The Role of Neutral Endopeptidase in Caerulein-Induced Acute Pancreatitis

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The Role of Neutral Endopeptidase in Caerulein-Induced Acute Pancreatitis

Yung-Hua Koh,* Shabbir Moochhala,*† and Madhav Bhatia‡

Substance P (SP) is well known to promote inflammation in acute pancreatitis (AP) by interacting with neurokinin-1 receptor. However, mechanisms that terminate SP-mediated responses are unclear. Neutral endopeptidase (NEP) is a cell-surface enzyme that degrades SP in the extracellular fluid. In this study, we examined the expression and the role of NEP in caerulein-induced AP. Male BALB/c mice (20–25 g) subjected to 3–10 hourly injections of caerulein (50 μg/kg) exhibited reduced NEP activity and protein expression in the pancreas and lungs. Additionally, caerulein (10^{-7} M) also downregulated NEP activity and mRNA expression in isolated pancreatic acinar cells. The role of NEP in AP was examined in two opposite ways: inhibition of NEP (phosphoramidon [5 mg/kg] or thiorphan [10 mg/kg]) followed by 6 hourly caerulein injections) or supplementation with exogenous NEP (10 hourly caerulein injections, treatment of recombinant mouse NEP [1 mg/kg] during second caerulein injection). Inhibition of NEP raised SP levels and exacerbated inflammatory conditions in mice. Meanwhile, the severity of AP, determined by histological examination, tissue water content, myeloperoxidase activity, and plasma amylase activity, was markedly better in mice that received exogenous NEP treatment. Our results suggest that NEP is anti-inflammatory in caerulein-induced AP. Acute inhibition of NEP contributes to increased SP levels in caerulein-induced AP, which leads to augmented inflammatory responses in the pancreas and associated lung injury. The Journal of Immunology, 2011, 187: 5429–5439.

Acute pancreatitis (AP) is the sudden inflammation of the pancreas. In the United States, ~210,000 patients seek treatment for AP annually, placing a huge burden of >2 billion dollars in hospitalization costs (1, 2). In ~80% of cases, patients suffer mild symptoms and usually recover in a few days, whereas others experience a severe attack with a high mortality rate (2). The primary cause of mortality in severe AP is necrosis of the pancreas, often followed by systemic inflammatory response syndrome, which causes injuries to distant organs such as lungs and kidneys (3–5). Heavy alcohol consumption and gallstones are two main etiologies of severe AP, but up to 20% remain idiopathic (2). It is generally accepted that premature activation of zymogens in pancreatic acinar cells leads to autodigestion of the organ, causing subsequent liberation of proinflammatory mediators that intensify the inflammatory responses (6). Despite recent advances in understanding the pathogenesis of AP, cellular mediators that determine the severity of AP are complex and incompletely understood.

Substance P (SP) is widely held to exert its effects on target cells expressing neurokinin-1 receptor (NK1R). SP–NK1R interaction plays an early and important role in the inflammatory cascade and promotes excessive activation of inflammatory cells. Proinflammatory mechanisms of SP are thought to be its effects on plasma extravasation, neutrophil recruitment, and inflammatory mediator synthesis upon exogenous stimuli (7, 8). SP is encoded by the preprotachykinin-A (PPTA) gene, produced primarily by sensory nociceptive neurons, but it can also be produced by inflammatory cells and pancreatic acinar cells (9–11). SP–NK1R interaction was shown to be a key mediator in the pathogenesis of experimental AP. Pharmacological antagonism of NK1R, knock-out of PPTA gene, or disruption of SP release from the nerve endings protected mice against AP and associated lung injury (12–14). A rapid increase in SP levels and gene expression was observed in caerulein-treated mice or caerulein-treated murine pancreatic acinar cells (13, 15). Early upregulation of SP expression positions it to influence many of the early inflammatory responses.

