Delayed Neutrophil Elastase Inhibition Prevents Subsequent Progression of Acute Lung Injury Induced by Endotoxin Inhalation in Hamsters

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To define the role of neutrophil elastase (NE) in the progression of acute lung injury (ALI), we examined the effects of post-treatment with a specific NE inhibitor, sivelestat sodium hydrate (sivelestat), on ALI induced by endotoxin (ET) inhalation in hamsters. Inhalation of ET (300 μg/ml, 30 min) in conscious hamsters increased inflammatory cell count, protein concentration, and hemorrhage in bronchoalveolar lavage fluid (BALF) that peaked 24 h after ET inhalation. These changes were significant 2 h after ET inhalation and paralleled the increase in NE activity in BALF. When intravenously infused from 2 to 24 h post-ET inhalation, sivelestat (0.03 to 3 mg/kg/h) dose-dependently attenuated changes in these BALF parameters at 24 h post-ET inhalation in a manner dependent on the inhibition of NE activity in BALF. Histopathological analysis also indicated that sivelestat prevented the progression of lung inflammation such as alveolar neutrophil infiltration and hemorrhage. In contrast, dexamethasone (3 mg/kg, intravenously) was not effective in this model when administered 2 h after ET inhalation, although it was highly effective when applied before ET. We conclude that delayed inhibition of NE activity with sivelestat prevents subsequent progression of ALI in hamsters after ET inhalation. Thus NE may play an important role in the progression of ALI.

A adult respiratory distress syndrome (ARDS) consists of acute lung injury (ALI) that results from a variety of unrelated insults including infection by gram-negative bacteria. This syndrome, underlined by lung inflammation, is characterized by severe alveolar edema with large numbers of neutrophils in the alveoli and lung interstitium (1). Despite recent progress in understanding of the pathogenesis of ARDS, no effective drug treatment has yet been established (2). In addition to the complex pathogenesis of this syndrome, the lack of effective treatment regimens has resulted from lack of information regarding factors involved in the progression of ARDS.

Neutrophil-derived toxic products such as reactive oxygen species (ROS) and proteases have been long considered to be important in the pathogenesis of ARDS (1). A monoa these toxic products, neutrophil elastase (NE) may be of particular interest as a progression factor. This enzyme is a destructive enzyme capable of degrading many of the lung microvascular and tissue components such as elastin, collagen type I–IV, and proteoglycan (3). Increased NE activity has been found in bronchoalveolar lavage fluid (BALF) in patients with ARDS (4). Degradation of these proteins by increased NE activity would then disrupt the integrity of the cell surface and basement membrane, leading to increased lung microvascular permeability (5). In addition to these matrix-degrading activities, NE has also been implicated in transvascular neutrophil migration (6) and production of chemotactic peptides including interleukin-8 (IL-8) (7, 8). Furthermore, recent studies have shown that pretreatment with structurally different NE inhibitors attenuates various animal models of ALI (9–13). However, although these studies have suggested that NE has an important role at least in the onset of ALI, the role of NE in the progression of ALI remains unclear.

This study was designed to examine the NE in the progression of ALI. We studied the relationship between the progression of ALI and NE activity in BALF and the effects of post-treatment with a specific NE inhibitor, sivelestat sodium hydrate (sivelestat) (14), on ALI in hamsters after endotoxin (ET) inhalation. Glucocorticoids have been suggested to be less effective in animal models of ALI when the injury is completely established (15, 16). Therefore, we studied the effects of post-treatment with dexamethasone administered at the same timing as sivelestat to examine if the present model of ALI can be established upon post-treatment of sivelestat. In the present study, we used the hamster as a model animal as it has an anti-NE defense system relatively similar to that in humans (17, 18). Sivelestat was previously known as ONO-5046 Na (14).

METHODS

Animals

Male Syrian golden hamsters weighing approximately 100 g were used throughout this study. All animals were housed for 2 wk before use in air-conditioned room at 23 ± 2°C and 55 ± 10% humidity with a 12-h light/dark cycle. All animals were given food and water ad libitum.

