoxin infusion. Observation of systemic and pulmonary hemodynamics, Qlym, and blood samples was continued for 5 h after endotoxin in both groups.

Statistical Analysis

The data are expressed as mean ± SD. Changes measuring variables over time and between groups were analyzed by two-way analysis of variance, and the differences were tested by Fisher exact test. $P < 0.05$ was accepted as a significant difference.

Results

Pulmonary Hemodynamics

Pulmonary hemodynamic response to endotoxin in both with and without ONO1714 are shown in figure 1. In both groups, pulmonary artery pressure increased significantly within 1 h after the start of endotoxin administration (early phase) and then decreased gradually and returned to the baseline by 4–5 h (late phase). iNOS inhibition with ONO1714 resulted in slight increase in pulmonary artery pressure and decrease in cardiac output at baseline, but these changes were statistically not significant. In addition, ONO1714 did not cause any significant changes in early transient and late sustained pulmonary hypertension during endotoxemia. The time courses of cardiac output and left atrial pressure during endotoxemia were also not affected by ONO1714 treatment.

Systemic Artery Pressure

The time courses of systemic aortic pressure both with and without ONO1714 treatment were shown in figure 2. ONO1714 did not cause any change in systemic aortic pressure at baseline. Systemic arterial pressure decreased
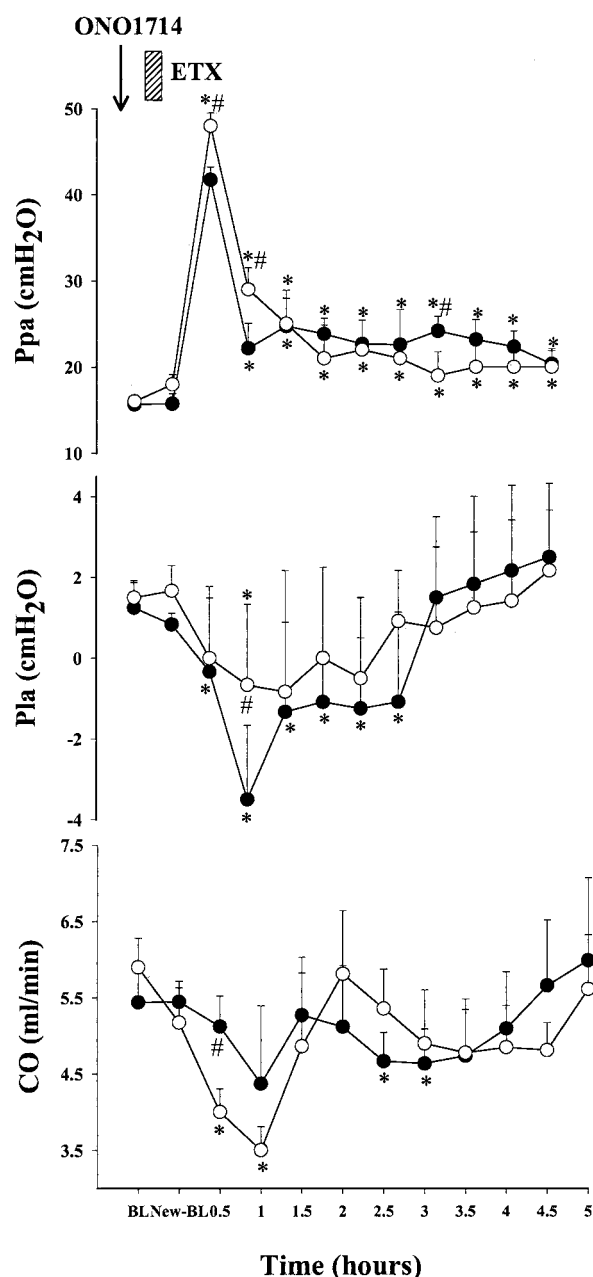
Fig. 1. Time course of pulmonary arterial pressure (Ppa), left atrial pressure (Pla), and cardiac output (CO) after endotoxin administration in sheep treated with and without ONO1714. Data are presented as mean ± SD. *Open circle* = endotoxin (ETX) plus ONO1714 treatment; *closed circle* = endotoxin alone. *$P < 0.05$ significant difference from baseline (BL). #$P < 0.05$, significant difference between groups with and without ONO1714 treatment.

significantly and transiently at the early phase after endotoxin administration. There were no significant differences in the decrease of systemic artery pressure between groups with and without ONO1714 treatment.

