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Neutrophil elastase inhibitor (sivelestat) attenuates subsequent ventilator-induced lung injury in mice

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Abstract

Mechanical ventilation can paradoxically cause acute lung injury, which is termed ventilator-induced lung injury. Neutrophil recruitment and neutrophil elastase release play a central role in the pathogenesis of ventilator-induced lung injury including cell damage, extracellular matrix degradation and alveolar-capillary hyperpermeability. We therefore speculated that neutrophil elastase inhibition ameliorates ventilator-induced lung injury. Anesthetized C57/BL6 mice received mechanical ventilation with a high tidal volume (V_T; 20 ml/kg) for 4 h. The neutrophil elastase inhibitor (sivelestat, 100 mg/kg) or saline was given intraperitoneally (i.p.) 30 min before ventilation. Sivelestat completely inhibited both neutrophil elastase and myeloperoxidase activities that were increased by ventilation, and attenuated the histopathological degree of lung damage, neutrophil accumulation and lung water content, as well as the concentration of macrophage inflammatory protein (MIP)-2, interleukin (IL)-6 and tumor necrosis factor (TNF)-α in bronchoalveolar lavage fluid and serum. Moreover, mechanical ventilation increased the phosphorylation of c-Jun NH2-terminal kinase (JNK) and the expression of early growth response gene-1 (Egr-1) mRNA, and these increases were also recovered by sivelestat. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining revealed apoptotic cells mainly in alveolar epithelial cells and their numbers corresponded to histological damage. These data suggested that sivelestat could protect against ventilator-induced lung injury by suppressing apoptotic responses through mechanical stress-induced cell signaling in addition to inhibiting neutrophil chemotaxis.

Keywords: Sivelestat; neutrophil elastase; mechanical ventilation; acute lung injury; mechanotransduction; apoptosis.

1. Introduction

Although an effective method of caring for patients with serious respiratory failure such as acute respiratory distress syndrome, mechanical ventilation can also induce lung injury, which is referred to as ventilator-induced lung injury. A large multicenter randomized trial has shown that mechanical ventilation with a lower tidal volume (V_T) results in lower mortality due to acute respiratory distress syndrome than conventional ventilation (The acute respiratory distress syndrome network, 2000), but the therapeutic approach to ventilator-induced lung injury has not been well elucidated.

Diffuse alveolar damage and initial vascular leak with a neutrophil-predominant inflammatory response are the key features of ventilator-induced lung injury (Pugin et al., 1998; Uhlig, 2002; Ware and Matthay, 2000). We and other investigators have applied high V_T ventilation in various animal models and have characterized ventilator-induced lung injury as neutrophil accumulation, vascular permeability and elevated chemoattractant levels (Belperio et al., 2002; von Bethmann et al., 1998; Takenaka et al., 2006). Neutrophils accumulate in the microvasculature of injured lungs and release various cytokines, chemokines and proteases. Among these proteins, levels of interleukin-8 (IL-8) and neutrophil elastase enzyme activity are increased in patients with adult respiratory distress syndrome (Donnelly et al., 1993; Rocker et al., 1989). Production of IL-8 or macrophage inflammatory protein-2 (MIP-2) (the rodent equivalent of human IL-8) is increased in the rat ventilator-induced lung injury model with high V_T ventilation (Chiumello et al., 1999; Choi et al., 2003). Furthermore, MIP-2 receptor knockout mice develop less lung damage than wild-type mice after mechanical ventilation (Dreyfuss and Saumon, 1998). The mechanisms of ventilator-induced lung injury with high V_T ventilation are not fully understood.

Nevertheless, the management of neutrophil activation should be an important therapeutic approach to ventilator-induced lung injury.

Sivelestat (ONO-5046; sodium N-[2-[4-(2,2-dimethylpropionyloxy) phenylsulfonylaminobenzoyl]aminoacetate tetrahydrate]) is a specific inhibitor of neutrophil elastase that was discovered and characterized by Ono Pharmaceutical Co. Ltd. in Japan (Kawabata et al., 1991). Some investigators have reported that neutrophil elastase inhibitor sivelestat plays protective roles in the lung with ischemia-reperfusion injury (Takayama et al., 2001), lipopolysaccharide-induced injury (Inoue et al., 2005), and bleomycin-induced fibrosis (Taooka et al., 1997). Sivelestat is commercially available in Japan as a treatment for acute lung injury associated with systemic inflammatory response syndrome. Its effect on acute lung injury in clinical trials remains controversial (Inoue et al., 2006; Zeiher et al., 2004).

We previously reported that high V_T ventilation induces neutrophilic inflammation that is controlled by nitric oxide overproduction (Takenaka et al., 2006). Here, we test the hypothesis that neutrophil elastase inhibitor contributes to the amelioration of lung inflammation in a mouse ventilator-induced lung injury model.

2. Materials and methods

2.1. *Animal preparation*

Seven to ten week-old C57BL/6 mice weighing 19-23 g were purchased from CLEA Japan (Osaka, Japan). Ono Pharmaceutical Co. Ltd. (Osaka, Japan) provided the sivelestat. All animal experiments proceeded according to the Guidelines for Animal

Experimentation at Kobe University Graduate School of Medicine.

2.2. *Murine model of ventilator-induced lung injury*

We modified the ventilation protocol that we previously described (Takenaka et al., 2006). Mice were anesthetized intraperitoneally (i.p.) with pentobarbital sodium (50 mg/kg; Abbott Laboratories, IL, USA). After inserting a 22-gauge ventilation cannula into the trachea, the mice were placed in the supine position on a warming device and then connected to a ventilator. The ventilator-induced lung injury model was defined as a high V_T of 20 ml/kg, a respiratory rate of 80 breaths per min and mechanical ventilation maintained for 4 h at an inspiratory oxygen fraction of 0.21. The ventilator was a model 683 from Harvard Apparatus Co. (South Natick, MA, USA).

To examine the inhibitory effect of neutrophil elastase inhibitor, the mice were injected with sivelestat (100 mg/kg body, i.p.) or saline (10 ml/kg, i.p.) 30 min before ventilation.

The mice were monitored every 30 min during ventilation to ensure adequate sedation and pentobarbital sodium was administered as required. The V_T was also calculated by integrating airway flow during inspiration using a murine respiratory monitor (WinPULMOS II, M.I.P.S. Co., Osaka, Japan). Blood pressure was measured by the tail-cuff method (model MK-1100; Muromachi Kikai, Tokyo, Japan) every 30 min during ventilation. The respiratory rate was adjusted to maintain pH between 7.30 and 7.45 according to arterial blood analysis. Control mice underwent tracheostomy without ventilation.

Arterial blood gas data of high V_T ventilation (pH 7.369 ± 0.008, PaO_2 100.5 ±

5.2 Torr, $PaCO_2$ 30.9 ± 1.9 Torr, $n = 5$) were not significantly changed even after administering sivelestat (pH 7.362 ± 0.019, $P = 0.73$, PaO_2 99.8 ± 8.0 Torr, $P = 0.92$, $PaCO_2$ 35.3 ± 1.5 Torr, $P = 0.11$, respectively; $n = 5$) at the end of ventilation. The blood pressure of the mice administered beforehand with saline or sivelestat was not significantly changed or different (104.8 ± 1.6 vs. 105 ± 3.9 mmHg, $P = 0.97$, respectively; $n = 6$) and was maintained for 4 h. Final blood pressure did not significantly differ between the two groups (72.5 ± 1.6 vs. 75.5 ± 1.6 mmHg, $P = 0.22$, respectively; $n = 6$).

2.3. Histopathology

After mechanical ventilation for 4 h, the mice were sacrificed, and their lungs and trachea were removed. The right main bronchus was tightly bound, and the ratio of wet-to-dry weight of the entire right lung was determined. The left lung was infused at 20 cm of H_2O pressure with 10% buffered formalin, embedded in paraffin blocks and cut into at 5 μm sections before staining with hematoxylin and eosin (H & E). Five or more random tissue sections from each group were histologically examined. A modified ventilator-induced lung injury histological scoring system was applied as described (Belperio et al., 2002). Briefly, the following pathological processes were scored on a scale of 0-4: (a) alveolar congestion, (b) hemorrhage, (c) leukocyte infiltration or neutrophil aggregation in the airspace or vessel walls and (d) alveolar wall thickness. A score of 0 represented normal findings and scores of 1, 2, 3 and 4 represented mild (<25%), moderate (25 - 50%), severe (50 - 75%) and very severe (>75%) lung involvement, respectively. The overall score was based on the sum of all

scores. Two pathologists who were not informed of the treatment groups reviewed the degree of injury in three random sections of at least 5 lungs from each group of mice.

