Effects of neutrophil elastase inhibitor (ONO-5046) on lung injury after intestinal ischemia-reperfusion

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Twelve hours before the study, food was withdrawn, but the Sprague-Dawley rats weighing 387.6 ± 49 g (means ± SD). Twelve hours before the study, food was withdrawn, but the

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METHODS

Animals. Studies were performed using adult male Sprague-Dawley rats weighing 387 ± 49 g (means ± SD). Twelve hours before the study, food was withdrawn, but the

THE GASTROINTESTINAL TRACT and liver become ischemic because of the hypovolemic shock associated with both trauma and extensive burns. Intestinal perfusion during the resuscitation procedure causes local ischemia-reperfusion (I/R) injury of the intestine (primary injury). In addition, this challenge produces a general systemic inflammatory response syndrome (SIRS), which can result in acute respiratory distress syndrome (ARDS) (1). It is well known that neutrophil elastase causes tissue injury directly and may also amplify inflammatory responses in SIRS by causing the production of chemokines at local inflammatory sites. It is also well documented that lung injury and increased pulmonary microvascular permeability occur after intestinal I/R associated with neutrophil elastase (4, 26). In addition, we have found that cytokine-induced neutrophil chemoattractant (CINC)-1 (a CXC chemokine) is involved in the neutrophil-mediated remote lung injury associated with 60 min of intestinal ischemia followed by reperfusion in rats (15). CINC-1 is produced by both rat peritoneal macrophages (32) and Kupffer cells (36) in vitro when these cells are challenged with neutrophil elastase. Therefore, neutrophil elastase may have a critical role in the progression of acute lung injury.

In several experimental models of SIRS and ARDS, pretreatment with cytokine receptor antagonists (26) or their specific antibodies (5) prevents lung injury. However, these agents have not proven to be effective in preventing ARDS in clinical trials (18). This discrepancy between experimental and clinical results may be related to the time course of intervention, because clinical treatment of ARDS usually occurs after the development of SIRS and ARDS. Several cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, are released during the early phase of SIRS, whereas other cytokines and chemokines are released at a later stage (3). These inflammatory proteins can cause neutrophil-mediated tissue injury in several remote organs such as the lungs, liver, and kidneys (3).

In the present study, we examined the effect of a specific neutrophil elastase inhibitor (NEI; ONO-5046) in preventing the progression of lung injury associated with intestinal I/R, together with reducing chemokine production, even if it is administered after the primary intestinal injury and the development of inflammatory response.
rats were allowed free access to water. Our study was approved by the animal ethics committee of Fukuoka University.

**Experimental protocol.** Pathogen-free rats were anesthetized with sodium pentobarbital (50 mg/kg ip) and placed onto a heating mat warmed to 37°C. Rats were then ventilated through a tracheotomy, and anesthesia was maintained by inhalation of 1% halothane. The jugular vein was cannulated by using polyethylene tubing. A midline laparotomy was performed, and the superior mesenteric artery (SMA) was exposed. Rats were assigned to five groups, as shown in Fig. 1. The intestinal I/R groups, designated as I/R(60) and I/R(240), were subjected to 90 min of intestinal ischemia followed by either 60 or 240 min of reperfusion, respectively. To produce ischemia, the SMA was occluded by using a microvascular clip for 90 min, and reperfusion was induced by removing the clip during a second laparotomy. Reperfusion was confirmed by the reappearance of pulsation to the mesenteric artery. The sham-operated control groups, designated as sham(60) and sham(240), underwent identical procedures but without SMA occlusion. Rats of the NEI treatment (I/R+NEI) group were also subjected to 90 min of intestinal ischemia followed by 240 min of reperfusion. In these rats, 2 mg/kg NEI (ONO-5046, Ono Pharmaceutical, Osaka, Japan) were injected at 60 min after interstitial reperfusion and then continuously infused at a rate of 10 nmol/kg h−1 of NEI into the jugular vein over the following 180 min.