Lung Lymph Balance

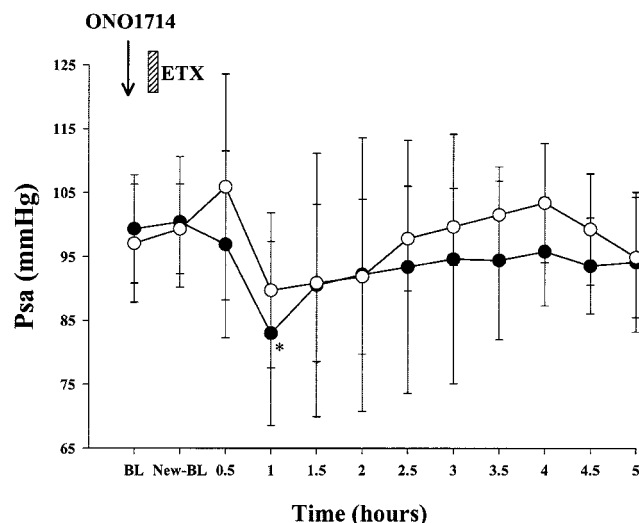The time courses in Qlym, L/P, and Clym are summarized in figure 3. There were no significant differences at

Fig. 2. Time course of systemic arterial pressure (Psa) after endotoxin administration in sheep treated with and without ONO1714. Data are presented as mean ± SD. *Open circle* = endotoxin (ETX) plus ONO1714 treatment; *closed circle* = endotoxin alone. *$P < 0.05$, significant difference from baseline (BL).

baseline lung lymph balance in either group. During the early phase of endotoxemia, concomitant with the peak in pulmonary hypertension, Qlym increased approximately threefold, and L/P decreased slightly in the endotoxin-alone group. Subsequently, Qlym decreased slightly but remained significantly increased during the late phase with an increased L/P. Increased Qlym in ONO1714 treatment was low, compared with that in the endotoxin-alone group. Increased Qlym during the late phase in sheep treated with ONO1714 was significantly lower than that in endotoxin-alone sheep at one point. However, increased levels of Clym during the late phase in the ONO1714 group were significantly lower than those in the endotoxin-alone group.