Reagents

Sivelestat (sodium N-[2-[4-(2, 2-dimethylpropionyloxy)phenylsulfonyl]amino-acetate tetrahydrate) was synthesized in our laboratory. Endotoxin (Escherichia coli, 055:B5) was purchased from DIFCO Laboratories (Detroit, MI). Zymosan, dexamethasone, and N-methoxy succinyl-Ala-Ala-Pro-Val-p-nitroaniline (Suc-Ala-Ala-Pro-Val pNA) were from Sigma Chemical Co. (St. Louis, MO). Sivelestat was dissolved in saline with a small amount of Na2CO3 solution (2 μl of 0.5 M Na2CO3/mg sivelestat). Suc-Ala-Ala-Pro-Val-pNA was dissolved in 1-methyl-2-pyrrolidone. Endotoxin was dissolved in saline. Zymosan which had previously been opsonized by hamster plasma was suspended at a concentration of 10 mg/ml in Hanks’ buffer (pH 7.4) containing 0.1% gelatin and was stored at −20°C until use.

Ex Vivo NE Inhibition

Groups of 7 to 8 animals were used under pentobarbital sodium anesthesia (60 mg/kg, intraperitoneally). This level of anesthesia was maintained by intraperitoneal injection of additional pentobarbital sodium (30 mg/kg) as needed. All animals were kept in the supine position and a small polyethylene tube was inserted into the right femoral vein for continuous infusion of agents with infusion pumps (Model S5-111: Harvard Apparatus Inc., South Natick, MA). A fer 2-h infusion of sivelestat (0.03, 0.1, 0.3, or 1 mg/0.2 ml/kg/h) or vehicle (saline) alone, 1 ml of citrated blood was taken from the abdominal aorta of each animal. Blood samples (0.8 ml) were then mixed with 0.1 ml of

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opsonized zymosan (OZ, 10 mg/ml) and saline, and the mixtures were incubated for 30 min at 37°C with gentle shaking. A 10 μl aliquot of these mixtures was centrifuged at 1,700 g for 10 min at 4°C. NE activity in the supernatants was measured by the method described subsequently. As with controls, animals were infused with saline and their citrated blood samples were stimulated with OZ vehicle alone (Hanks’ buffer containing 0.1% gelatin).

Animal Model
A total of 11 animals inhaled ET (300 μg/ml) for 30 min in a transparent box (width: depth: height = 22:22:35 cm) using an ultrasonic nebulizer (NE-U 12; Omorin, Tokyo, Japan) with a flow volume of 0.9 ml/min and a droplet size of 1 to 5 μm. The transparent box was placed in a negative draft chamber with a negative air flow velocity of 0.4 m/s. After inhalation, a small-diameter polyethylene tube was inserted into the left femoral vein, tunneled under the skin, and exteriorized in the dorsal neck of each animal under pentobarbital sodium anesthesia (60 mg/kg, intraperitoneally) and were used for continuous infusion at a rate of 0.2 ml/h. Oils of the polyethylene tube in the dorsal neck were connected with infusion pumps (Model 55-1111; Harvard Apparatus Inc.) and were used for continuous infusion at a rate of 0.2 ml/h. This allowed continuous infusion of agents under free movement of animals after recovery from anesthesia. Animals usually recovered from anesthesia within 3 h and were maintained in the conscious state with free access to food and water during the experimental period. Twenty-four hours after ET inhalation, animals were killed by exsanguination via transection of the abdominal aorta under pentobarbital sodium anesthesia (60 mg/kg, intraperitoneally) and their whole lungs were lavaged 5 times with a single volume (2.8 ml) of citrated saline via a tracheal cannula. Inflammatory cell counts, protein concentration, and NE activity in the BALF were then analyzed by the methods described subsequently. For histopathological examination, the right lungs were fixed with 10% buffered formalin.

Statistical Analyses
V values are expressed as means ± SE. Data were analyzed by either two-tailed Student’s t test or Dunnett’s t test followed by one-way analysis of variance (ANOVA), and p values of less than 0.05 were considered statistically significant.