Each group was also divided into three subgroups, each comprising five to seven rats. In the first subgroup, the lungs and intestinal segments were removed for measurement of pulmonary and intestinal endothelial permeability indexes. In the second subgroup, bronchoalveolar lavage was performed by using three aliquots of 5 ml of sterile PBS warmed to 37°C, and then bronchoalveolar lavage fluid (BALF) and serum were collected for measurement of cytokine levels. In the third subgroup, the left lung was used to measure extravascular lung water volume, whereas the right lung was used to perform pulmonary vascular lavage (PVL). Blood and PVL fluid (PVLF) were collected to measure neutrophil oxidative production. After the PVLF was obtained, the right lung and intestinal segments were used to measure tissue myeloperoxidase (MPO) activity and intestinal wet-to-dry weight ratio.

PVL was performed using a modification of the technique described previously (35). After injection of heparin (1,000 IU/kg) into the jugular vein, the thoracic cavity was opened, and the heart was quickly clamped using a large hemostat to arrest blood flow. The left lung was then ligated at the hilus and harvested to measure the extravascular lung water volume. The right lung and heart were also excised en bloc. The pulmonary artery was cannulated with a polyethylene tube, and the left atrium was cannulated with a larger tube. The pulmonary vasculature was then perfused with 100 ml of a mixture of 6% (wt/vol) hydroxyethyl starch solution (34 ml) and 0.9% (wt/vol) saline (66 ml) containing 1,000 IU of heparin warmed to 37°C. The right lung was perfused at a constant pressure of 20 cmH2O, and effluent from the left atrium (PVLF) was collected.

**Lung and intestinal injury.** The left lung and intestinal segments were used to measure extravascular lung water volume and intestinal wet-to-dry weight ratio. Extravascular lung water volume was measured by using a modification of the technique described previously by Pearce et al. (22). The left lung was weighed and homogenized in the same volume of distilled water. This specimen was centrifuged at 10,000 g for 20 min. The supernatant was then assayed for Hb concentration. Lung and blood wet-to-dry weight ratios were measured simultaneously. Dry weights were obtained by freeze-drying. Extravascular lung water volume (Qwl) was calculated using the following formula: Qwl (ml/g BFDW) = (l lung W/D − [blood W/D × (tissue Hb/blood Hb)]) / [1 − (tissue Hb/blood Hb)], where BFDW is the blood-free dry weight, and W/D is the wet-to-dry weight ratio.

Pulmonary and intestinal microvascular permeabilities were also measured by using a modification of the Evans blue dye extravasation technique described by Colletti et al. (7). Rats received an injection of 20 mg/kg of Evans blue dye into the jugular vein 30 min before death. At the end of the experiments, a blood sample, lungs, and a segment of the intestine were obtained, and the lungs were perfused with 20 ml of saline through a right ventriculotomy. The intestinal segment was washed with saline, and the contents were removed. The lung and intestinal tissue were freeze-dried and weighed. Evans blue was then extracted from the lung and intestinal tissue in deionized formamide at 60°C for 12 h. The levels of Evans blue in the lung, intestine, and serum (0.5 ml) were quantified by using dual-wavelength spectrophotometry. This method measures absorbance at 620 nm while correcting for any contaminating heme pigments, with the use of the following formula: corrected absorbance at 620 nm = actual absorbance at 620 nm − [1.426 (absorbance at 740 nm) + 0.03]. The endothelial permeability indexes of the lungs and intestine were then calculated by dividing the corrected lung and intestinal Evans blue absorbance at 620 nm/g dry tissue by the corrected serum Evans blue absorbance at 620 nm.

**Neutrophil oxidative production.** To evaluate neutrophil oxidative production in systemic blood and pulmonary vasculature blood (PVB), neutrophil H2O2 production in the systemic blood and PVLF was measured using a modification of the flow cytometric technique (2). At the end of the experimental protocol, a heparinized blood sample was obtained from the abdominal aorta and depleted of erythrocytes by
sedimentation on 6% (wt/vol) dextran for 30 min. The leukocyte-rich supernatants of plasma and PVLF were then diluted to obtain a cell count of 2.0 × 10^6 cell/ml and were stored at 4°C for later determination of neutrophil H₂O₂ production. For these measurements, specimens were preincubated with 5 μM 2',7'-dichlorofluorescein diacetate (Sigma Immunocumano, St. Louis, MO) at 37°C for 15 min and then incubated with 10⁻⁶ M N-formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma Immunocumano) at 37°C for 15 min. Relative fluorescence intensity was measured by flow cytometry (488 nm; Becton Dickinson, Mountain View, CA) and analyzed using Consort 30 software. Relative fluorescence intensity was presented as the mean 2',7'-dichlorofluorescein fluorescence per cell.

