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ORIGINAL ARTICLE

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# The effects of a specific neutrophil elastase inhibitor (ONO-5046) in pulmonary ischemia–reperfusion injury

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**Abstract** Ischemia–reperfusion injury is a significant problem in lung transplantation. Polymorphonuclear elastase derived from neutrophils plays a major mechanistic role in this process. Hence, we have investigated the effects of ONO-5046, a neutrophil elastase inhibitor, on ischemia–reperfusion injury. Fifteen rabbits were divided into three groups: 2 h of single left-lung perfusion (control group, $n=3$); 2 h of ischemia followed by 2 h of reperfusion (ischemic group, $n=6$); and drip intravenous administration of ONO-5046 during the 2 h of ischemia and 2 h of reperfusion (ONO-5046 group, $n=6$). Hemodynamic parameters were determined and a histopathological examination of the lung was performed. In the ONO-5046 group, arterial oxygen pressure, cardiac output, and tissue blood perfusion were higher and pulmonary vascular resistance was lower than in the ischemic group. The ONO-5046 group also showed large decreases in neutrophil infiltration, pulmonary edema, and intra-alveolar hemorrhage. Treatment with ONO-5046 improves lung function in a rabbit-lung ischemia–reperfusion model.

**Keywords** Ischemia–reperfusion injury · Polymorphonuclear elastase · Lung transplantation · Neutrophil infiltration · Specific neutrophil elastase inhibitor

## Introduction

When organs lapse into ischemia for a certain period, tissues are injured and cellular metabolic processes are disturbed. Furthermore, severe injury of the ischemic cells and tissues is induced because a sudden oxygen load is added through reperfusion [1, 8]. This phenomenon is referred to as ischemia–reperfusion injury and it remains a significant problem in current methods used to preserve lungs for transplantation. Since the initial observation by Tate et al. [16] of the involvement of superoxides in ischemia–reperfusion injury, the importance of these species in the process has been recognized for many organs. The hypoxanthine–xanthine oxidase cascade [11, 16], the nicotinamide–adenine dinucleotide phosphate-oxidase cascade [14], and the arachidonic-acid cascade, have all drawn attention, and it has been suggested [15] that cytokines derived from neutrophils are related to superoxide production. Hence, superoxides and neutrophils are considered to be major etiological factors in ischemia–reperfusion injury.

Among the proteases released from activated neutrophils, polymorphonuclear elastase (PMN-E), which was shown some time ago by Janoff and Zeligs [6] to be an extremely active protease, is one of the enzymes most associated with the onset and progression of ischemia–reperfusion injury. PMN-E is a 33-kDa serine protease that can hydrolyze peptide bonds in many different proteins. It has a powerful decomposition effect on bacterial and other foreign proteins and plays an

extremely important role in biophylaxis [2]. However, PMN-E possesses a low substrate specificity, and its defense mechanism activity can also result in damage to normal cells [12]. For example, PMN-E activity in the lungs can result in the decomposition of elastin fiber, an interstitial component [3]. Hence, there are several inhibitors in the blood and interstitial fluid that can suppress an excessive PMN-E response. Three kinds of serum proteases, α1-antitrypsin (α1-AT), α2-macroglobulin, and inter-α-trypsin inhibitor, and a secretory leukocyte protease inhibitor that exists in a variety of tissues, are all known to be endogenous PMN-E inhibitors [4].

Inhibition of PMN-E may be an effective strategy for the control of ischemia–reperfusion injury, but there has been no application of a synthetic drug as a PMN-E inhibitor. In this paper, we report the effects of ONO-5046, a new, specific neutrophil elastase inhibitor [17], in a rabbit-lung model of ischemia–reperfusion injury. ONO-5046 has previously been used for the treatment of acute circulatory failure, and here we show that this drug is also effective against ischemia–reperfusion injury.

## Materials and methods

### ONO-5046

Sodium *N*-[2-[4-(2,2-dimethylpropionyloxy) phenylsulfonylamino]-benzoyl] amino acetate tetrahydrate (ONO-5046; $C_{20}H21N2O7S$-$Na{\cdot}4H_20$; mol. wt. 528.51) was provided by the ONO Pharmaceutical Company, Osaka, Japan.