2.4. Bronchoalveolar lavage

After mechanical ventilation for 4 h, bronchoalveolar lavage was performed twice using 0.75 ml of saline (n = 6-8). Total cell counts were determined using standard hematological procedures in 40 μL aliquots of each sample. The remaining fluid was centrifuged at 3,000 ×g for 5 min (at 4°C) and the supernatants were stored at -80°C.

The cell pellets were resuspended in saline and slides were prepared by centrifugation at 400 ×g for 3 min in a Cytospin 2 (Shandon Inc., Pittsburgh, PA, USA). Specimens of bronchoalveolar lavage fluid were stained with Diff Quik (Kokusai Shiyaku Co., Kobe, Japan) and differential cell counts were determined from 400 cells per slide.

2.5. Neutrophil elastase activity in bronchoalveolar lavage fluid and serum

Neutrophil elastase activity in bronchoalveolar lavage fluid and serum was determined spectrophotometrically using *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide, a highly specific synthetic substrate for neutrophil elastase as described (Yoshimura *et al*., 1994). Briefly, 20 μl of bronchoalveolar lavage fluid or serum was incubated with 200 μl of 0.1 M Tris-HCl buffer pH 8.0 containing 0.5 M NaCl and 1 mM substrate at 37°C for 24 h and the amount of liberated *p*-nitroanilide was

determined spectrophotometrically at 405 nm. Neutrophil elastase activity was calculated from the standard curve of *p*-nitroanilide absorbance.

2.6. Neutrophil accumulation in lung tissue

After mechanical ventilation for 4 h, myeloperoxidase (MPO) activity was determined (Yamashita et al., 2000) as an index of neutrophil accumulation in lung tissue. In brief, the right lung was surgically removed, externally rinsed with saline, blotted dry and weighed. The lung tissue was homogenized and sonicated in 2 ml of 50 mM potassium phosphate-buffered solution (PBS) (pH 7.4) containing 0.5% hexadecyltrimethylammonium bromide. The homogenate was centrifuged at 25,000 ×*g* for 15 min at 4°C, and then the supernatant was decanted and the pellet was resuspended in 1 ml of hexadecyltrimethylammonium bromide-PBS. This procedure was repeated 3 times. The supernatants were diluted 1:30 (vol./vol.) with 50 mM PBS (pH 6.0) containing 0.167 mg/ml of o-dianisidine and 0.0005% hydrogen peroxide. Absorbance was measured at 460 nm for 5 min and then MPO activity was calculated as a change in absorbance over time based on wet lung weight.

2.7. Lung water content

Lung water content related to lung injury was measured using the lung wet-to-dry weight ratio as described (Quinn et al., 2002). The lungs were weighed immediately after removal (wet weight) and again after drying in an oven at 80°C for 48 h (dry weight). The lung wet-to-dry weight ratio was calculated as the ratio of wet

weight to dry weight (n = 6).

2.8. Enzyme-linked immunosorbent assay (ELISA) for cytokine analysis

Levels of TNF-α, IL-6 and MIP-2 were determined in serum and in *bronchoalveolar lavage fluid* supernatants using ELISA kits. The TNF-α and IL-6 kits were from Biosource International (Camarillo, CA, USA) and the MIP-2 kit was from TECHNE Co. (Minneapolis, MN, USA). The sensitivity of these kits for TNF-α, IL-6 and MIP-2 were 3.0, 3.0 and 1.5 pg/ml, respectively, and the values were specific for mice. The absorbance of each sample was measured at 450 nm using a Multiskan JX microplate reader (Thermo Labsystems, Thermo Bio-analysis, Tokyo, Japan).

2.9. Western blot analysis of mitogen-activated protein kinases

To determine the amount of mitogen-activated protein kinases (MAPKs) phosphorylation in the injured lungs, we analyzed the left lungs after lavage. Lungs from C57BL/6 mice were excised and homogenized in lysis buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10% glycerol, and protease inhibitor cocktails (Sigma)) on ice and centrifuged at 15,000 ×g for 20 min. Lung homogenates (100 μg) were separated on 10% SDS-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane (Millipore, Tokyo, Japan). Non-specific binding was blocked with 2% gelatin in PBS for 1 h, and the membrane was incubated with antibodies to total and phosphorylated forms of extracellular signal regulated kinase (ERK), p38 MAPK, and c-Jun NH2-terminal kinase (JNK) (diluted

1:1,000) (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. The membranes were washed and incubated with each secondary antibody (1:2,000) conjugated to horseradish peroxidase for 1 h at room temperature and developed with the enhanced chemiluminescence detection kit (Pierce, Rockford, IL, USA). Antigen–antibody complexes were visualized and quantified using a LAS 3000 imaging system (Fujifilm, Tokyo, Japan).

2.10. Analysis of early growth response gene-1 (Egr-1) expression

We measured relative levels of Egr-1 gene expression by quantitative real-time PCR. Total RNA from mouse lungs was isolated with the ISOGEN reagent (Nippon Gene, Tokyo, Japan) and then first strand cDNA was synthesized from 1 μg using ExScript^{TM} RT reagent Kits (Takara, Otsu, Japan) and random hexamer primers. Quantitative PCR was performed using real-time SYBR Green PCR technology and an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The primers were: Egr-1, FP: AGG TTC CCA TGA TCC CTG ACT, RP: GGT ACG GTT CTC CAG ACC CTG, and β-actin, FP: CCC TAA GGC CAA CCG TGA A, RP: GTT GAA GGT CTC AAA CAT GAT CTG. Duplicate amplification reactions proceeded with SYBR Premix Ex Taq^{TM} (Takara, Otsu, Japan) under thermal cycling conditions of 10 s at 95°C, followed by 40 cycles of 5 s at 95°C and 34 s at 60°C. The expression of mouse Egr-1 was normalized to that of β-actin mRNA.

2.11. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining

Exposed 3′-OH ends of DNA fragments in paraffin-embedded tissue were end-labeled using the *in situ* apoptosis detection TUNEL kit (Takara, Shiga, Japan) according to the manufacturer's instructions (Tornusciolo et al., 1995). The specimens were further stained with diaminobenzidine and counterstained by methyl green and then 20 fields of alveoli were randomly selected and 2,000 nuclei were counted per lung. The frequency of apoptosis was calculated as an apoptotic index, and the proportion of cells undergoing apoptosis is expressed as a ratio (%) of all observed cells.

2.12. Statistical analysis

Data are expressed as means ± S.E.M and differences were considered statistically significant at $P < 0.05$. Arterial blood gas parameters and blood pressure were evaluated using Student's *t*-test. Differences among groups were analyzed using a one-way analysis of variance or the Kruskal-Wallis test. When differences were statistically significant ($P < 0.05$), individual results were compared using the Bonferroni/Dunn test.

3. Results

3.1. Sivelestat attenuates neutrophil activity in ventilated lungs

To explore the effect of neutrophil elastase inhibition in a mouse ventilator-induced lung injury model, we initially examined the inhibitory effect of

different doses of sivelestat and assessed the level of neutrophil elastase activity in both bronchoalveolar lavage fluid (Fig. 1A) and serum (Fig. 1B). High V_T ventilation increased neutrophil elastase activity in both bronchoalveolar lavage fluid (0.22 ± 0.028 nmol/ml, P <0.01) and serum (0.909 ± 0.083 nmol/ml, P <0.01) and these increases were significantly reduced by sivelestat at the doses of 100 mg/kg (0.055 ± 0.020 nmol/ml, 0.463 ± 0.010 nmol/ml, respectively) and 200 mg/kg (0.055 ± 0.016 nmol/ml, 0.514 ± 0.122 nmol/ml, respectively). Since mechanical ventilation group injected with sivelestat at 50 mg/kg did not show significant inhibition of neutrophil elastase activity (0.169 ± 0.040 nmol/ml in bronchoalveolar lavage fluid, 0.836 ± 0.122 nmol/ml in serum), we treated with sivelestat at the dose of 100 mg/kg for subsequent studies.

We measured lung myeloperoxidase (MPO) activity as an index of neutrophil activity (Fig. 1C). The degree of MPO activity after mechanical ventilation was significantly increased in high V_T mice (P < 0.01), and significantly decreased in the high V_T-sivelestat group compared with the high V_T group. These data showed that intraperitoneal injection of sivelestat (100 mg/kg) suppressed neutrophil activity during 4 h of ventilation.

3.2. Sivelestat inhibits cell infiltration within high V_T-ventilated lungs

There was no pathological difference between sivelestat-treated (sivelestat group) and saline-treated mice (control group) without mechanical ventilation (Fig. 2A and 2B). Compared with normal lungs, cell infiltration of the alveolar wall and interstitial oedema were obviously increased in the high V_T ventilation (high V_T) group

(Fig. 2C). The mice treated with sivelestat in the high V_T (high V_T-sivelestat) developed less pulmonary inflammation than the high V_T group (Fig. 2D).