RESULTS
Ex Vivo NE Inhibition by Sivelestat
A preliminary experiment using sivelestat at 1 mg/kg/h indicated that inhibition of plasma NE activity reached a plateau value in the plasma was measured. In the control group, blood samples were incubated with opsonized zymosan (OZ) for 30 min at 37°C. NE activity in the plasma was measured. In the control group, blood samples were incubated with OZ vehicle alone. Each bar represents the mean ± SEM of 7 to 8 animals. **p < 0.001 versus Cont group. *p < 0.05 and **p < 0.01 versus OZ group.
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Therefore, we infused sivelestat for 2 h in the following ex vivo study. As shown in Figure 1, incubation of whole blood with opsonized zymosan increased plasma NE activity in saline-infused animals (Figure 1, OZ group). Two-hour intravenous infusion of sivelestat dose-dependently inhibited the increase in NE activity, with significant inhibition at doses of 0.1 mg/kg/h or higher (Figure 1, sivelestat group).

Another preliminary ex vivo study indicated that the NE inhibitory effect of sivelestat was saturated at doses higher than 1 mg/kg/h.

Time Course of Lung Injury

Parameters in BALF. Endotoxin (300 μg/ml) inhalation caused ALI in hamsters as indicated by increases in inflammatory cell count, protein concentration, and hemorrhage in BALF with significant changes 2 h after ET inhalation. These changes peaked 24 h after inhalation and declined thereafter. Changes in these parameters were associated with an increase in NE activity in BALF (Figure 2). A nalysis of differential cell counts in BALF indicated that the majority of infiltrating cells were neutrophils (89.1 ± 1.4%) 24 h after ET inhalation when changes of bronchoalveolar lavage (BAL) parameters peaked. Inhalation of ET at lower concentrations (30 and 100 μg/ml) caused no obvious changes in any of the parameters under our experimental conditions (data not shown).

Lung neutrophil sequestration. In a separate experiment, we also measured MPO activity of lavaged lungs to study neutrophil sequestration in lung tissue. As shown in Figure 3, lung MPO activity rapidly increased after ET inhalation with high levels between 2 to 12 h post-ET inhalation, indicating that a substantial number of neutrophils were sequestered into lung tissue during this period. The activity, however, declined 24 h after ET inhalation.

Effects of Post-treatment with Sivelestat

Dose–response study. Because the time course study indicated that changes in BALF parameters became statistically significant and peaked 2 and 24 h post-ET inhalation, respectively, we intravenously infused hamsters with sivelestat from 2 to 24 h post-ET inhalation. Inflammatory cell count, protein concentration, and hemorrhage in BALF with significant changes 2 h after ET inhalation. These changes peaked 24 h after inhalation and declined thereafter. Changes in these parameters were associated with an increase in NE activity in BALF (Figure 2). Analysis of differential cell counts in BALF indicated that the majority of infiltrating cells were neutrophils (89.1 ± 1.4%) 24 h after ET inhalation when changes of bronchoalveolar lavage (BAL) parameters peaked. Inhalation of ET at lower concentrations (30 and 100 μg/ml) caused no obvious changes in any of the parameters under our experimental conditions (data not shown).

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Histopathological study. The effects of sivelestat (3 mg/kg/h) on ET-induced ALI were histopathologically studied using a different set of animals. Typical light micrographs of sections from each group are shown in Figure 5. A nimals killed 2 h post-ET inhalation already had alveolar neutrophil infiltration and hemorrhage (ET2 group). These histopathological...
Changes were more consistent and severe in animals killed 24 h post-ET inhalation (ET24 group). However, these changes were only minimal in sivelestat (3 mg/kg/h)-treated animals (EI group).

Dexamethasone efficacy. We compared the effects of post-treatment with sivelestat (3 mg/kg/h) with those of pre- and post-treatment with dexamethasone. The efficacy of post-treatment with sivelestat (Figure 6; EI group) was similar to that observed in the dose-response study. When administered 2 h after ET inhalation, dexamethasone failed to attenuate the changes in BALF parameters (alveolar cell infiltration, edema, and hemorrhage) and the increase in NE activity in BALF (Figure 6; Dexa-post group), although it markedly attenuated these parameters as well as NE activity in BALF by pretreatment 3 h before ET inhalation (Figure 6; Dexa-pre group).