Increased expression of SP in SP-expressing cells and increased release of SP from nerve endings may contribute to elevated SP levels in experimental AP. However, inhibitory mechanisms that terminate the effect of SP were not clearly understood. The cell-surface enzyme neutral endopeptidase (NEP; also called enkephalinase, neprilysin, common acute lymphoblastic leukemia Ag, or CD10) is known to degrade a variety of short peptides in the extracellular fluid (16, 17). NEP is capable of modulating inflammation, but was prevented by cotreatment with NK1R antagonists (18). Additionally, administration of exogenous recombinant NEP to animals is protective against inflammatory disorders, such as intestinal inflammation and burns (18, 20, 21). Current evidence supports that NEP plays an anti-inflammatory role.

To date, the role of NEP in the pathogenesis of AP is not thoroughly understood. Genetic deletion of NEP exacerbated pancreatitis-associated lung injury and greatly increased mortality rate in choline-deficient and ethionine-supplemented diet-induced AP (22). However, physiological changes of NEP expression and
activity in caerulein-induced AP are not known. Therefore, in the present study, we investigated the potential regulatory role of caerulein on NEP activity and expression. Subsequently, NEP inhibitors and exogenous NEP were used to examine the effects of NEP on the outcome of AP. Although the primary focus of this article is pancreatic injury, lung injury is often observed in severe AP (13, 22). Hence, lungs have also been investigated for pathological changes.

Materials and Methods

Animals and chemicals

All experimental procedures were approved by the Animal Ethics Committee of the National University of Singapore and carried out in accordance with established International Guiding Principles for Animal Research. BALB/c mice (male, 20–25 g) were acclimatized in a controlled environment with an ambient temperature of 23°C and a 12-h light/dark cycle. Caerulein was purchased from Bachem (Torrance, CA). Glucose, HEPES, and soybean trypsin inhibitor were obtained from Sigma-Aldrich (St. Louis, MO). Type IV collagenase was purchased from Worthington Biochemical (Freehold, NJ). Recombinant mouse NEP was purchased from Sino Biological (Beijing, China). Phosphoramidon and thiorphan (NEP inhibitors) were purchased from Sigma-Aldrich. All chemicals were purchased with the highest purity available.

Preparation of pancreatic acini

Pancreatic acini were obtained from mouse pancreas by collagenase treatment as described previously (11). Briefly, mice were euthanized by a lethal dose of sodium pentobarbitone (150 mg/kg). Fresh pancreas was infused with buffer A (140 mM NaCl, 4.7 mM KCl, 1.13 mM MgCl2, 1 mM CaCl2, 10 mM glucose, 10 mM HEPES, and 0.5 mg/ml soybean trypsin inhibitor) containing 200 IU/ml type IV collagenase. The pancreas was then minced and digested with buffer A containing 200 IU/ml type IV collagenase (10 min, 37°C). To obtain dispersed acini, the digested tissue was passed through small pipette tips. The viability of pancreatic acinar cells was determined by trypan blue exclusion assay. Cell preparations with at least 95% viability were used for further experiments. Isolated pancreatic acinar cells were treated with caerulein (10−7 M) for 0–120 min for investigation of NEP activity and mRNA expression.

Induction of AP

BALB/c mice were randomly assigned to control or experimental groups by using 10 animals for each group. Animals were given hourly i.p. injections of saline containing caerulein (50 μg/kg) for 3, 6, or 10 h to induce mild, moderate, or severe AP, respectively (in terms of relative severity of caerulein-induced models). Control mice received hourly normal saline injections. To investigate whether NEP inhibition exacerbates AP, mice received a pretreatment of phosphoramidon (5 mg/kg, i.v.) 1 h before six hourly caerulein injections. These concentrations were chosen in reference to previous studies (23, 24). Additionally, (10 mg/kg, i.v.) 1 h before six hourly caerulein injections. These concentrations were chosen in reference to previous studies (23, 24). Additionally, to investigate the protective effects of NEP, mice received 10 hourly caerulein injections and received posttreatment of recombinant mouse NEP (1 mg/kg, i.v.) during the second injection of caerulein. One hour after the last caerulein injection, animals were killed by a lethal dose of sodium pentobarbitone (150 mg/kg, i.p.). Samples of pancreas, lung, and blood were collected. Plasma was prepared from anticoagulated blood samples by centrifugation (10,000 × g, 5 min, 4°C). Random sections of the head, body, and tail of the pancreas and samples of the right lung were fixed in 10% neutral buffered formalin (Sigma-Aldrich). A small section of pancreas and lung was weighed and then dried for 72 h at 55°C and reweighed to determine pancreatic water content. Remaining samples were then stored at −80°C for subsequent analysis.