Figure 2E shows the histopathological grades of ventilator-induced lung injury using the scoring system. The ventilator-induced lung injury scores were significantly increased in the high V_T group compared with control mice (6.5 ± 0.22 and 2.0 ± 0.26, respectively, P <0.01). In contrast, the scores in the high V_T -sivelestat group were significantly lower than those in the high V_T group (5.2 ± 0.22, P <0.01 vs. high V_T group).

The bronchoalveolar lavage fluid from control and sivelestat groups contained only alveolar macrophages (Table 1). High V_T ventilation significantly increased total cell counts in bronchoalveolar lavage fluid (7.61 ± 0.68 x 10^4/ml, P <0.01). The total cell counts were lower in the high V_T-sivelestat group (4.48 ± 0.29 x 10^4/ml, P <0.01) than in the high V_T group. Moreover, neutrophils were higher in the high V_T group and significantly decreased by sivelestat (0.789 ± 0.113 x 10^4/ml, 0.123 ± 0.033 x 10^4/ml, respectively, P <0.01). These findings suggest that sivelestat inhibits the subsequent development of ventilator-induced lung injury including neutrophil accumulation.

3.3. Sivelestat decreases lung oedema induced by ventilator-induced lung injury

The ratio of wet-to-dry weight, a parameter of lung oedema, was significantly increased in high V_T mice (Fig. 3; 6.47 ± 0.30 pg/ml, P <0.01). This increase was significantly attenuated in the high V_T-sivelestat group compared with the high V_T group (5.55 ± 0.15 pg/ml, P <0.05). The ratio of wet-to-dry weight in the sivelestat

and control groups did not differ. These data indicate that sivelestat inhibited lung oedema induced by high V_T ventilation.

3.4. Cytokine levels in serum and bronchoalveolar lavage fluid

Ventilation-induced lung injury is known to increase various inflammatory chemokines and cytokines (Tremblay et al., 1997; Takenaka et al., 2006) similar to other injurious lung strategies. Among these, we measured IL-6 and TNF-α levels as markers of systemic inflammation by ELISA (Fig. 4A and 4D, 4B and 4E). Both TNF-α and IL-6 levels were increased in bronchoalveolar lavage fluid and serum from the high V_T group. The increased IL-6 levels in the bronchoalveolar lavage fluid (606 ± 34 pg/ml, $P < 0.01$) and serum (2,536 ± 503 pg/ml, $P < 0.01$) from the high V_T group were significantly reduced by sivelestat (329 ± 55 pg/ml in bronchoalveolar lavage fluid, $P < 0.01$; 1,004 ± 261 pg/ml in serum, $P < 0.01$). The increased TNF-α levels in the bronchoalveolar lavage fluid (25.0 ± 1.0 pg/ml) and serum (297.5 ± 111.5 pg/ml, $P < 0.05$) of high V_T mice were significantly reduced by sivelestat (to 3.1 ± 1.3 pg/ml, $P < 0.05$ and 41.3 ± 16.0 pg/ml, $P < 0.05$, respectively). Thus, sivelestat significantly decreased levels of IL-6 and TNF-α in bronchoalveolar lavage fluid and in serum.

Figure 4C and 4F show that serum and bronchoalveolar lavage fluid MIP-2 concentrations were significantly increased in the high V_T group (162.2 ± 42.4 pg/ml, $P < 0.01$ and 1,675.6 ± 262.4 pg/ml, $P < 0.01$, respectively) compared with control mice. These increases were obviously attenuated in the high V_T -sivelestat group (19.1 ± 3.2 pg/ml in bronchoalveolar lavage fluid and 267.8 ± 77.3 pg/ml in serum, $P < 0.01$) compared with the high V_T group.

These data indicated that sivelestat improves ventilator-induced lung injury by reducing the amounts of circulating inflammatory chemokines and cytokines.

3.5. Sivelestat influences on mechanical stress signals induce by ventilator-induced lung injury

Mechanical ventilation is known to provoke various overstretch-induced signals in lung epithelial cells. We measured activity of three members of the MAPKs families, JNK, ERK and p38 MAPK, in lung homogenates of ventilator-induced lung injury by western blotting. Phosphorylated bands of JNK p54 protein were significantly increased in the high V_T group, whereas total JNK was not changed in both p54 and p46 protein (Fig. 5). The phosphorylation of ERK was provoked by mechanical ventilation, however there was no significant difference between the high V_T group and high V_T -sivelestat group. On the other hand, the phosphorylation of p38 MAPK was not significantly changed by the mechanical ventilation.

We then measured the relative levels of Egr-1 gene expression generated by mechanical stress signals (Fig. 6). Quantitative real-time PCR revealed that obviously increased Egr-1 gene expression in the high V_T group compared with control mice (7.82 ± 1.84 and 2.27 ± 0.23 arbitrary units, respectively, P <0.01). In contrast, sivelestat considerably decreased Egr-1 gene expression (1.52 ± 0.14 arbitrary units, P <0.01 vs. high V_T group). These data indicated that sivelestat inhibits the overstretch-induced signaling pathway in addition to neutrophil activity.

3.6. Sivelestat attenuates ventilator-induced lung injury-induced apoptosis

We examined whether sivelestat attenuates apoptosis in the ventilated lung using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay. Nuclei/apoptotic bodies were TUNEL-positive in inflammatory and alveolar epithelial cells in the lungs of all groups (Fig. 7A-D). The apoptotic indices were significantly increased in the high V_T group compared with control mice (62.3 ± 2.9% and 6.7± 0.8%, respectively, P <0.01) (Fig. 7E). In contrast, the apoptotic indices in the high V_T -sivelestat group were significantly lower than those in the high V_T group (39.0 ± 2.5%, $P < 0.01$). The proportion of apoptosis cells was not significantly different among inflammatory and alveolar epithelial cells. These findings suggested that sivelestat inhibits apoptotic changes in pulmonary cells after ventilation injury.

4. Discussion

Mechanical ventilation causes the activation of macrophages, which promotes the migration of neutrophils from blood vessels into the alveolar space and discharges neutrophil elastase and oxidants. Alveolar-capillary membranes are subsequently damaged and activated neutrophils induce an increase in lung vascular permeability that causes protein-rich lung oedema (Dreyfuss *et al*., 1998). Kotani *et al.* (2004) demonstrated that mechanical ventilation with high V_T (20 ml/kg) for 4 h induces diffuse infiltration by activated neutrophils in the normal rabbit lung, whereas such changes are attenuated in animals depleted of neutrophils. Thus, neutrophil infiltration might play a pivotal role in the pathogenesis of ventilator-induced lung injury.

Macrophages are also one of important factors in the lung inflammation. However, the number of neutrophils was dramatically changed by ventilation compared with macrophages in our model.

Neutrophil elastase is a protease that can degrade key structural elements of connective tissues such as elastin, collagen, and proteoglycan (Campbell and Campbell, 1998). Recent studies have suggested that cell junction proteins such as cadherins in endothelial and epithelial cells, thrombomodulin (Miyazaki et al., 1998) and proteoglycans in the basement membrane (Raats et al., 2000) and in the lung interstitium (Passi et al., 1998) are responsible for the increased alveolocapillary permeability either by disrupting or directly damaging the lung permeability barrier. Neutrophil elastase has been implicated in the increase of permeability in both vascular endothelial (Suttorp et al., 1993) and alveolar epithelial (Peterson et al., 1995) cells that are involved in lung oedema. In addition, it was reported that sivelestat indirectly reduces neutrophil sequestration by suppressing release of neutrophil chemotactic factors (IL-8) (Takayama et al., 2001), inflammatory cytokines (IL-6, TNF-α) (Kawabata et al., 2002), and other adhesion-promoting molecules (P-selectin, intercellular adhesion molecule-1) (Doerschuk 1996). These findings have strengthened the notion that mechanical ventilation can lead to the infiltration of neutrophils, and that neutrophils become targets of neutrophil elastase during the pathogenesis of ventilator-induced lung injury. On the other hand, a recent study demonstrated the controversial data that neutrophil elastase is not required for successful neutrophil transendothelial migration (Hirche et al., 2004).

Injurious ventilatory strategies increase the concentrations of various inflammatory cytokines (Tremblay et al., 1997). MIP-2 plays a pivotal role in

ventilator-induced lung injury in terms of mediating neutrophil recruitment, and in promoting neutrophil adherence to endothelial cells and transendothelial migration into lung tissue (Belperio et al., 2002). MIP-2/IL-8 is a neutrophilic chemotactic factor and its cellular source in bronchoalveolar lavage fluid is multiple cell types in the lungs including lung epithelial cells and alveolar macrophages (Kotani et al., 1998). A study in vitro has shown that cyclic stretch stimulation increases IL-8 release from alveolar epithelial cells and macrophages (Pugin et al., 1998**;** Vlahakis et al., 1999). Chen et al. (2004) demonstrated that neutrophil elastase dose-dependently increases IL-8 release from cultured type 2 alveolar epithelial cells and that the specific elastase inhibitor, elastase inhibitor II, dose-dependently inhibits subsequent IL-8 release induced by neutrophil elastase. On the other hand, MIP-2 generated by macrophages in response to LPS (Chiumello et al., 1999, Yamashita et al., 2000) might be an important factor for neutrophil recruitment to the lung during acute lung injury. Although both vascular endothelial and smooth muscle cells might also secrete IL-8 in response to inflammatory signals, their responses to mechanical stress generated by ventilation have not been fully clarified.