**Neutrophil accumulation in lungs and intestine.** To evaluate neutrophil sequestration in the pulmonary vessels and alveolar spaces, neutrophils in the PVLF and BAF were counted by Diff-Quick staining. Leukocytes in systemic blood were also counted in rats used for PVL. Neutrophil accumulation within the lung and intestinal tissues was quantified by using the MPO assay described by Goldblum et al. (10). The right lung, obtained after PVL, and the intestinal segment were washed with saline, weighed, and immediately homogenized in potassium phosphate buffer (10 mM, pH 7.4) containing 0.5% (vol/vol) hexadecyltrimethylammonium bromide (Sigma Immunocumano). The pellet was rehomogenized and sonicated and then centrifuged at 10,000 g at 4°C for 20 min. The supernatant was centrifuged in potassium phosphate buffer (50 mM, pH 6.0) containing 0.167 mg/ml O-dianisidine hydrochloride (Sigma Immunocumano) and 0.0005% (wt/vol) hydrogen peroxide. Absorbance of the solution was measured over 3 min at 460 nm. MPO activity was expressed as international units per gram of dry tissue. One unit of enzyme activity was defined as the amount of peroxidase that produced an absorbance change of 1.0 optical density unit/min at 25°C.

**Cytokine levels in blood and BAF.** Serum and BAF samples stored at −80°C were used to measure cytokine levels. CINC-1, CINC-3, and TNF-α levels in the serum and BAF were measured by ELISA using commercially available ELISA kits for rat CINC-1 and CINC-3 (biotin-conjugated anti-rat CINC-1 and CINC-3 rabbit IgG; Immuno-Biological Laboratories, Gunma, Japan) and an ELISA kit for rat TNF-α (horseradish peroxidase-conjugated anti-TNF-α rabbit IgG; Genzyme, Cambridge, MA).

**Neutrophil elastase activity in BAF.** Neutrophil elastase activity was measured with the synthetic substrate Suc-Ala-Ala-Pro-Val p-nitroaniline by using the method described by Yoshimura et al. (38). Samples were incubated in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl and 1 mM substrate for 24 h at 37°C. After incubation, the amount of released p-nitroaniline was measured spectrophotometrically at 405 nm, which reflected neutrophil elastase activity.

**Statistical analysis.** All data are presented as means ± SE. Parametric data were subjected to ANOVA followed by Fisher’s protected least significant difference test as a multiple comparison procedure. To analyze the correlation between chemokines and neutrophils in BAF, we estimated Pearson’s correlation coefficient and assessed its significance by Fisher’s z conversion. All analyses were performed using Stat View 4.0 for Macintosh (Abacus Concepts, Berkeley, CA). P values < 0.05 were considered statistically significant.

**RESULTS**

**Lung and intestinal injuries.** The intestinal wet-to-dry weight ratio, endothelial permeability index, and MPO activity were all significantly greater in the I/R group than in the sham group at both 60 and 240 min after reperfusion (P < 0.01). However, these increases were not different from those in the I/R + NEI group (Table 1). As shown in Fig. 2A, the lung endothelial permeability index in the I/R group was greater than in the sham group at 240 min (P < 0.01) but not at 60 min after intestinal reperfusion. Although the permeability index in the I/R + NEI group was slightly higher than that in the sham group, it was significantly smaller than that in the I/R(240) group (P < 0.05). There were no significant differences in extravascular lung water volume in all groups (Fig. 2B).