### Surgical preparation

Fifteen New Zealand white female rabbits (body weight 3.0–3.5 kg) were anesthetized with sodium pentobarbital. A tracheostomy and endotracheal intubation (with a tube 6 mm in diameter) were performed, followed by ventilation at 50% inspiratory oxygen with a respirator (SN-480-5, Shinano, Tokyo). After a median sternotomy had been performed, 4-0 Prolene ligatures were passed around both sides of the pulmonary hilum and heparin sodium was injected into the ear vein. Catheters were inserted into the right carotid artery, the pulmonary artery, and the left ventricle, for the measurement of pressure.

### Experiment protocol

The 15 rabbits were divided into three groups, which are referred to as the control group, the ischemic group, and the ONO-5046 group. The experiment protocol is summarized in Fig. 1. In the control group ($n=3$), immediate single left-lung perfusion was performed for 2 h by the clamping of the right hilum. In the ischemic group ($n=6$), ischemia was performed for 2 h and then single left-lung reperfusion was performed for 2 h by the simultaneous release of the left hilum clamp and the clamping of the right hilum. In the ONO-5046 group ($n=6$), ischemia was performed for 2 h with the simultaneous administration of ONO-5046 as an intravenous drip (25 mg/kg per h) from the beginning of the ischemia period. This was followed by reperfusion, performed as for the ischemic group. ONO-5046 administration was continued during the reperfusion period.

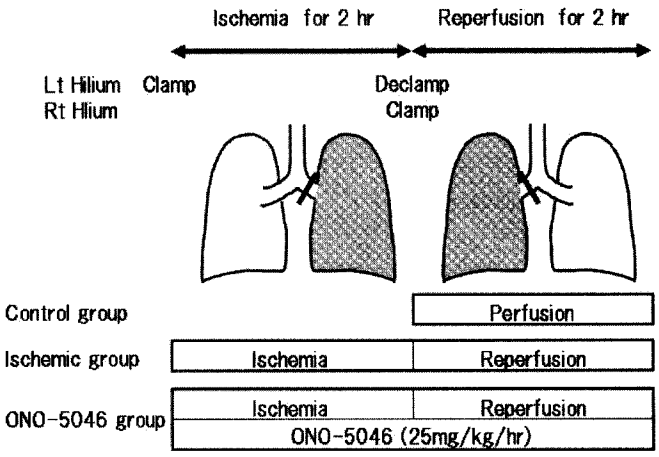


**Fig. 1** Schematic illustration of the experiment protocol. In the control group, 2 h of single left-lung perfusion was performed, with no ischemia. In the ischemic group, 2 h of single left-lung reperfusion was performed after a 2 h ischemia period. In the ONO-5046 group, ONO-5046 was administered as a drip intravenous injection during both the 2 h of ischemia and 2 h of single left-lung reperfusion periods

### Measurements of hemodynamic parameters

The arterial oxygen pressure ($PaO_2$) and oxygen saturation ($SaO_2$) were analyzed from the carotid artery blood pressure, pulmonary pressure (PA), and left ventricle pressure (LVP) by an electrical recorder (Polygraph System RM-6000, Nihon Koden, Tokyo). Cardiac output (CO) was measured with a Doppler blood perfusion recorder (T101, Advance, Tokyo) positioned on the ascending aorta. Tissue blood perfusion was measured with a laser tissue blood perfusion recorder (ALF21, Advance, Tokyo). Pulmonary vascular resistance (PVR) was calculated, as the value before the clamping of the right hilum, from the following equation: (mean PAP–diastolic LAP)/CO×K, where PAP is the pulmonary arterial pressure, LAP is the left atrial pressure, K is a constant, and LVP⇒LAP. The hemodynamic parameters were measured at intervals of 30 min.

### Histopathological examination of the lungs

The animals of the control group were killed after the blocking of the left hilar region for 2 h, and those in the ONO-5046 and ischemic groups were killed after reperfusion for 2 h. Tissue from the left lung was collected from all the animals, fixed in 10% buffered formalin, and then subjected to hematoxylin–eosin staining. Neutrophil infiltration and intra-alveolar changes were graded by use of the following scale: −, nothing; +, moderate; + +, severe.