DISCUSSION
This study shows that the increase in alveolar NE activity after ET inhalation paralleled the progression of ALI in hamsters (Figure 2), and post-treatment with the NE inhibitor sivelestat (also known as ONO-5046 Na) (14) prevented subsequent development of this injury (Figure 4). The efficacy of sivelestat was also evidenced by histopathological analysis, showing a reduction of lung inflammation (Figure 5). In the present model, parameters of ALI such as the increase in protein concentration in BALF (Figure 2), lung neutrophil sequestration (Figure 3), and lung histopathological changes (Figure 5) were already evident 2 h after ET inhalation when sivelestat treatment was started. Furthermore, dexamethasone, a glucocorticoid which has been suggested to be ineffective in established ALI (15, 16), was not effective when administered 2 h after ET.
The protective effect of sivelestat might be largely attributable to the specific inhibition of NE activity. As has been reported previously, sivelestat inhibits hamster NE activity with an inhibitory concentration of 50% (IC50) value of 37 ± 4 nM but does not inhibit other neutrophil-derived proteases such as cathepsin G (14). In addition to its specificity among proteases, sivelestat neither affects the production of ROS (21) nor inhibits the activities of lipooxygenase (5 and 15-lipooxygenase) or cyclooxygenase (cyclooxygenase I and II) (unpublished data) which have been implicated in the development of ALI (1). Furthermore, sivelestat administration at doses of 0.1 mg/kg/h or higher inhibited endogenous NE activity in hamsters (Figure 1). Indeed, sivelestat reduced the increase in NE activity in BALF which was associated with ALI in the present model (Figure 4), although part of this effect may have been caused by the reduction of inflammatory cells in BALF. These findings collectively with the present results suggest that increased NE activity after ET inhalation significantly contributes to the progression of ALI in hamsters.

After ET inhalation, there was a sequential change of lung tissue MPO activity and inflammatory cell count in BALF. Because we measured MPO activity of the whole lung after lavage, the MPO activity reflects only neutrophil number in lung tissue not that in the alveolar space. Therefore, this result may reflect neutrophil migration from lung tissue to alveolar space. Interestingly, the increase in MPO activity was associated with the first-phase increase in protein concentration and hemorrhage in BALF. The decline of MPO activity in lung tissue was then followed by an increase in inflammatory cell count and was again associated with the second-phase increase in protein concentration and hemorrhage in BALF. It is likely that the neutrophils sequestered in lung tissue and those infiltrated into the alveolar space, respectively play an important role in the development of ALI in this model. Changes of NE activity closely associated with the number of neutrophils in lung tissue and inflammatory cell count in BALF, suggest that NE is particularly important as a toxic mediator from neutrophils.

Our findings are in accordance with the recent suggestion that NE at inflammatory sites can injure tissues even in the presence of endogenous protease inhibitors (22, 23) such as alpha-1 protease inhibitor (alpha-1-PI) (24). Several mechanisms by which local balance between NE and these protease inhibitors is disturbed have been proposed. First, these protease inhibitors have high molecular weights and cannot enter the microenvironmental spaces between neutrophils and their substrate tissues owing to structural constraints. Second, a major endogenous inhibitor, alpha-1-PI, is inactivated via oxidation of the active center by neutrophil-derived ROS (25). Finally, they are not fully effective in inhibiting tissue-bound NE (26, 27). In contrast, sivelestat, a low-molecular-weight synthetic NE inhibitor (14), may be able to access such microenvironmental spaces, is structurally resistant to ROS inactivation, and is effective in inhibiting tissue-bound NE (27). It is likely, therefore, that sivelestat effectively inhibits NE activity at inflammatory sites, resulting in a protection from ALI.