**Neutrophil oxidative production.** The mean value of neutrophil H₂O₂ production for each treatment group is shown in Table 2. Levels of H₂O₂ production in systemic blood and PVB (both unstimulated and stimulated by FMLP) in the I/R group were greater than those in the sham group after 240 min of reperfusion, although the differences at 60 min were not significant. There was a similar rise in production in the systemic blood of the I/R + NEI group, but the increase in PVB H₂O₂ production was markedly suppressed in this group (P < 0.05).

**Neutrophil accumulation in lungs.** The leukocyte count in systemic blood was decreased in the I/R group compared with the sham group at both 60 (P < 0.05) and 240 min (P < 0.05) after reperfusion (Fig. 3A). In contrast, the neutrophil count in PVB was higher in the I/R group compared with the sham group at both 60 (P < 0.01) and 240 min (P < 0.05) after reperfusion. A similar increase in neutrophil count was present in the I/R + NEI group (Fig. 3B).

**Table 1. Intestinal injury**

<table>
<thead>
<tr>
<th></th>
<th>Sham(60)</th>
<th>I/R(60)</th>
<th>Sham(240)</th>
<th>I/R(240)</th>
<th>I/R + NEI</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wet/dry weight</td>
<td>2.9 ± 0.13</td>
<td>3.82 ± 0.23†</td>
<td>3.23 ± 0.09</td>
<td>3.95 ± 0.19†</td>
<td>3.91 ± 0.15</td>
</tr>
<tr>
<td>Permeability index, tissue/plasma</td>
<td>0.19 ± 0.02</td>
<td>0.72 ± 0.16†</td>
<td>0.16 ± 0.03</td>
<td>0.47 ± 0.07‡</td>
<td>0.45 ± 0.06</td>
</tr>
<tr>
<td>MPO activity, unit/g DW</td>
<td>0.39 ± 0.18</td>
<td>4.15 ± 0.81†</td>
<td>0.13 ± 0.05</td>
<td>4.30 ± 0.76‡</td>
<td>3.54 ± 0.75</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of animals. I/R, ischemia-reperfusion; NEI, neutrophil elastase inhibitor; wet/dry weight, ratio of wet to dry weight. MPO, myeloperoxidase; DW, dry weight. See METHODS for explanation of groups. Intestinal wet/dry weight ratio, permeability index, and MPO activity increased at 60 and 240 min after intestinal I/R. These increases were unaffected by posttreatment with NEI. Significant difference compared with sham group: †P < 0.05, ‡P < 0.01.
MPO was significantly suppressed in the I/R+NEI group ($P < 0.05$) (Fig. 3C). The neutrophil count in BALF in the I/R group was also higher than in the sham group at 240 min ($P < 0.01$) but not at 60 min, and this increase was also markedly suppressed in the I/R+NEI group ($P < 0.01$) (Fig. 3D).

Cytokine levels and neutrophil elastase activity. After 60 min of reperfusion, CINC-1 levels were not significantly increased in either blood (Fig. 4A) or BALF (Fig. 4B). However, after 240 min of reperfusion, CINC-1 levels in both blood and BALF were markedly elevated in the I/R group ($P < 0.01$). These elevations in CINC-1 were significantly attenuated in the I/R+NEI group compared with no treatment in both blood and BALF ($P < 0.05$). CINC-3 levels in blood (Fig. 5A) were not significantly altered by I/R or I/R+NEI treatment, but CINC-3 levels in BALF (Fig. 5B) were significantly elevated in the I/R group ($P < 0.01$). Importantly, CINC-3 levels in the I/R+NEI group were significantly lower compared with those of the I/R group ($P < 0.05$). The levels of both CINC-1 and CINC-3 correlated significantly with neutrophil count in BALF at 240 min after reperfusion ($r = 0.73$ and 0.75, respectively; both $P < 0.01$). TNF-α levels in the blood were not increased in the I/R group at either 60 or 240 min after reperfusion (data not shown) and also TNF-α was not detected in BALF in any group. Neutrophil elastase activity could not be detected in BALF in any group.