### Statistical analysis

Values are expressed as means ± SD. Parametric data were analyzed by a repeated measure ANOVA and by Scheffe's method, after a one-factor ANOVA was used to test for significant differences between and within groups. Significant differences were accepted at a level of $P<0.05$.

## Results

### Hemodynamic parameters

#### *$PaO_2$*

In the control group there was no significant change in $PaO_2$, whereas in the ischemic and ONO-5046 groups, $PaO_2$ decreased progressively (Table 1). The ischemic group showed significantly lower $PaO_2$ levels after reperfusion than the control group, and the ONO-5046 group showed significantly higher $PaO_2$ levels after reperfusion than the ischemic group (Fig. 2A).

#### *$SaO_2$*

There were no significant changes in $SaO_2$ within each group, and there were no significant differences in $SaO_2$ between the groups (Fig. 2B).

#### *mPAP*

There was no significant change in mPAP in the control group, but in the ischemic group the mPAP increased progressively (Table 1). The ischemic group showed significantly higher mPAP levels after reperfusion than the control group, and the ONO-5046 group tended to show lower mPAP levels after reperfusion than the ischemic group (Fig. 2C).

#### *CO*

There was also no significant change in CO in the control group, but in the ischemic and ONO-5046 groups, CO decreased progressively (Table 1). The ischemic group showed significantly lower CO levels after reperfusion than the control group, and the ONO-5046 group showed significantly higher CO levels after reperfusion than the ischemic group (Fig. 2D).

#### *PVR*

Similarly to the mPAP and CO, there was no significant change in PVR in the control group, but in the ischemic and ONO-5046 groups, the PVR increased progressively (Table 1). The ischemic group showed significantly higher PVR values after reperfusion than the control group, and the ONO-5046 group showed significantly lower PVR values after reperfusion than the ischemic group (Fig. 2E).

#### *Tissue blood perfusion*

There was also no significant change in tissue blood perfusion in the control group, but in the ischemic and ONO-5046 groups, tissue blood perfusion decreased progressively (Table 1). The ischemic group showed significantly lower tissue blood perfusion after reperfusion than the control group, and the ONO-5046 group showed significantly higher tissue blood perfusion after reperfusion than the ischemic group (Fig. 2F).

### Histopathological examination of the lungs

As shown in Table 2, in the control group there were no significant changes in neutrophil infiltration, or hemorrhage (Fig. 3A). In the ischemic group, the interstitium and intra-alveolar septa were diffusely infiltrated by neutrophils, and severe edema and hemorrhage were