Blood Gas Analysis

The time courses of Po_2, A-aDo_2, and shunt ratio (Q_S/Q_T) are summarized in figure 4. Po_2 after endotoxin administration was significantly decreased within 1 h and gradually recovered to baseline at 5 h after endotoxin in both groups. However, the values of Po_2 in the ONO1714 group during 2–4 h were significantly higher than those in the endotoxin-alone group. In the endotoxin-alone group, A-aDo_2 immediately increased from 0.53 ± 0.11 at baseline to 0.87 ± 0.24 at 0.5 h. The values of A-aDo_2 then sustained the increased levels during the late phase and returned to the baseline level approximately 5 h after the start of endotoxin infusion. In sheep treated with ONO1714, the values of A-aDo_2 were significantly lower than those in the endotoxin-alone group. Likewise, the time courses of Q_S/Q_T in both groups were almost similar to those in A-aDo_2. The increased values of Q_S/Q_T during 2–4 h after endotoxin administra-
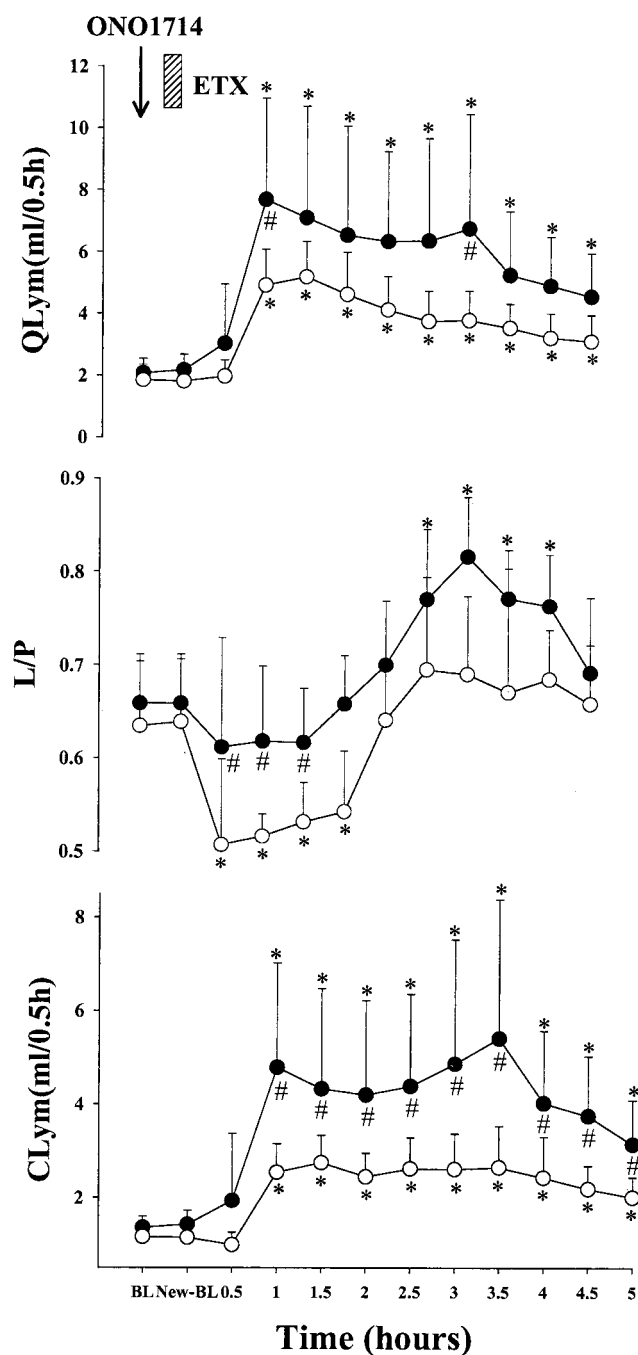
Fig. 3. Time course of lung lymph flow (Qlym), lymph–to–plasma protein concentration ratio (L/P), and lung lymph protein clearance (Clym) after endotoxin administration in sheep treated with and without ONO1714. Data are presented as mean ± SD. *Open circle* = endotoxin (ETX) plus ONO1714 treatment; *closed circle* = endotoxin alone. * $P < 0.05$, significant difference from baseline (BL). # $P < 0.05$, significant difference between groups with and without ONO1714 treatment.

Fig. 4. Time course of arterial partial blood gas tension (Po_2), alveolar–arterial oxygen difference (A-aDo_2), and intrapulmonary shunt ratio (Q_s/Q_T) after endotoxin administration in sheep treated with and without ONO1714. *Open circle* = endotoxin (ETX) plus ONO1714 treatment; *closed circle* = endotoxin alone. Data are presented as mean ± SD. * $P < 0.05$, significant difference from baseline (BL). # $P < 0.05$, significant difference between groups with and without ONO1714 treatment.

tion in animals treated with ONO1714 were significantly lower than those in the endotoxin-alone group.