The present study confirms recent findings that pretreatment with NE inhibitors attenuates pulmonary edematous changes in various animal models of ALI (9–13). We extend these findings by showing that post-treatment with sivelestat attenuated an increase in protein concentration in BALF and lung inflammation in hamsters following ET inhalation. Because the increase of protein concentration in BALF can be considered as an index of alveolar edema, the present results suggest that NE plays an important role in the progression of alveolar edema in ALI. We speculate that NE disrupts the integrity of the transalveolar permeability barrier such as endothelial and alveolar epithelial cell and basement membrane via its capability to degrade a variety of matrix proteins (4). This is supported by the findings that NE increases the permeability of lung microvascular endothelial (28) and alveolar epithelial cells (29). The target proteins through which NE disrupts the integrity of the transalveolar permeability barrier are not yet clear. However, cell junctional proteins, cadherins, may be one of the potential target proteins of NE as has been suggested recently (13).

Sivelestat also attenuated inflammatory cell infiltration into BALF. This attenuation is mostly due to reduction of neutrophil infiltration as indicated by histopathological analysis (Figure 5). Our results are consistent with the recent findings that the beneficial effects of NE inhibitors are accompanied by reduction of neutrophil infiltration in tissues not only in models of ALI (12, 13) but also in other models of inflammation such as ischemia-reperfusion injury (30, 31). These results suggest that their effects are also mediated by the reduction of neutrophil infiltration, thereby reducing levels of neutrophil-derived toxic mediators. However, the effects of sivelestat could not be completely attributed to the secondary reduction of neutrophil-derived toxic mediator levels because a submaximal number of neutrophils had already infiltrated lung tissue when sivelestat treatment was started (2 h post-ET inhalation) (Figure 3). Rather, it appears that both of the effects of sivelestat, i.e., reduction of edematous changes and neutrophil infiltration, are mediated by NE.

There is accumulating evidence that NE directly and/or indirectly plays a role in neutrophil migration. Directly, NE may degrade components of the basement membrane thereby allowing neutrophil migration into the extravascular space (6). Recently, it has been demonstrated that NE is an endogenous ligand for integrin complement receptor type 3 (CR3) and expression of NE on the neutrophil surface allows neutrophil migration by eluting immobilized ligands such as intercellular adhesion molecule 1 (ICAM 1) (32). Indirectly, NE may facilitate the migration of neutrophils via its ability to produce chemotactic factors such as fibrin fragments (7) and IL-8 (8). On the other hand, sivelestat does not affect the expression of adhesion molecules such as ICAM 1, vascular cell adhesion molecule 1 (VCAM 1), and expression of a leukocyte-adhesion molecule 1 (ELAM 1) or neutrophil chemotaxis (33). Moreover, it did not inhibit gelatinase even at 300 μM (unpublished data), one of the metalloproteases which has recently attracted attention in relation to inflammatory cell migration (34). Thus, we speculate that reduction of alveolar neutrophil infiltration by sivelestat is also mediated by the inhibition of NE activity, at least under the present experimental condition, although the possibility of contributions of other as yet unidentified pharmacological effects of sivelestat cannot be excluded.

ROS are other potential toxic mediators produced by neutrophils (25). Weiss has suggested that ROS and NE synergistically contribute to ALI by a mechanism in which these ROS inactivate protease inhibitors, thereby allowing NE to attack and degrade tissues (25). In this respect, selection of appropriate animal species may be important to study the roles of ROS and/or NE on ALI, as anti-NE activity and susceptibility to oxidative inactivation of protease inhibitors vary widely between species. It has been reported that protease inhibitors in some rodents such as the mouse, rat, and guinea pig have two- to fourfold higher anti-NE activity than those in humans (17), whereas those in the sheep, rabbit, and mini pig are much
more susceptible to oxidant inactivation than those of humans (18). In contrast, anti-NE activities of endogenous protease inhibitors and their susceptibility to oxidative inactivation in hamster are relatively similar to those in humans (17, 18). Thus, the relative contributions of these mediators to ALI may be different depending on animal species. Further studies are required to pharmacologically clarify the roles of NE and/or ROS in the progression of ALI.

In summary, the delayed inhibition of NE activity after the onset of ALI appears to prevent subsequent progression of lung edema and alveolar neutrophil infiltration associated with ALI. NE, thus, may have an important role in the progression of ALI.

References