**Table 1** Comparison of lung-function parameters in the three groups. Data are shown as mean ± SD

| Group | $PaO_2$ (mmHg) | $SaO_2$ (%) | mPAP (mmHg) | CO (ml/min) | PVR | Tissue blood perfusion (ml/min per 100 g) |
|---|---|---|---|---|---|---|
| Control ($n=3$) | | | | | | |
| Pre | 326 ± 6 | 99.9 ± 0 | 13 ± 2.6 | 463 ± 58 | 1.0 ± 0 | 98 ± 8 |
| 30 min | 318 ± 5 | 99.9 ± 0 | 13 ± 0.5 | 450 ± 62 | 1.0 ± 0 | 97 ± 6 |
| 60 min | 324 ± 12 | 99.9 ± 0 | 13 ± 0.5 | 468 ± 67 | 1.0 ± 0 | 97 ± 11 |
| 90 min | 323 ± 17 | 99.9 ± 0 | 13 ± 1.2 | 450 ± 65 | 1.0 ± 0 | 93 ± 11 |
| 120 min | 322 ± 18 | 99.8 ± 0.1 | 12 ± 1.2 | 433 ± 49 | 1.0 ± 0 | 90 ± 7 |
| Ischemic ($n=6$) | | | | | | |
| Pre | 294 ± 36 | 99.9 ± 0.1 | 14 ± 2.0 | 424 ± 44 | 1.1 ± 0.2 | 89 ± 3 |
| 30 min | 243 ± 39 | 99.9 ± 0.1 | 15 ± 0.8 | 328 ± 62 | 1.7 ± 0.4 | 81 ± 6 |
| 60 min | 166 ± 48** | 99.9 ± 0.1 | 17 ± 1.7 | 243 ± 63** | 2.6 ± 0.8 | 75 ± 4 |
| 90 min | 132 ± 26** | 99.9 ± 0.1 | 19 ± 2.9 | 181 ± 64** | 3.9 ± 0.9** | 73 ± 6* |
| 120 min | 80 ± 13** | 99.8 ± 0.1 | 21 ± 4.1** | 147 ± 57** | 5.8 ± 1.5** | 68 ± 5** |
| ONO-5046 ($n=6$) | | | | | | |
| Pre | 301 ± 12 | 99.9 ± 0.1 | 14 ± 0.8 | 417 ± 38 | 1.1 ± 0.1 | 93 ± 3 |
| 30 min | 270 ± 25 | 99.9 ± 0.1 | 15 ± 1.2 | 343 ± 51 | 1.5 ± 0.2 | 92 ± 5 |
| 60 min | 219 ± 56 | 99.9 ± 0.1 | 16 ± 1.7 | 294 ± 18** | 1.9 ± 0.2 | 84 ± 5 |
| 90 min | 192 ± 64* | 99.9 ± 0.1 | 18 ± 3.3 | 258 ± 26** | 2.3 ± 0.4** | 80 ± 7* |
| 120 min | 162 ± 59** | 99.8 ± 0.1 | 18 ± 4.4 | 240 ± 24** | 2.7 ± 0.8** | 76 ± 7** |