Measurement of NEP activity

NEP enzymatic activity was determined spectrophotometrically from extracted protein samples as described previously (25). Briefly, treated pancreatic acinar cells, pancreas, and lung tissue were homogenized in T-PER tissue protein extraction reagent (Pierce, Rockford, IL) and centrifuged (12,000 × g, 15 min, 4°C). Cell-free extracts (30 μg protein) were incubated with 1 mM succinyl-Ala-Ala-Phe-p-nitroanilide (Succ-Ala-Ala-Phe-pNA; Bachem) and 0.25% C218 carboxylic columns (Bachem) as substrate in 0.1 M Tris-HCl (pH 7.6) in the presence of 1 U porcine kidney aminopeptidase (AP-N, Sigma-Aldrich). This coupled activity assay, NEP cleaves Succ-Ala-Ala-Phe-pNA between Ala and Phe, yielding Phe-pNA. AP-N subsequently cleaves Phe-pNA, generating pNA as the final product. The kinetic change in absorbance at 405 nm due to the accumulation of free pNA was determined each minute at 37°C for 60 min using a microplate reader. Substrate alone and substrate with AP-N and Tris buffer blanks were run in parallel. The rate of absorbance change was normalized with the control and expressed as fold change to control. Protein concentrations were determined by a Bradford protein assay (26).

SP extraction and detection

Pancreatic and lung tissues were homogenized in 1 ml ice-cold SP assay buffer (Bachem). The homogenates were centrifuged (13,000 × g, 20 min, 4°C) and the supernatants collected. They were adsorbed on C18 cartridge columns (Bachem) as previously described and eluted with 1.5 ml 75% acetonitrile (27). Samples were freeze-dried overnight and reconstituted with SP assay buffer. SP content was then determined with an ELISA kit (Bachem) according to the manufacturer’s instructions. The results were quantified by spectrophotometry at 450 nm. The results were then nor-

Measurement of myeloperoxidase activity

Neutrophil sequestration in the pancreas and lung was quantified by measuring tissue myeloperoxidase (MPO) activity. Tissue samples were thawed, homogenized in 20 mM phosphate buffer (pH 7.4), centrifuged (13,000 × g, 10 min, 4°C), and the resulting pellet was resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% (w/v) hexadecyltrimethylammonium bromide (Sigma-Aldrich). The suspension was subjected to four cycles of freezing and thawing and was further disrupted by sonication (40 s). The sample was then centrifuged (13,000 × g, 5 min, 4°C) and the supernatant used for MPO assay. The reaction mixture consisted of the supernatant (50 μl), 1.6 mM tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD), 80 mM sodium phosphate buffer (pH 5.4), and 0.3 mM hydrogen peroxide (reagent volume, 50 μl). This mixture was incubated at 37°C and terminated with 50 μl 0.18 M H2SO4. The absorbance was measured at 450 nm. This absorbance was then corrected for the DNA content of the tissue sample and results were expressed as fold change over control (28).

Histopathological examination

A small portion of pancreas was excised and fixed with 10% neutral buffered formalin (Sigma-Aldrich), dehydrated through a graded ethanol series, and embedded in paraffin wax for routine histology. Sections of 5 μm thickness were stained with H&E and examined by light microscopy using a Nikon Eclipse 80i microscope (objective lens magnification ×20; eye-piece magnification ×10).