Common mechanosensors include stretch-activated ion channels, integrin receptors, focal adhesion complex and growth factor receptors (Uhlig and Uhlig, 2004). These mechanosensors subsequently activate the MAPKs, such as ERK, JNK and p38 MAPK, which activate transcriptional factors such as activator protein 1 (AP-1), activating transcription factor-2 (ATF-2), ETS-like protein-1 (ELK-1) and Egr-1 to activate gene transcription (Uhlig and Uhlig, 2004). A member of the immediate-early gene family, Egr-1 is a zinc finger transcription factor that might function in mediating cellular responses to environmental stress, such as ischemia, mechanical injury, and

ionizing radiation (Yan et al., 2000). Egr-1 binding to the TNF-α promoter is required for the full activation of TNF-α transcription (Yao et al., 1997). Moreover, some investigators reported that there were different magnitudes of JNK and ERK1/2 activation between high and low V_T-ventilated rats, but p38 was not activated (Uhlig et al., 2002; Li et al., 2004). In same ways, our mechanical ventilation provoked the significant activation of JNK and ERK, whereas p38 MAPK was not apparently phosphorylated in total lung homogenates (Fig. 5).

One pro-inflammatory pathway is the activation of JNK and subsequently of the transcription factor AP-1. Over-ventilation in vivo activates JNK in alveolar type 2 epithelial cells (Uhlig et al., 2002) and JNK inhibition prevents the production of MIP-2 and ventilator-induced lung injury (Li et al., 2004). In addition, stretch-induced AP-1 binding to the IL-8 promoter is dependent on JNK activation in vitro (Li et al., 2003). In our model, western blotting analysis showed that sivelestat attenuated JNK activation accompanied by MIP-2/IL-8 release induced by mechanical ventilation. We also found that the mechanical ventilation provoked an increase of phosphorylated JNK staining in alveolar epithelial cells (data not shown). These results are compatible with the fact that neutrophil elastase inhibitor prevents neutrophil activation and accumulation in the lungs of ischemia-reperfusion injury while reducing chemokine production (Takayama et al., 2001).

Mechanical stress causes apoptotic changes in alveolar epithelial cells during mouse ventilator-induced lung injury (Li et al., 2005) and in cyclic stretch models in vitro (Hammerschmidt et al., 2004). Increased neutrophil elastase induces the apoptosis of human lung epithelial cells mediated via proteinase-activated receptor-1 (Suzuki et al., 2005). The activation of JNK is important in stress responses including

apoptosis, cell survival decisions and cell transformation (Janssen-Heininger et al., 2002). A causal relationship between JNK activation and the induction of apoptosis has been identified in various tissues in response to multiple stresses (Tournier et al., 2000). Eichhorst et al. (2000) reported that JNK activation with subsequent phosphorylation of c-Jun appears to regulate Fas ligand transcription through an AP-1 site in the promoter. We discovered that sivelestat has the additional benefits of decreasing JNK activity and Egr-1 mRNA expression that result in a reduction in the number of apoptotic cells including inflammatory and alveolar epithelial cells. These findings indicated that this inhibition is due to direct pharmacological effects through cell membranes or mediated via the inhibition of neutrophil activity. Misumi et al. (2006) recently reported that high dose of sivelestst regulated the production of IL-8 and monocyte chemoattractant protein-1 induced by TNF-α or endotoxin in alveolar epithelial cell line A549. However this dose (100 μg/ml) was 10 to 100 times higher than the plasma concentration for clinical use. In this point, we suppose that sivelestat could affect ventilation-induced signaling pathway as a result of the specific inhibition of neutrophil elastase at the dose we used, and play a protective role against cell death in injured lungs.

Fujita et al. (2006) recently reported that another neutrophil elastase inhibitor, Ono-EI-600 could not protect against lung injury induced by high V_T ventilation in a rat model. They constantly infused Ono-EI-600 during ventilation, whereas we intraperitoneally administered sivelestat before ventilation. They did not show whether the inhibitor altered neutrophil elastase activity in their model, therefore we cannot determine whether the infused inhibitor concentration was sufficient to inhibit the elastase activity.

The STRIVE study (Zeiher et al., 2004) discovered that sivelestat does not confer beneficial effects on mortality and ventilation-free days among acute lung injury patients mechanically ventilated with a low V_T. However, recent clinical studies have shown that sivelestat reduces the neutrophil elastase concentration, neutrophil stiffness and improves pulmonary oxygenation in patients with acute lung injury (Inoue et al., 2006). The administrated dose of sivelestat is different between these two studies, in addition we have to take into consideration the difference of the disease background underlying acute lung injury and ventilation settings in these clinical studies. Although animal studies have a difficulty to maintain ventilation strategies for longer periods, our results from a mouse ventilator-induced lung injury model indicate a therapeutic potential of neutrophil elastase inhibitor on ventilator-induced lung injury. Further investigation is required to clarify which patients can benefit from sivelestat and to determine the most effective way to use neutrophil elastase inhibitor in the clinical approach to treating ventilator-induced lung injury.

In conclusion, we report the novel finding that a specific neutrophil elastase inhibitor suppressed subsequent neutrophilic inflammation induced by ventilator-induced lung injury with high V_T ventilation. Since sivelestat affected the cellular signals as well as inhibited neutrophil elastase activity in *in vivo* models, our results offer a different approach to understanding the role of neutrophils in ventilator-induced lung injury.

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Figure Legends

Figure 1. Assessment of neutrophil inflammation after ventilator-induced lung injury.

After high V_T ventilation for 4 h, neutrophil elastase activity in mouse bronchoalveolar lavage fluid (**A**) and serum (**B**) (n = 6 per group) was measured. Lung homogenate was used to measure lung myeloperoxidase (MPO) activity (**C**) (n = 5 per group). Values are means ± S.E.M. *****$P < 0.01$.

Figure 2. Hematoxylin and eosin-stained lung sections and semi-quantitative analysis of ventilator-induced lung injury.

Normal mice lungs without (**A**) or with (**B**) prior sivelestat administration. Ventilated mouse lungs without (**C**) or with (**D**) prior sivelestat administration. Magnification, x400; bar, 50 μm. (**E**). Ventilation-induced lung injury (VILI) scores are based on leukocyte infiltration, exudative edema, hemorrhage and alveolar wall thickness as described in Materials and methods. Values are means ± S.E.M. of at least 5 mice per group. *****$P < 0.01$ vs. control. $^{\#}P < 0.05$ vs. high V_T group.

Figure 3. Assessment of lung water content in ventilator-induced lung injury.

Wet to dry weight ratio of lungs was measured in whole lungs by dividing wet weight by dry weight. Values are means ± S.E.M. of at least 5 mice per group. *****$P < 0.01$, $^{\#}P < 0.05$.

Figure 4. Cytokine concentrations in bronchoalveolar lavage fluid and serum.

Levels of IL-6 (**A**), TNF-α (**B**) and MIP-2 (**C**) in bronchoalveolar lavage fluid after mechanical ventilation measured using ELISA kits (n = 5-7 per group). Serum concentrations of IL-6 (**D**), TNF-α (**E**) and MIP-2 (**F**). All levels were elevated in high V_T group and obviously reduced in high V_T-sivelestat group. $^{*}P < 0.01$, $^{\#}P < 0.05$.

Figure 5. Western blot analysis of mitogen activated protein kinases (MAPKs) phosphorylation in ventilated lungs.

Representative figures of total and phosphorylated forms of c-Jun NH2-terminal kinase (JNK) (upper panel), extracellular signal regulated kinase (ERK) (middle panel), and p38 MAPK (lower panel) in lung homogenates were shown (A). Arbitrary units were expressed as relative phosphorylation (B). Level of phosphorylated JNK was increased in high V_T group and significantly reduced in high V_T-sivelestat group (n = 6 per group). $^{\#}P < 0.05$.

Figure 6. Expression of early growth response gene-1 (Egr-1).

Quantitative real time PCR determined mRNA levels of early growth response gene-1 (Egr-1). Level of Egr-1 mRNA was increased in high V_T group and significantly reduced in high V_T-sivelestat group (n = 4 per group). $^{*}P < 0.01$.