**DISCUSSION**

Our study clearly demonstrated that intestinal I/R caused neutrophil-mediated remote lung injury and that this injury was related to increased levels of CINC-1 and CINC-3 in the lung. Our results also showed that NEI posttreatment attenuated the lung injury. These results confirm the recent findings that NEI attenuates lung injury in various experimental models (16, 21, 24, 30, 33, 37). Our study extended these findings by showing that NEI prevented neutrophil activation and accumulation in the lung, together with reducing chemokine production, even when it was administered after intestinal injury and the development of inflammatory response. Although the lung endothelial permeability index was significantly increased after intestinal I/R, indicating lung injury with increased microvascular permeability, the extravascular lung water volume did not increase significantly. However, edema fluid may be cleared by lymphatics in this instance, thus preventing the formation of a significant amount of alveolar edema (13).

At 60 min after reperfusion, the leukocyte count in systemic blood was decreased, whereas the neutrophil count in PVB increased significantly and neutrophil H$_2$O$_2$ production also tended to increase in PVB. Caty et al. (5) showed that TNF-α increased in the blood 30 min after intestinal I/R but was cleared within 60 min after reperfusion. In our model, activated neutrophils could be sequestered in the pulmonary vessels within 60 min after reperfusion, and some nonactivated neutrophils might remain in the systemic circulation. Our results showed significant increases in neutrophil H$_2$O$_2$ production in both systemic blood and PVB (both unstimulated and stimulated by fMLP) at 240 min after reperfusion and marked increases in both lung

**Table 2. Neutrophil H$_2$O$_2$ production (fluorescence/cell)**

<table>
<thead>
<tr>
<th></th>
<th>Sham(60)</th>
<th>I/R(60)</th>
<th>Sham(240)</th>
<th>I/R(240)</th>
<th>I/R + NEI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 5</td>
<td>n = 6</td>
<td>n = 5</td>
<td>n = 7</td>
<td>n = 6</td>
</tr>
<tr>
<td>Systemic blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated</td>
<td>46 ± 9</td>
<td>25 ± 5</td>
<td>45 ± 9</td>
<td>96 ± 14*</td>
<td>83 ± 24</td>
</tr>
<tr>
<td>Stimulated by fMLP</td>
<td>87 ± 11</td>
<td>47 ± 9</td>
<td>96 ± 16</td>
<td>151 ± 18*</td>
<td>119 ± 29</td>
</tr>
<tr>
<td>PVB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated</td>
<td>670 ± 212</td>
<td>895 ± 117</td>
<td>437 ± 101</td>
<td>1,319 ± 106†</td>
<td>907 ± 170‡</td>
</tr>
<tr>
<td>Stimulated by fMLP</td>
<td>1,054 ± 274</td>
<td>1,317 ± 128</td>
<td>755 ± 158</td>
<td>1,869 ± 131‡</td>
<td>1,196 ± 153§</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$ = no. of animals. PVB, pulmonary vasculature blood; fMLP, N-formyl-methionyl-leucyl-phenylalanine. See METHODS for explanation of groups. Neutrophil H$_2$O$_2$ production in both systemic blood and PVB increased significantly after intestinal I/R. The increase in neutrophil H$_2$O$_2$ production in PVB, but not that in systemic blood, was prevented by posttreatment with NEI. Significant difference compared with sham group: *$P < 0.05$ and †$P < 0.01$. Significant difference compared with the I/R(240) group: ‡$P < 0.05$ and §$P < 0.01$. 

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MPO activity and neutrophil counts in BALF. We speculate that neutrophils in the pulmonary vessels are further activated and migrate into the lung interstitial and alveolar spaces.