*$P<0.05$, **$P<0.01$ vs pre-clamp data

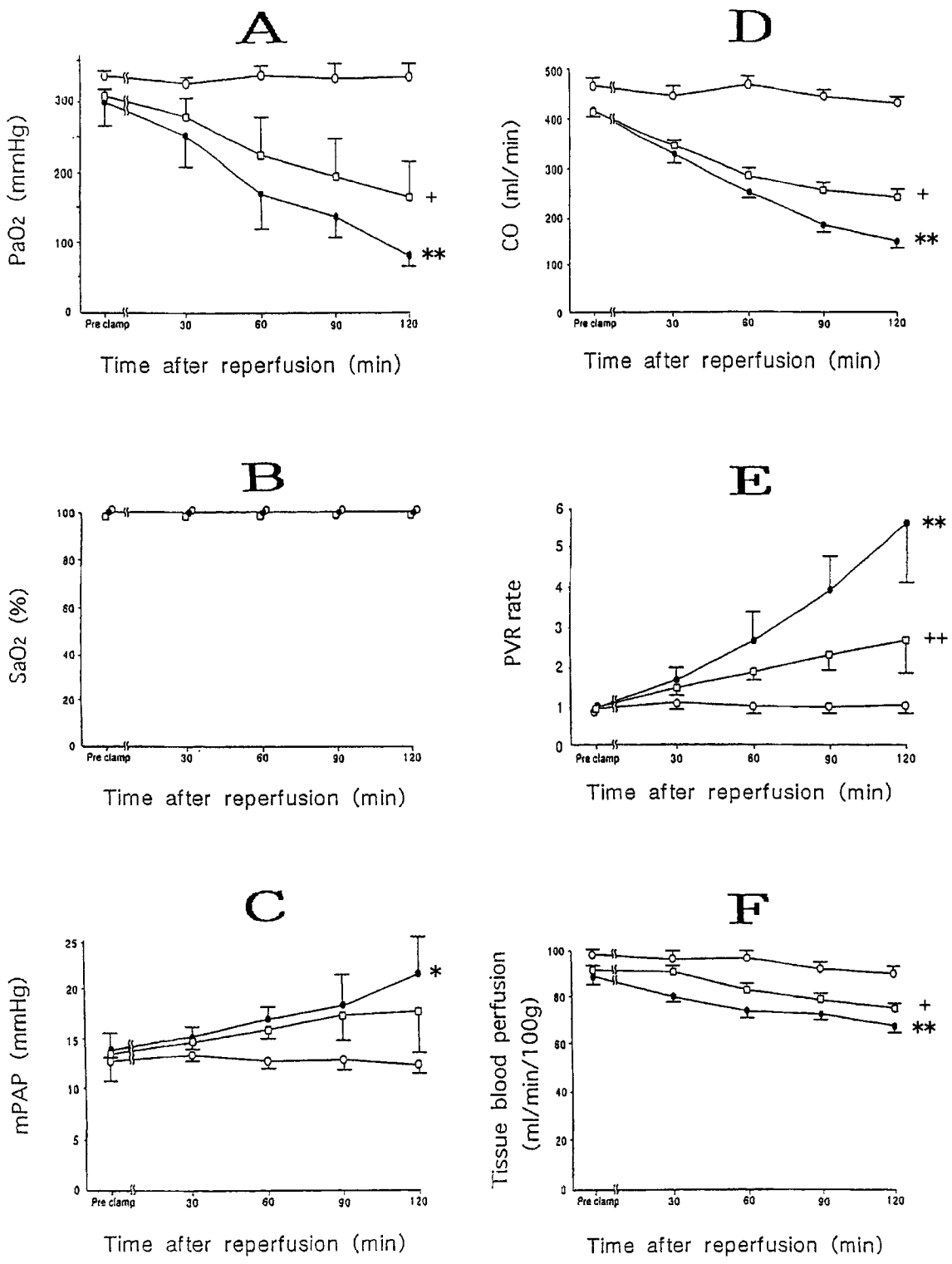


**Fig. 2A–F** Changes in hemodynamic parameters. **A** $PaO_2$, **B** $SaO_2$, **C** mPAP, **D** CO, **E** PVR, and **F** tissue blood perfusion. *Each point* shows the mean ± SD. *Open circle* control group, *closed circle* ischemic group, *open square* ONO-5046 group. $*P < 0.05$ vs control group, $**P < 0.01$ vs control group, $+P < 0.05$ vs ischemic group, $++P < 0.01$ vs ischemic group, by repeated measure ANOVA

seen in the alveoli (Fig. 3B). In the ONO-5046 group, the interstitium and the alveoli contained moderately scattered neutrophils, and mild edema and hemorrhage were present in the alveoli (Fig 3C).

**Table 2** Histopathological findings in the lung in the three study groups

| Group | Number of rabbits used | Neutrophil infiltration | | Intra-alveolar change | |
|---|---|---|---|---|---|
| Interstitium | Alveoli | Edema | Hemorrhage | | |
| Control | 3 | – | – | – | – |
| Ischemic | 6 | + + | + + | + + | + + |
| ONO-5046 | 6 | + | + | + | + |

## Discussion

During ischemia, pulmonary venules in the most fragile reperfusion areas, at junctions between vascular tunica intimas, are damaged [12] and activated neutrophils produce superoxides and release proteases, such as PMN-E, from lysosomes. As a result, it is believed that elastin fibers (constitutive components of the pulmonary connective tissues, pulmonary alveoli, and the basal membrane of vascular endothelial cells) are degraded [9]. Extravasation of serum containing proteins induces osmotic pressure, and this leads to enhanced permeability and pulmonary edema [5, 13]. Edematous and hemorrhagic changes, caused by a decrease in oxygen concentration in pulmonary tissues that is induced by endothelial cell damage, result in the development of a non-ventilated area in the lungs. We concluded that the anoxemia in the ischemic group was caused mainly by the pulmonary edema in this area. We further suggest that the increased PVR was caused by the swelling of the endothelial cells and vasospasm at an early stage, and by adhesion to endothelial cells and aggregation of the neutrophils at a later stage.