Figure 7. TUNEL assay.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling of pulmonary cells. Normal mice lungs without (**A**) or with (**B**) prior sivelestat administration. Ventilated mice lungs without (**C**) or with (**D**) prior sivelestat administration. Magnification is x400. Apoptotic index (ratio (%) of

TUNEL-positive nuclei) (**E**). Values are means ± S.E.M. of 5 mice per group. ***P* < 0.01.

Table 1

Bronchoalveolar lavage fluid analysis

	total cell counts ($\times 10^4$/ml)	macrophages ($\times 10^4$/ml)	neutrophils ($\times 10^4$/ml)	lymphocytes ($\times 10^4$/ml)
control	3.000 ± 0.289	2.990 ± 0.287	0.002 ± 0.001	0.003 ± 0.002
sivelestat	3.900 ± 0.557	3.890 ± 0.556	0.005 ± 0.004	0.004 ± 0.002
Mechanical ventilation	**a** 7.890 ± 0.475	**a** 7.030 ± 0.400	**a** 0.789 ± 0.113	0.011 ± 0.004
Mechanical ventilation-sivelestat	**b** 4.480 ± 0.293	**b** 4.350 ± 0.275	**b** 0.123 ± 0.033	0.005 ± 0.002

All values are means ± S.E.M. of at least 5 mice per group.

[a] $P < 0.01$ versus control group

[b] $P < 0.01$ versus mechanical ventilation group

Fig. 1

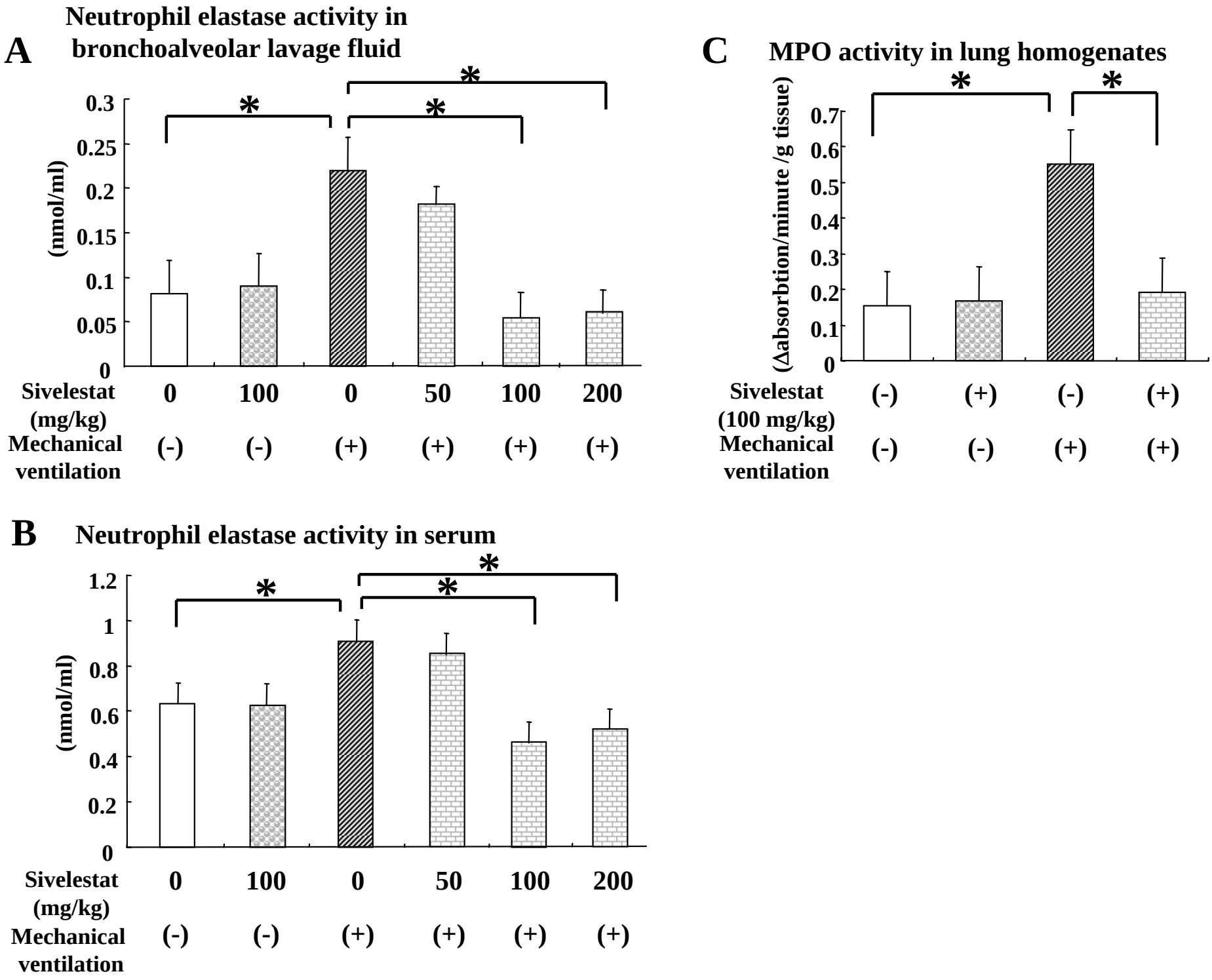

A
Neutrophil elastase activity in bronchoalveolar lavage fluid
(nmol/ml)
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
*
Sivelestat (mg/kg)
0 100 0 50 100 200
Mechanical ventilation
(-) (-) (+) (+) (+) (+)
B
Neutrophil elastase activity in serum
(nmol/ml)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
Sivelestat (mg/kg)
0 100 0 50 100 200
Mechanical ventilation
(-) (-) (+) (+) (+) (+)
C
MPO activity in lung homogenates
(Δabsorbtion/minute /g tissue)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
Sivelestat (100 mg/kg)
(-) (+) (-) (+)
Mechanical ventilation
(-) (-) (+) (+)

Fig. 2

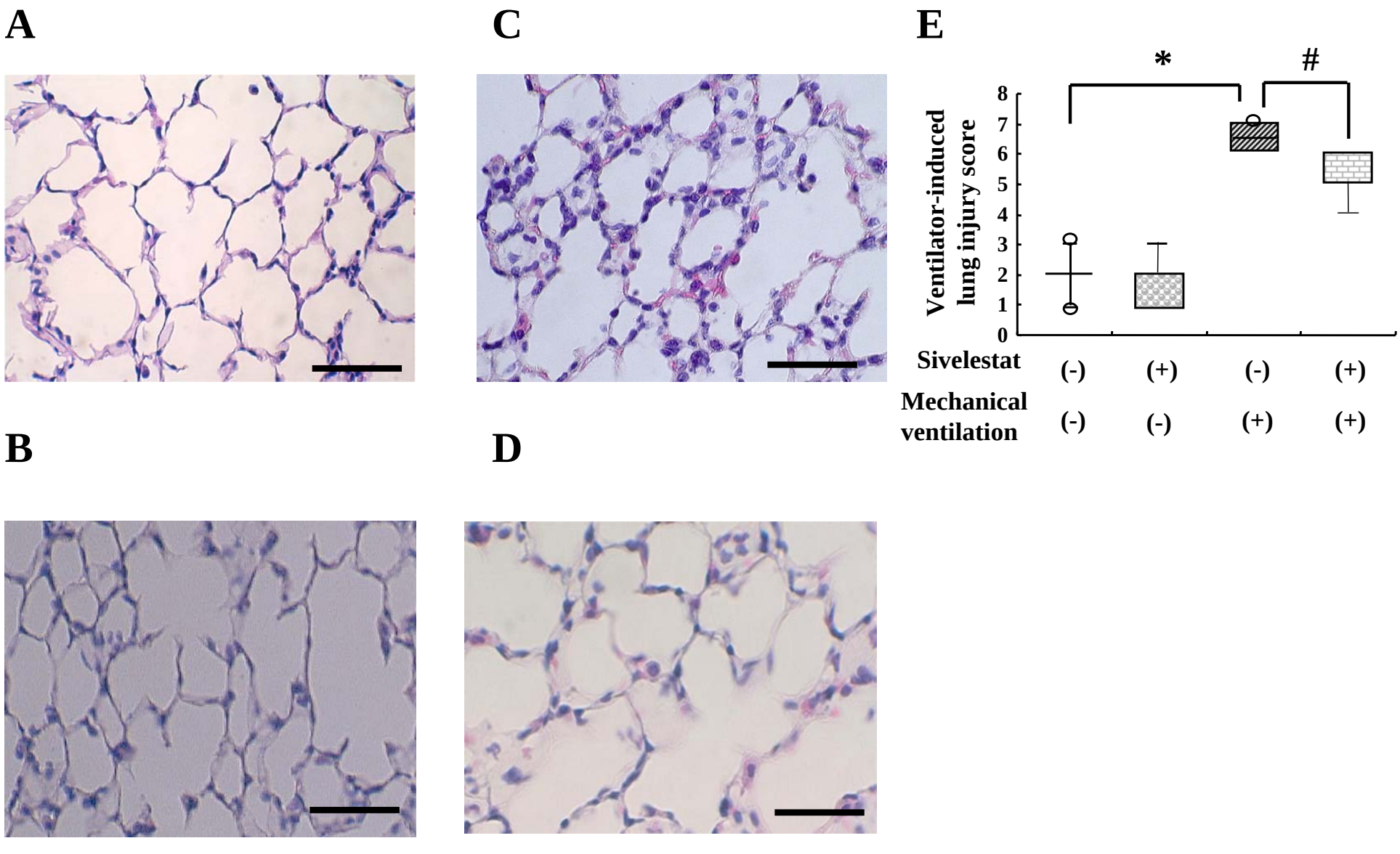

A
C
E
B
D
*
#
Ventilator-induced lung injury score
0
1
2
3
4
5
6
7
8
Sivelestat
(-)
(+)
(-)
(+)
Mechanical ventilation
(-)
(-)
(+)
(+)