CINCs, including CINC-1, CINC-2α, CINC-2β, and CINC-3, are known to induce neutrophil chemotaxis and activation (25). In our study, CINC-1 levels in blood and CINC-1 and CINC-3 levels in BALF were significantly elevated after 240 min of reperfusion. Because CINCs in BALF were diluted by PBS, CINC alveolar levels could actually be greater than those in the blood. Although CINC-3 levels in BALF were lower than those of CINC-1, the CINC-3 concentration gradient acting between blood and alveolar spaces was greater than that of CINC-1. CINC-1 and CINC-3 levels correlated significantly with neutrophil counts in BALF after 240 min of reperfusion, suggesting that these chemokines play critical roles in promoting remote lung injury after intestinal I/R. It has been shown that alveolar macrophages (9, 27), alveolar type II cells (8), epithelial cells (9), and fibroblasts (9, 12) can produce CINCs after exposure to endotoxin, TNF-α, and IL-1β. Therefore, in our experimental model, CINCs in BALF are likely to be released from these cells in response to inflammatory cytokines and intestine-derived endotoxin.

Fig. 3. Neutrophil accumulation within the lung tissue and alveoli. A: the leukocytes in systemic blood decreased at 60 and 240 min after intestinal I/R. B: the neutrophil count in pulmonary vascular blood (PVB) increased at 60 and 240 min after intestinal I/R. This increase was unaffected by NEI treatment. Lung myeloperoxidase (MPO) activity (C) and neutrophil count in bronchoalveolar lavage fluid (BALF) (D) increased at 240 min after intestinal I/R, and these increases were similarly prevented by posttreatment with NEI. Values are means ± SE. WBC, white blood cell.

Fig. 4. Levels of cytokine-induced neutrophil chemoattractant (CINC)-1 in blood and BALF. Levels of CINC-1 in blood (A) and BALF (B) were increased at 240 min after intestinal I/R. These increases were prevented by posttreatment with NEI. Values are means ± SE.
In this study, lung injury was attenuated by post-treatment with NEI, suggesting that neutrophil elastase might play an important role in promoting the remote lung injury after intestinal I/R. In humans, increased neutrophil elastase activity has been found in BALF of patients with ARDS after trauma, shock, or sepsis (28). Furthermore, experimental studies showed significantly elevated levels of neutrophil elastase in BALF of isolated rat lungs that had been perfused with blood obtained from animals subjected to 120 min of intestinal ischemia followed by reperfusion (4). The presence of a high neutrophil elastase activity in BALF could not be confirmed in our model. This was either due to insensitivity of the method used for analysis or reflected the presence of low levels of elastase activity in BALF. The latter possibility could be due to the duration of ischemia used in our study (90 min) or sequestration of intestinal I/R-activated neutrophils in our in vivo model in not only the lungs but also other remote organs, such as the liver and kidneys. ONO-5046 competitively inhibits neutrophil elastase in humans, rats, hamsters, and mice (17). It also inhibited lung injury in various experimental models, such as endotoxin-induced lung injury (16, 21, 24, 30, 33, 37). However, the compound itself has no direct effects on the production of monocyte chemoattractant protein-1 (a CC chemokine) production be-
tween neutrophils and Kupffer cells in a dose-dependent manner and that such production of CINC is reduced by ONO-5046 (NEI) in vitro (32, 36). Thus, in our model, NEI probably reduced neutrophil elastase-induced production of CINCs by alveolar macrophages through the inhibition of neutrophil elastase activity. NEI might possibly reduce monocyte chemotactic protein-1 (a CC chemokine) production because neutrophil elastase has been reported to enhance the production of monocyte chemoattractant protein-1 by peritoneal macrophages in vitro (14). Hyaluronan, fragmented by oxygen radicals, is known to induce IL-8 gene expression in human alveolar macrophages (20). Therefore, the observed decrease in the generation of oxygen radicals by NEI could also reduce chemokine production in the lungs. Suppression of chemokine release in the alveolar spaces would attenuate both neutrophil activation and their migration into the lungs.
In conclusion, posttreatment of intestinal I/R with NEI reduced neutrophil activation in pulmonary vessels and neutrophil accumulation in the lungs and attenuated the increased pulmonary microvascular permeability. Furthermore, attenuation of lung injury is related to the suppression of chemokine production in the lungs. Our results indicate that treatment with NEI, even when administered after the primary intestinal insult and the development of inflammatory response, can prevent the progression of lung injury. These findings also suggest that administration of NEI after the onset of ARDS might be therapeutically beneficial, although further clinical studies are needed to confirm the results reported in our experimental rat model.

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