NOx Concentrations in Plasma and Lung Lymph

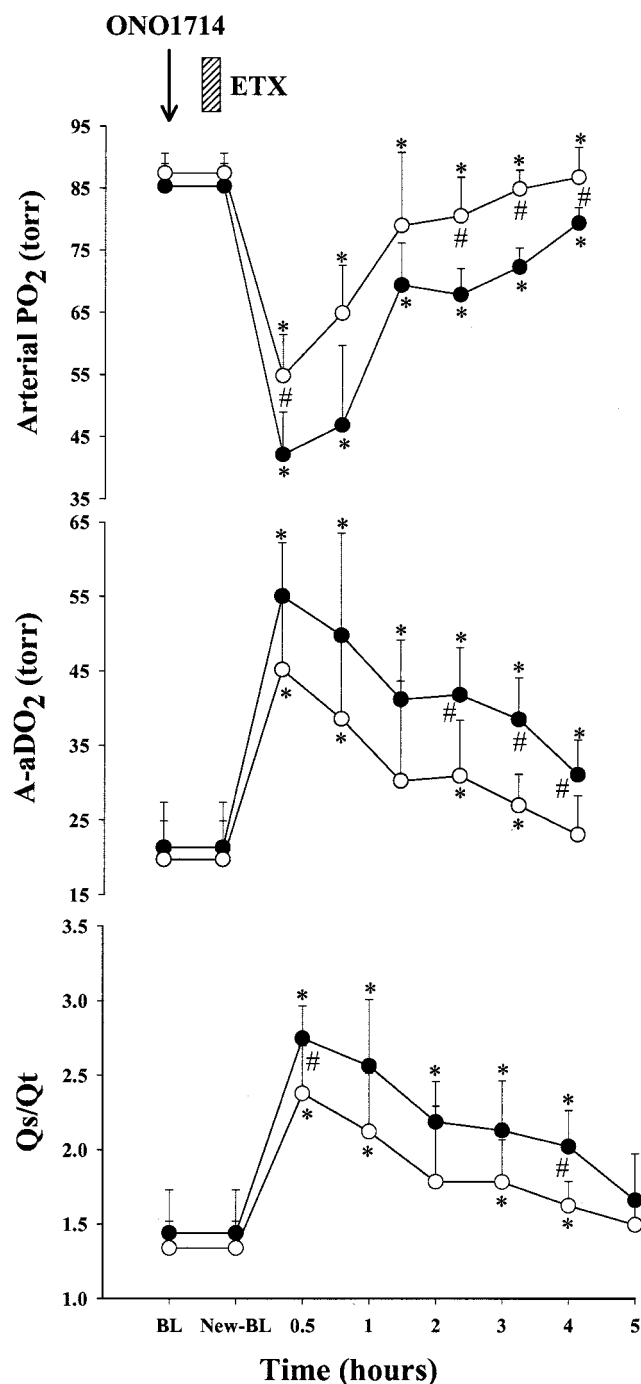There were no significant differences at baseline values of NOx in plasma (2.78 ± 0.4 pg/ml in endotoxin alone, 3.38 ± 0.4 pg/ml in endotoxin plus ONO1714; P = not significant) and lung lymph (4.66 ± 0.6 pg/ml in endotoxin alone, 6.31 ± 0.6 pg/ml in endotoxin plus ONO1714; P = not significant) between groups with and without ONO1714 treatment. We estimated NOx
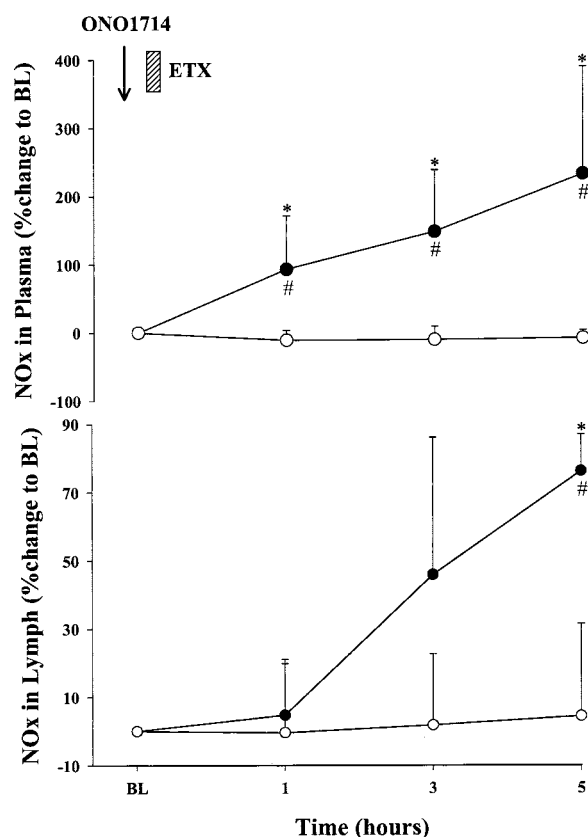
Fig. 5. Percent change in nitrate–nitrite (NOx) concentrations in plasma and lung lymph after endotoxin administration in sheep treated with and without ONO1714. Data are presented as mean ± SD. * $P < 0.05$, significant difference from baseline (BL). # $P < 0.05$, significant difference between groups with and without ONO1714 treatment.