The ONO-5046 group showed a significantly higher $PaO_2$, CO, and pulmonary tissue blood perfusion and a significantly lower PVR than the ischemic group. In addition, the histopathological features of the ONO-5046 group showed a lower degree of neutrophil infiltration into the intra-alveolar septa and the alveoli than did those of the ischemic group, and erythrocyte infiltration into the alveoli and pulmonary edema were suppressed. From these results, we concluded that administration of ONO-5046 directly suppressed the increase in PMN-E level, leading to an indirect suppression of ischemia–reperfusion injury. It has been reported that among the proteases, PMN-E has the potential to increase superoxide production [7]. These superoxides inhibit α1-AT binding to PMN-E by oxidizing methionine residues in the active site of α1-AT [10]. Hence, we suggest that when PMN-E is specifically inhibited by the administration of ONO-5046, superoxide production is also suppressed and the effect of ONO-5046 is further increased.

In conclusion, we have shown that ONO-5046, a low-molecular-weight, specific neutrophil elastase inhibitor, can have a significant effect on ischemia–reperfusion injury in a rabbit-lung injury model. Hence, following treatment with ONO-5046, the $PaO_2$, CO, and blood flow volume in lung tissues were significantly higher than in the ischemic group, but the PVR was

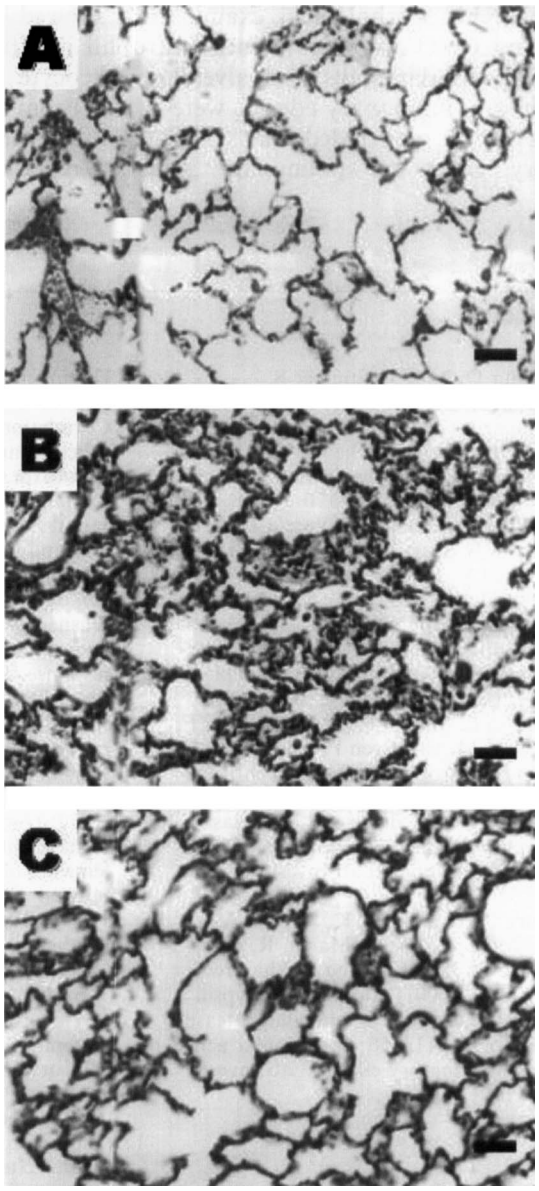


**Fig. 3A–C** Histological results following hematoxylin–eosin staining of the rabbit lung. **A** Control group, after 2 h of blocking of the right hilum, showing no significant change and no evidence of neutrophil infiltration or hemorrhage. **B** Ischemic group, after 2 h of ischemia, 2 h of reperfusion, and no ONO-5046 treatment. The interstitium and intra-alveolar septa are diffusely infiltrated by neutrophils, and severe edema and hemorrhage are seen in the alveoli. **C** ONO-5046 group, after 2 h of ischemia, 2 h of reperfusion, and drip intravenous administration of ONO-5046. Neutrophils are moderately scattered in the interstitium and alveoli. Mild edema and hemorrhage are present in the alveoli. *The scale bar* indicates a distance of 50 μm

lower. A histopathological examination showed that, following ONO-5046 treatment, neutrophil infiltration into the alveolar walls and alveoli, erythrocyte infiltration, and pulmonary edema, were slight. From these results it was concluded that ONO-5046 had an inhibitory effect on pulmonary ischemia–reperfusion injury and that this effect was probably due to inhibition of polymorphonuclear elastase.