ELISA analysis

ELISA assays were performed for the measurement of cytokines (IL-1, IL-6, TNF-α), chemokines (MIP-1α, MIP-2), and adhesion molecules (ICAM-1,VCAM-1, P-selectin, E-selectin) in pancreas and lung tissue homogenates, according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Results were then corrected for the DNA content of the tissue homogenates and were expressed as picograms per microgram of DNA (28). In this study, tissue content of DNA was chosen over tissue weight or tissue protein content as a normalization factor in ELISA analysis. In the pancreas, it has been shown that total DNA content (milligrams DNA per pancreas) remained relatively stable, but total protein content (milligrams protein per pancreas) was decreased in caerulein-induced pancreatitis models. Pancreatic AP also alters the proportion of water in the tissue, which might cause bias when total pancreatic weight was used as a normalization factor.

Whole cell lysate preparation and Western blot analysis

Pancreatic tissue was homogenized in ice-cold radioimmunoprecipitation assay lysis buffer supplemented with protease inhibitor mixture (Sigma-
and phosphatase inhibitor mixture (Sigma-Aldrich). Protein concentrations were determined by Bradford protein assay. Western blotting was performed using rabbit monoclonal anti-NEP (1:2500 dilution, Abcam) or hypoxanthine-guanine phosphoribosyltransferase (1:1000 dilution; Santa Cruz Biotechnology) in a buffer containing 2.5% nonfat dry milk in 0.05% Tween 20 in PBS. Afterwards, the membranes were incubated with goat anti-rabbit HRP-conjugated secondary Ab (1:2000 dilution; Santa Cruz Biotechnology). Membranes were washed and then incubated in SuperSignal West Pico chemiluminescent substrate (Pierce) before exposure to x-ray films (CL-Xposure; Pierce). Hypoxanthine-guanine phosphoribosyltransferase was applied as an internal control to normalize protein loading. The intensity of bands was quantified using LabWorks image analysis software (UVP).

Quantitative real-time PCR analysis

Total RNA from snap-frozen pancreatic tissue was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. All the steps were done in ice-cold conditions. The integrity of RNA was verified by ethidium bromide staining for the presence of distinct 28S and 18S bands on a 1.2% agarose gel. One microgram total RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) in a total volume of 20 μl, according to the manufacturer’s instructions. cDNA (2 μl) was used as a template for PCR amplification by using a SYBR Green PCR Master Mix from Roche Diagnostics (Singapore). No template controls and intron spanning primers were used. All reactions were done in duplicates. PCR reaction mix was first subjected to 95˚C for 5 min, followed by 45 cycles of amplification. Each cycle consisted of 95˚C for 30 s, annealing temperature of 55˚C for 15 s, and elongation temperature of 72˚C for 15 s. β-actin was used as a housekeeping gene to normalize the mRNA expression. Expression of NEP, PPTA, NK1R, and β-actin was determined using the “crossing point” of the sample, which is the point (cycle number) at which the fluorescence of a sample rises above the background fluorescence.

Results

Caerulein suppress NEP activity and mRNA expression in isolated pancreatic acinar cells

Pancreatic acinar cell NEP activity was significantly decreased as early as 30 min after treatment with caerulein (10^{-7} M), when compared with the control. A time-dependent decrease of NEP enzymatic activity was revealed, reaching a minimum after 2 h caerulein stimulation (Fig. 1A). Moreover, NEP mRNA expression was significantly downregulated in pancreatic acinar cells that were stimulated with caerulein for >60 min (Fig. 1B, Table I). Cells that were incubated in control buffer did not show a significant change in both NEP activity and mRNA expression (Fig. 1A, 1B).

Caerulein-induced AP suppress endogenous NEP activity

The suppression of NEP activity in caerulein-treated pancreatic acinar cells prompted us to investigate whether similar observations could be reproduced in animal models of AP. BALB/c mice were given hourly i.p. injections of caerulein for 3, 6, or 10 h to induce mild, moderate, or severe AP, respectively, in terms of relative severity. Caerulein-treated mice showed a rapid decrease of...
pancreas NEP activity, with maximal suppression observed at severe AP conditions (Fig. 1C). Consistent with NEP activity results, Western blot analysis confirmed early and sustained decrease of NEP protein expression in the pancreas (Fig. 1D). In the lungs, similar decreases of NEP activity and protein expression were observed after induction of AP (Fig. 1E, 1F). Notably, protein expression of NEP in the lung showed a large magnitude of decrease when compared with the control (Fig. 1F).