Fig. 3

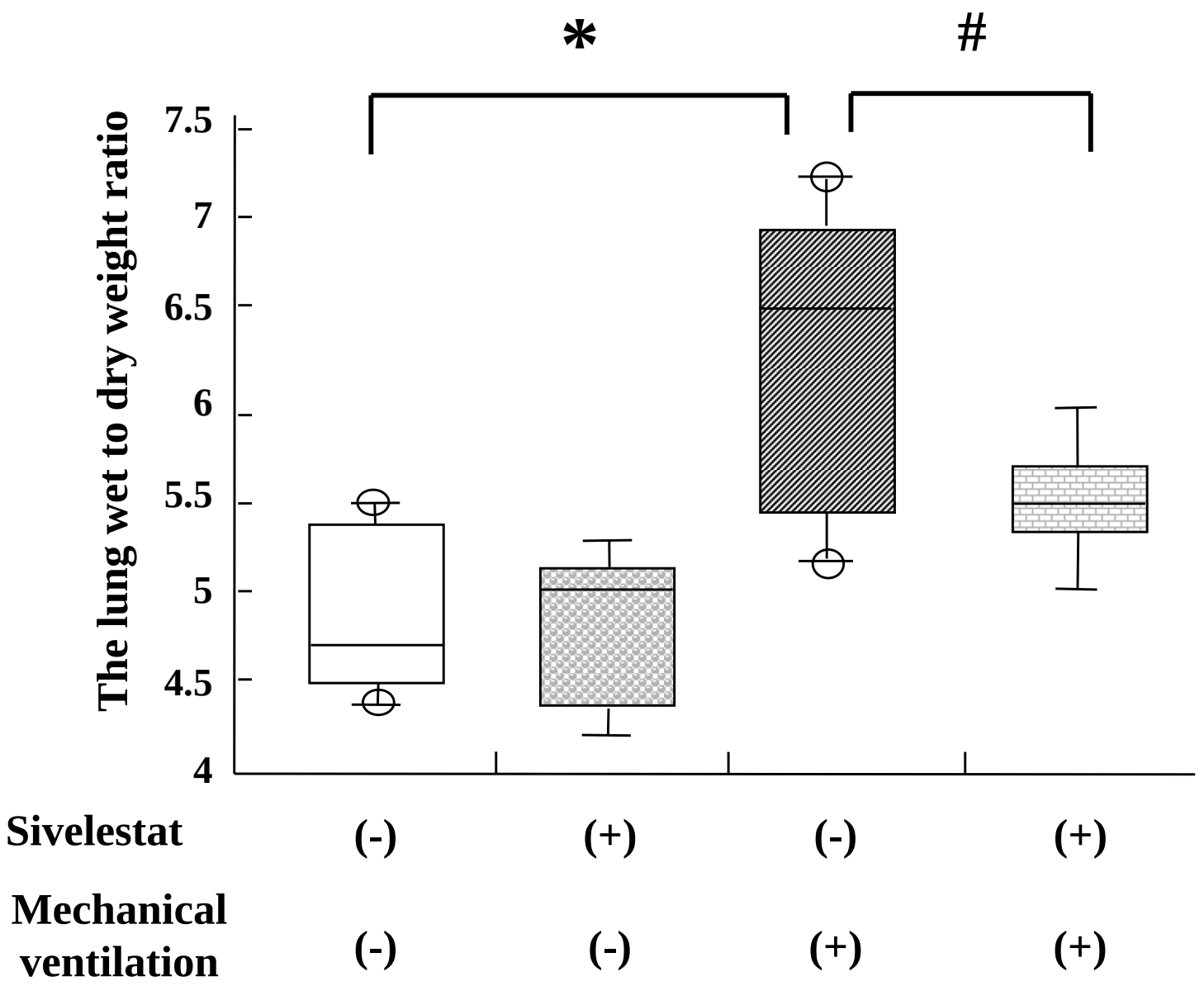

*
#
The lung wet to dry weight ratio
7.5
7
6.5
6
5.5
5
4.5
4
Sivelestat
(-)
(+)
(-)
(+)
Mechanical ventilation
(-)
(-)
(+)
(+)