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## References


1. Al Nita D, Nita V, Spulber S, Moldovan M, Popa DP, Zagrean AM, Zagrean L (2001) Oxidative damage following cerebral ischemia depends on reperfusion—a biochemical study in rat. J Cell Mol Med 5:163–170
2. Alegre J, Surinach JM, Varela E, Armadans L, Marti R, Segura R, Aleman C, Falco V, Fernandez de Sevilla T (2000) Diagnostic accuracy of pleural fluid polymorphonuclear elastase in the differentiation between pyogenic bacterial infectious and non-infectious pleural effusions. Respiration 67:426–432
3. Cavarra E, Martorana PA, Gambelli F, de Santi M, van Even P, Lungarella G (1996) Neutrophil recruitment into the lungs is associated with increased lung elastase burden, decreased lung elastin, and emphysema in α1 proteinase inhibitor-deficient mice. Lab Invest 75:273–280
4. Churg A, Dai J, Zay K, Karsan A, Hendricks R, Yee C, Martin R, MacKenzie R, Xie C, Zhang L, Shapiro S, Wright JL (2001) Alpha-1-antitrypsin and a broad spectrum metalloprotease inhibitor, RS113456, have similar acute anti-inflammatory effects. Lab Invest 81:1119–1131
5. Dahinden C, Galanos C, Fehr J (1983) Granulocyte activation by endotoxin. I. Correlation between adherence and other granulocyte function, and role of endotoxin structure on biologic activity. J Immunol 130:857–862
6. Janoff A, Zeligs JD (1968) Vascular injury and lysis of basement membrane in vitro by neutral protease of human leukocytes. Science 161:702–705
7. Kusner DJ, King CH (1989) Protease-modulation of neutrophil superoxide response. J Immunol 143:1696–1702
8. Li C, Jackson RM (2002) Reactive species mechanisms of cellular hypoxia–reoxygenation injury. Am J Physiol Cell Physiol 282:227–241
9. Mainardi CL, Dixit SN, Kang AH (1980) Degradation of type IV (basement membrane) collagen by a proteinase isolated from human polymorphonuclear leukocyte granules. J Biol Chem 255:5435–5441
10. Matheson NR, Wong PS, Travis J (1979) Enzymatic inactivation of human α1-proteinase inhibitor by neutrophil myeloperoxidase. Biochem Biophys Res Commun 88:402–409
11. McCord JM (1985) Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med 312:159–163
12. Schneeberger EE, Karnovsky MJ (1976) Substructure of intercellular junctions in freeze-fractured alveolar–capillary membranes of mouse lung. Circ Res 38:404–411
13. Senior RM, Tegner H, Kuhn C, Ohlsson K, Starcher BC, Pierce JA (1977) The induction of pulmonary emphysema with human leukocyte elastase. Am Rev Respir Dis 116:469–475
14. Shasby DM, Vanbenthuysen KM, Tate RM, Shasby SS, McMurtry I, Repine JE (1982) Granulocytes mediate acute edematous lung injury in rabbits and in isolated rabbit lungs perfused with phorbol myristate acetate: role of oxygen radicals. Am Rev Respir Dis 125:443–447
15. Stachura J, Taranawski A, Ivey KJ, Mach T, Bogdal J, Szczudrawa J, Klimczyk B (1981) Prostaglandin protection of carbon tetrachloride-induced liver cell necrosis in the rat. Gastroenterology 81:211–217
16. Tate RM, Vanbenthuysen KM, Shasby DM, McMurtry IF, Repine JF (1982) Oxygen-radical-mediated permeability edema and vasoconstriction in isolated perfused rabbit lungs. Am Rev Respir Dis 126:802–806
17. Zeiher BG, Matsuoka S, Kawabata K, Repine JE (2002) Neutrophil elastase and acute lung injury: prospects for sivelestat and other neutrophil elastase inhibitors as therapeutics. Crit Care Med 30: S281–S287