values as the percent changes to baseline and graphed this in figure 5. The plasma NOx in endotoxin group gradually increased after endotoxin administration and reached approximately 100% increase at 5 h. Likewise, NOx in lung lymph increased significantly after endotoxin administration. In contrast, NOx in both plasma and lung lymph remained unchanged after endotoxin administration in animals treated with ONO1714. Therefore, ONO1714 treatment could prevent the increased NOx in plasma and lung lymph during endotoxemia.

Discussion

In the current study, we have shown in unanesthetized sheep that a selective iNOS inhibitor, ONO1714, reduces lung lymph filtration and improves oxygenation during endotoxemia in awake sheep. These data suggest that NOS activation through inducible isoform contributes to the development of endotoxin-induced lung injury in sheep.

Nonselective NOS inhibitors such as *N*-nitro-L-arginine methyl ester (or *N*-monomethyl-L-arginine) aggravated endotoxin-induced pulmonary hypertension and in-

creased extravascular water in sheep.[19,20] These physiologic findings by nonselective NOS inhibitors were due to the pharmacologic effects of constitutive NOS and seemed to be unsuitable in the situation of increased pulmonary permeability. Although Evgenov *et al.*[18] demonstrated that aminoguanidine administration improved oxygenation during the late phase of endotoxemia in sheep, aminoguanidine caused significant increases in pulmonary arterial pressure and Qlym during the simultaneous phase in the same sheep model. These findings suggested that pharmacologic effects of aminoguanidine on constitutive NOS during endotoxemia were present even in aminoguanidine. In the current study, ONO1714 did not cause any pulmonary or systemic hemodynamic changes induced by endotoxin. The finding suggested that ONO1714 was superior in selectivity against iNOS as compared with aminoguanidine. Our results *in vivo* were therefore similar to those *in vitro*.[21] Furthermore, ONO1714 significantly reduced Qlym and improved abnormal oxygenation during endotoxemia, suggesting that ONO1714 could attenuate endotoxin-induced lung injury in sheep. Based on the current results as well as published data,[15-17] it seems that selective inhibition of iNOS leads to improvement in lung edema and gas exchange during endotoxemia.

We used a newly synthesized iNOS inhibitor, ONO1714, which is more potent and selective both *in vitro* and *in vivo* than the previously evaluated iNOS inhibitors. ONO1714 is 10-fold more selective for iNOS than constitutive NOS in the human cell line, which is still higher than aminoguanidine. The iNOS selectivity of ONO1714 was approximately 2-fold higher than aminoguanidine in human cell lines.[22] In addition, inhibitory activity of ONO1714 is more than 20,000-fold more potent than aminoguanidine. In an *in vivo* study, which was performed in endotoxin-stimulated mice, ONO1714 inhibited the increases in NOx accumulation in plasma 2,600-fold more than *N*[G]-monomethyl-L-arginine monoacetate. These data indicated that ONO1714 was one of the most potent iNOS inhibitors *in vitro* and *in vivo* to date. We confirmed that ONO1714 could completely prevent NOx concentrations in plasma and lung lymph in response to endotoxin, whereas plasma NOx was not prevented by the treatment of aminoguanidine in the same endotoxemic sheep model[18] and other studies.[15,16] These observations suggest that the dose of 0.1 mg/kg ONO1714 was adequate to inhibit the iNOS activity during endotoxemia and that the inhibitory activity of ONO1714 was more potent than that of aminoguanidine in sheep.