Fig. 4

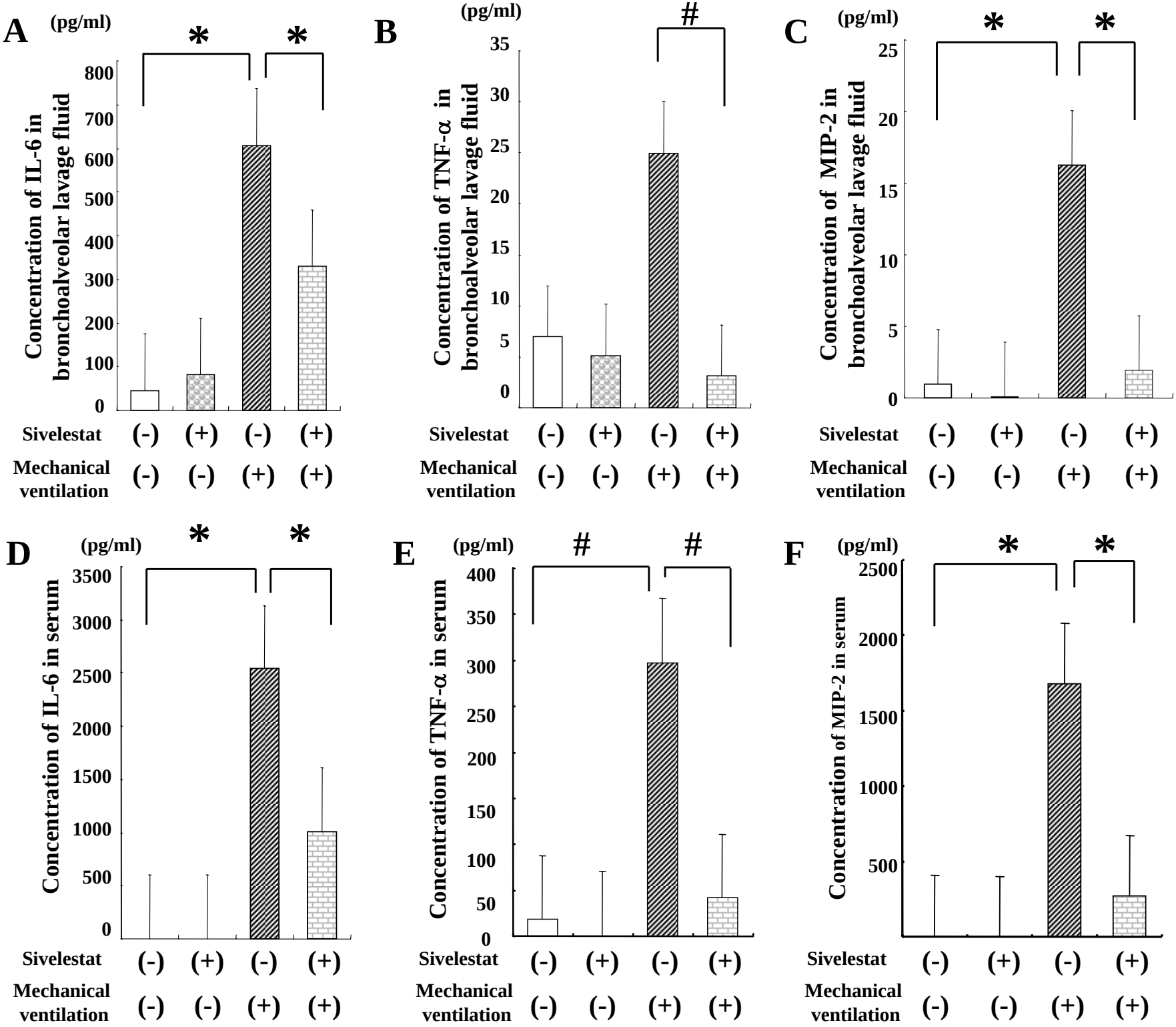
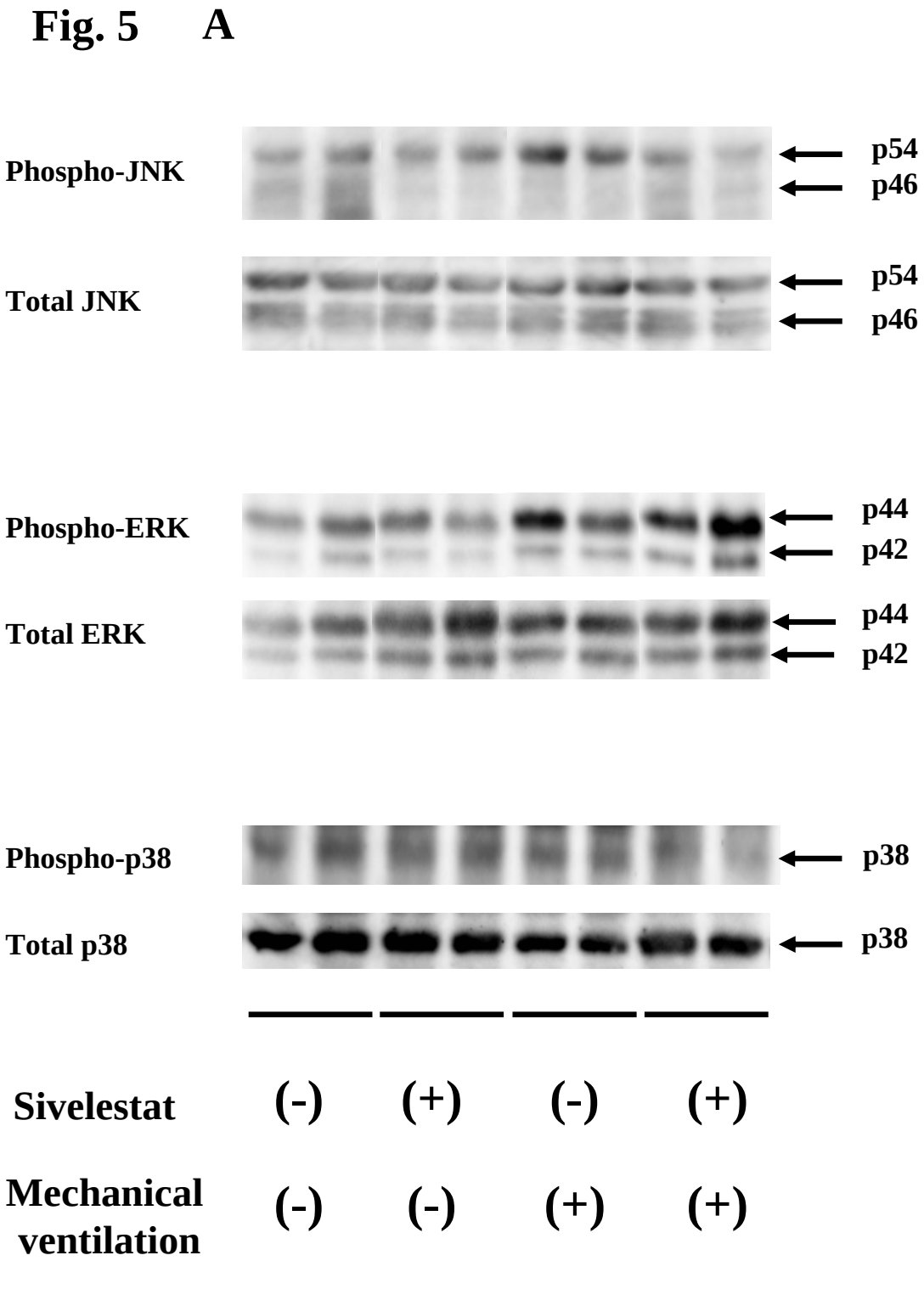

A
(pg/ml)
Concentration of IL-6 in bronchoalveolar lavage fluid
800
700
600
500
400
300
200
100
0
*
*
B
(pg/ml)
Concentration of TNF-α in bronchoalveolar lavage fluid
35
30
25
20
15
10
5
0
#
C
(pg/ml)
Concentration of MIP-2 in bronchoalveolar lavage fluid
25
20
15
10
5
0
*
*
Sivelestat (-) (+) (-) (+)
Mechanical ventilation (-) (-) (+) (+)
D
(pg/ml)
Concentration of IL-6 in serum
3500
3000
2500
2000
1500
1000
500
0
*
*
E
(pg/ml)
Concentration of TNF-α in serum
400
350
300
250
200
150
100
50
0
#
#
F
(pg/ml)
Concentration of MIP-2 in serum
2500
2000
1500
1000
500
*
*
Sivelestat (-) (+) (-) (+)
Mechanical ventilation (-) (-) (+) (+)

Fig. 5


A
Phospho-JNK
p54
p46
Total JNK
p54
p46
Phospho-ERK
p44
p42
Total ERK
p44
p42
Phospho-p38
p38
Total p38
p38
Sivelestat (-) (+) (-) (+)
Mechanical ventilation (-) (-) (+) (+)

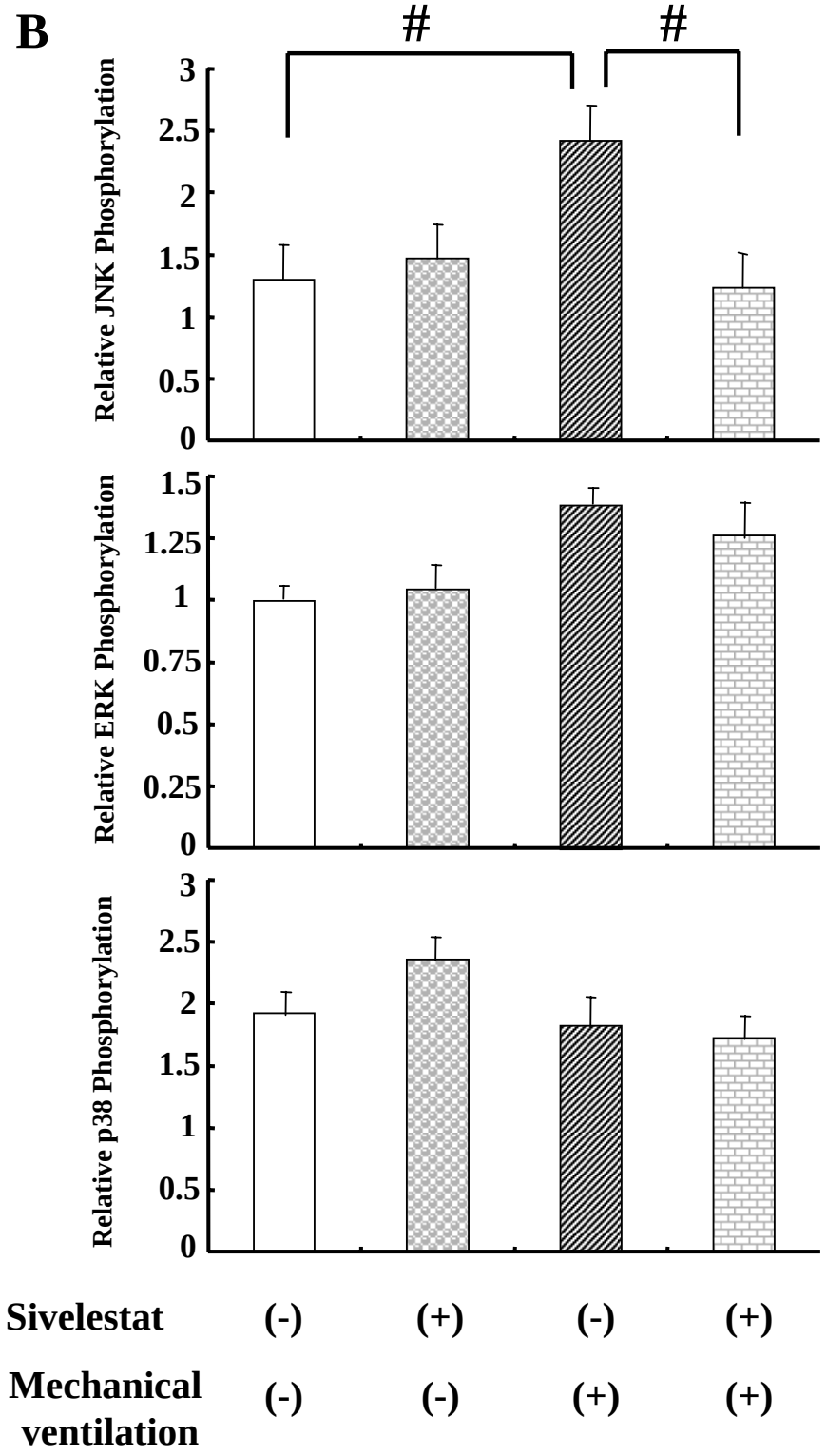

B
#
#
Relative JNK Phosphorylation
3
2.5
2
1.5
1
0.5
0
Relative ERK Phosphorylation
1.5
1.25
1
0.75
0.5
0.25
0
Relative p38 Phosphorylation
3
2.5
2
1.5
1
0.5
0
Sivelestat (-) (+) (-) (+)
Mechanical ventilation (-) (-) (+) (+)