**Phosphoramidon and thiorphan increase SP levels in the pancreas, lung, and plasma**

As NEP was previously shown to be protective against excessive inflammation, we anticipated that further suppression of NEP activity should exaggerate inflammatory conditions in AP (18, 19). Therefore, we preinhibited NEP before induction of moderate AP to investigate whether it would degenerate into a more severe form of AP. To do this, mice received a single dose of phosphoramidon (5 mg/kg, i.v.) or thiorphan (10 mg/kg, i.v.), followed by six hourly caerulein or saline injections. Both NEP inhibitors decreased NEP activity in the pancreas by \( \sim 70\% \) 1 h posttreatment (data not shown). Control mice challenged with a single dose of phosphoramidon or thiorphan still showed a 20–30% reduction of NEP activity in the pancreas and lungs after six hourly saline injections, suggesting metabolism and clearance of the drug over time (Fig. 2A, 2C). In contrast, SP levels rose significantly (Fig. 2B, 2D).

Induction of moderate AP suppressed NEP activity in both pancreas and lungs, coupled with a strong increase of SP in the pancreas and a modest but insignificant increase in the lungs.

### Figure 2

Inhibition of NEP by phosphoramidon and thiorphan decreased NEP activity and increased SP levels. Mice were randomly given phosphoramidon (5 mg/kg, i.v.) or thiorphan (10 mg/kg, i.v.) before six hourly injections of caerulein (50 \( \mu \)g/kg, i.p.) or normal saline. One hour after the last caerulein injection, mice were sacrificed and NEP activity and SP ELISA assays were performed as described in Materials and Methods. A, Pancreas NEP activity. B, Pancreas SP levels. C, Lung NEP activity. D, Lung SP levels. E, Plasma NEP activity. F, Plasma SP levels. Values are means ± SEM; \( n = 10 \) mice/time point. *\( p < 0.05 \) versus control, †\( p < 0.05 \) versus caerulein group.

<table>
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<th>Gene</th>
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<td></td>
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<tr>
<td></td>
<td>Antisense 5’-GCAAAGGCCCTCCACACT-3’</td>
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Interestingly, pretreatment of NEP inhibitors followed by induction of AP provoked a further increase in SP levels (Fig. 2B, 2D) but did not further reduce NEP activity (Fig. 2A, 2C) in pancreas and lungs. Phosphoramidon or thiorphan administration did not alter background NEP activity in plasma, as NEP is a membrane-bound enzyme and not normally found in cell-free plasma (Fig. 2E). Despite this, plasma SP concentrations were significantly elevated in all mice that received NEP inhibitor treatments when compared with their respective untreated controls (Fig. 2F).

Effect of NEP inhibition on plasma amylase activity, MPO activity, tissue water content, and pancreatic histology

After confirming that phosphoramidon and thiorphan elevated SP levels in mice, we assessed how NEP inhibition influences the outcome of AP. Amylase is produced in the pancreatic acinar cells and released into the bloodstream when there is a pancreatic injury, and therefore it is often used clinically as a marker for the diagnosis of AP. Another important feature of AP is an elevated MPO activity in the pancreas and lungs, indicating infiltration of neutrophils into these tissues (27). Inhibition of NEP without induction of AP did not alter basal plasma amylase activity, tissue water content, or tissue MPO activity, despite elevated SP levels (Fig. 3). Additionally, NEP inhibitors further elevated plasma amylase activity, pancreas water content, and pancreas MPO activity in AP-induced mice (Fig. 3A–C). The lungs were less affected, as NEP inhibitors did not alter AP-induced increases in lung MPO activity and water content (Fig. 3D, 3E). Histological examination of the pancreas confirmed that inhibition of NEP worsened damage in AP-induced mice, characterized by increased pancreatic edema, neutrophil infiltration, and pancreatic necrosis (Fig. 4B, 4D, 4F). Normal pancreas architecture was observed in all control mice (Fig. 4A, 4C, 4E).