Hypoxemia during endotoxemia is thought to be primarily due to ventilation/perfusion mismatch with physiologic shunting through collapsed alveoli.[26] It seems that the degree of pulmonary edema is a major determinant of Q_S/Q_T. In the current study, iNOS inhibition with ONO1714 improved abnormal oxygenation during endo-

toxemia. The beneficial findings were most likely responsible for the reduction of lung fluid filtration, as assessed by Qlym and Clym. On the other hand, it is well known that endotoxemia impairs hypoxic pulmonary vasoconstriction.[27,28] The attenuated hypoxic pulmonary vasoconstriction during endotoxemia might cause increased ventilation/perfusion mismatching, augmented right-to-left shunting of venous blood. Nitric oxide contributes to the impairment of hypoxic pulmonary vasoconstriction during endotoxemia.[8,29,30] We previously reported in the same sheep model that ONO1714 could restore the decreased hypoxic pulmonary vasoconstriction during endotoxemia.[8] We speculate that restoration of decreased hypoxic pulmonary vasoconstriction by ONO1714 could contribute to improve abnormal gas exchanges in the current study.

Nitric oxide inhibition could restore the endotoxin-induced hypotension in animal models[6,17] and humans.[31] These findings suggested that overproduction of nitric oxide was responsible for the hypotension. Likewise, administration of nonselective NOS inhibitor could restore the endotoxin-induced hypotension in sheep.[32] However, it has been reported that a significant increase in systemic arterial pressure is also obtained before endotoxin administration in sheep[21] and that prominent iNOS expression is induced 6 h after endotoxin treatment.[12,33] Furthermore, ONO1714 did not restore the early hypotension induced by endotoxin, although the unanesthetized sheep model used in the current study was not typical for endotoxic shock. Therefore, taken together, we believe that early hypotension after endotoxin administration in sheep was at least independent for the iNOS pathway.

Although iNOS inhibition by ONO1714 prevented the increases in nitric oxide products after endotoxin, improvement of lung injury was not complete in sheep treated with ONO1714. Similarly, several studies showed that endotoxin-induced lung injury was not completely reversed by the other NOS inhibitors and even in iNOS-deficient animals.[12,13,15–17] These results indicate that a self-limited contribution of the iNOS pathway and some other mediators are involved in the development of endotoxin-induced lung injury. Especially reactive oxidant species, such as superoxide anion, interact with nitric oxide in the development of acute lung injury.[34] Peroxynitrite, a product of nitric oxide and superoxide, has been implicated in sepsis-induced lung injury.[35] Because there are no data about the direct effects of ONO1714 on reactive oxygen intermediates, further studies are needed to evaluate the pharmacologic interaction of ONO1714 with oxygen radical production during endoxemia.

Pharmacologic effects of aminoguanidine on the inflammatory disorders may vary by the dose of the agents, sepsis, or both.[15–17] The relatively high dose of aminoguanidine caused an increase in pulmonary artery pressure in sheep.[18] We previously showed that 0.2 mg/kg ONO1714 caused pulmonary vasoconstriction in normal sheep, which is thought to be an inhibitory influence on constitutive NOS.[8] We have not investigated the dose-related effects of ONO1714 on the degree of lung injury and gas exchanges in the current study. To further evaluate the usefulness of this agent in clinics, we need additional studies of differential dose settings, other types of inflammatory models, or both.

In summary, we have shown that a new selective iNOS inhibitor, ONO1714, attenuates increased pulmonary fluid flux and improves abnormal oxygenation during endotoxemia in unanesthetized sheep. The current study reveals that nitric oxide release by iNOS pathway partially contributes to the development of lung injury during endotoxemia and that iNOS inhibition by ONO1714, a newly developed selective iNOS inhibitor, may be a therapeutic approach in patients with sepsis, acute lung injury, or both.

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