Fig. 6

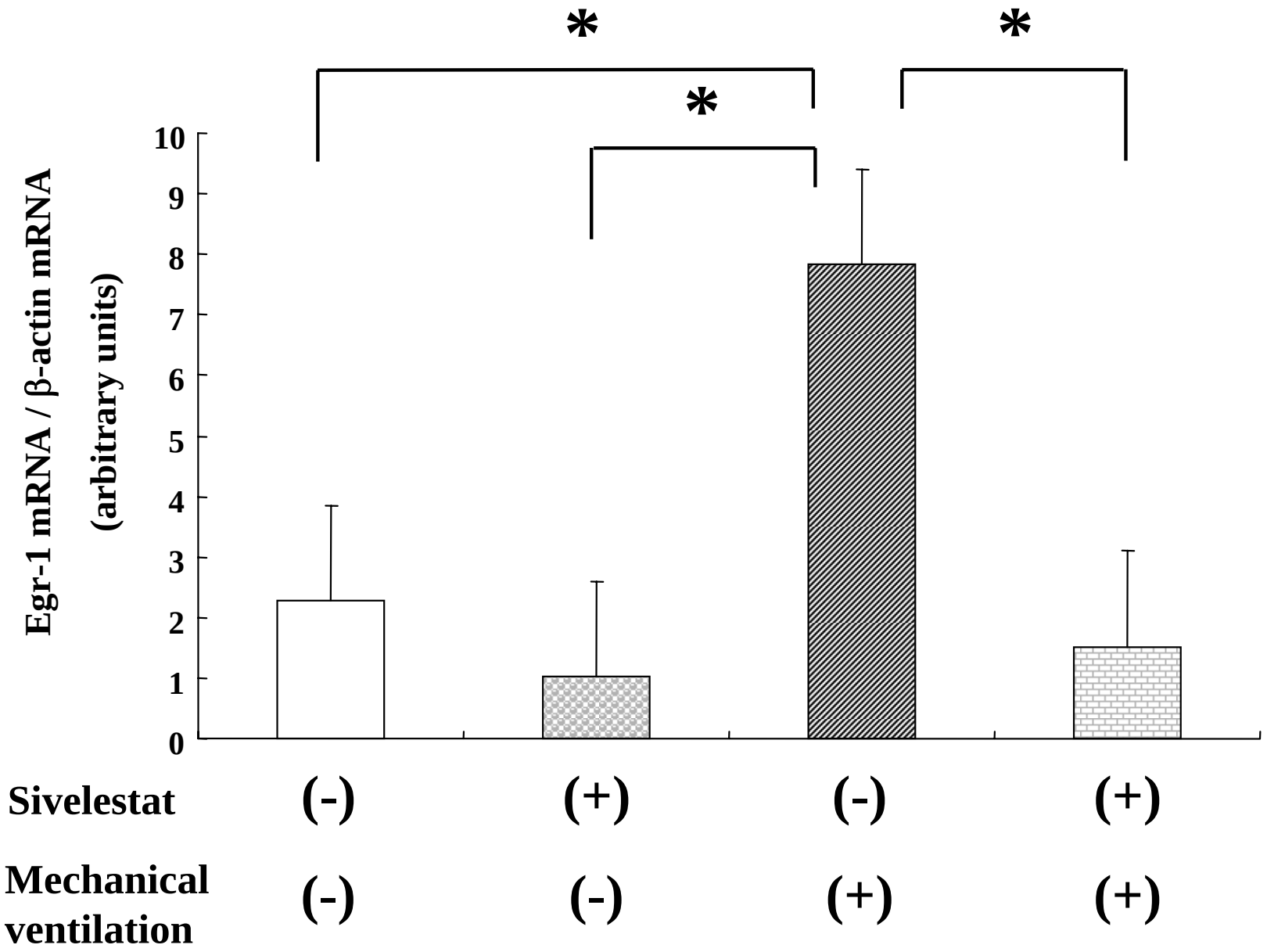
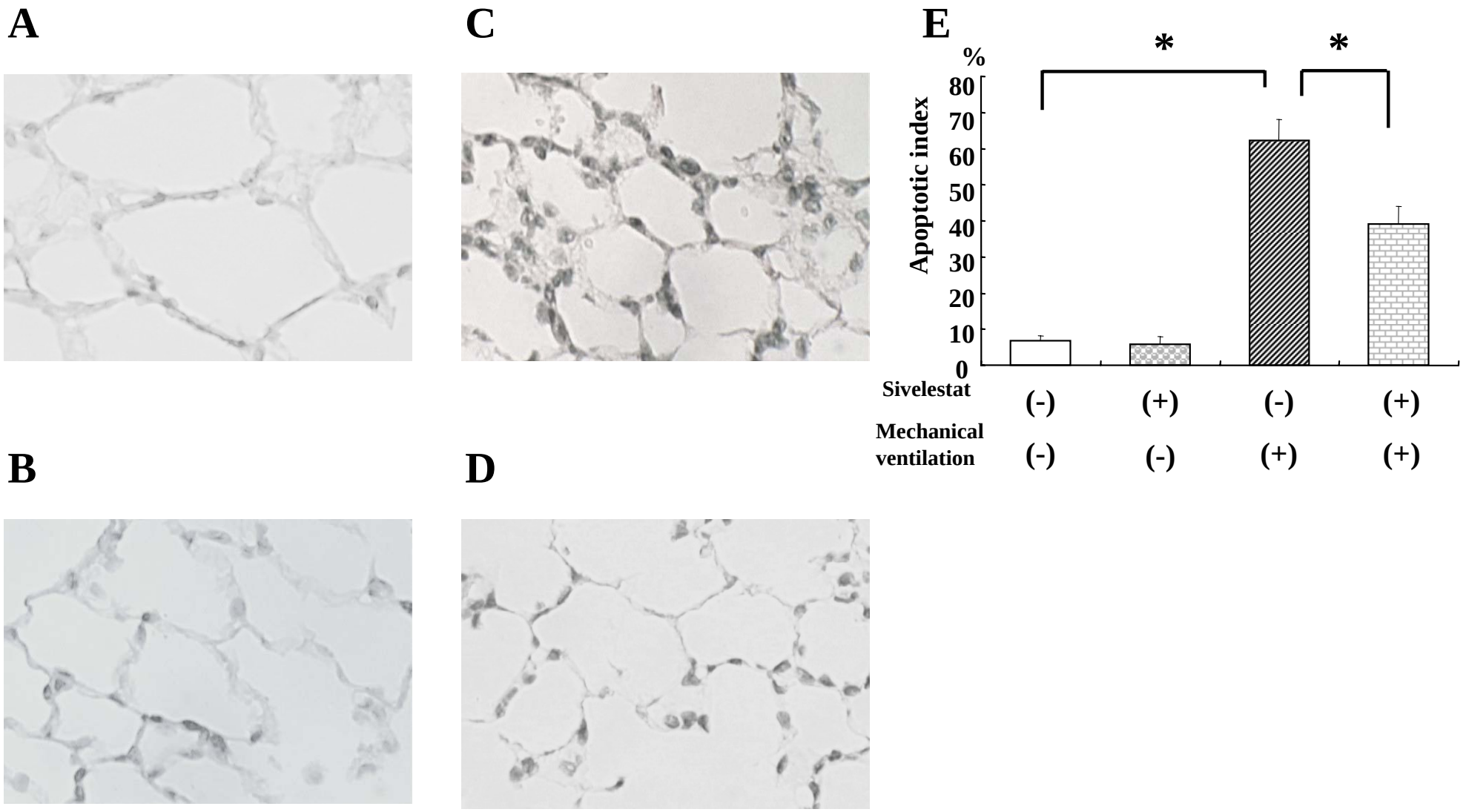

*
*
*
Egr-1 mRNA / β-actin mRNA
(arbitrary units)
10
9
8
7
6
5
4
3
2
1
0
Sivelestat
(-)
(+)
(-)
(+)
Mechanical ventilation
(-)
(-)
(+)
(+)

Fig. 7


A
C
E
B
D
%
80
70
60
50
40
30
20
10
0
Apoptotic index
*
*
Sivelestat
(-)
(+)
(-)
(+)
Mechanical ventilation
(-)
(-)
(+)
(+)