Effect of NEP inhibition on proinflammatory cytokine, chemokine, and adhesion molecule expression

Mice induced with moderate AP exhibited a significant elevation in pancreatic proinflammatory mediators, which include cytokines (IL-1, IL-6, TNF-α) and chemokines (MIP-1α, MIP-2) (Table II), compared with control mice, which received saline injections. Adhesion molecule (ICAM-1, VCAM-1, P-selectin, E-selectin) expression also showed modest increases, but they were not significantly different from the control. In the lungs, a milder up-regulation of proinflammatory mediators was observed, with only IL-1 and ICAM-1 significantly increased from control mice (Table II). Inhibition of NEP in AP-induced mice generally showed a further increase of proinflammatory mediator expression in both pancreas and the lungs when compared with the control groups (Table II). NEP inhibitors alone did not appear to raise tissue expression of any of the investigated molecules.

Mouse recombinant NEP decreases SP levels in the pancreas, lung, and plasma

We have observed suppression of NEP activity in caerulein-induced AP. Therefore, we investigated whether mice would be protected against severe AP by treatment of exogenous NEP. Mice that received 10 hourly caerulein injections developed severe AP and exhibited significantly suppressed NEP activity in the pancreas and lungs (Fig. 5A, 5C). Administration of exogenous NEP markedly increased NEP activity as well as decreased SP levels in the lung and pancreas (Fig. 5B, 5D). Inhibition of AP did not affect the background plasma NEP activity, but a 10-fold increase was observed after mice were treated with exogenous NEP (Fig. 5E). Treatment of mice with exogenous NEP did not completely abolish pathological SP levels in the pancreas and plasma. SP levels were still significantly higher in NEP-treated AP mice when compared with the control (Fig. 5B, 5F).
Exogenous NEP protects mice against caerulein-induced pancreatic injury

Plasma amylase, as well as MPO activity and water content in the pancreas and lungs, was strongly augmented after induction of severe AP (Fig. 6). Mouse recombinant NEP (1 mg/kg, i.v.), given during the second caerulein injection, significantly attenuated AP-induced MPO activity and water content (Fig. 6B–E). Exogenous NEP also protected mice against pancreatic injury, as shown by a decrease in plasma amylase activity (Fig. 6A). Histological examination of the pancreas confirmed the protective effects, as demonstrated by reduced neutrophil infiltration, pancreatic edema, and pancreatic necrosis (Fig. 4G, 4H). Notably, the therapeutic effect of exogenous NEP correlated with a decrease of SP levels. However, a single dose of exogenous NEP treatment did not completely protect mice from severe AP, as pancreatic morphology, plasma amylase activity, and tissue MPO activity were still significantly increased when compared with healthy control mice.

Effect of exogenous NEP treatment on proinflammatory cytokine, chemokine, and adhesion molecule expression

Mice that received 10 hourly caerulein injections (severe AP) showed a further increase of proinflammatory mediator levels in pancreas and lung tissue when compared with mice that received 6 hourly caerulein injections (moderate AP) (Tables II, III). Notably, NEP treatment decreased expression of proinflammatory cytokines (IL-1, IL-6, TNF-α), chemokines (MIP-1α, MIP-2), and adhesion molecules (ICAM-1, VCAM-1, E-selectin, P-selectin) in mice induced with severe AP (Table III). Consistent with MPO and histology results, exogenous NEP treatment did not completely protect mice from AP-induced upregulation of cytokines, chemokines, and adhesion molecules (Table III).

Exogenous NEP attenuates caerulein-induced NK1R mRNA upregulation in the pancreas

It was previously reported that disruption of SP–NK1R interaction by CP96,345, a selective NK1R antagonist, decreased PPTA and NK1R mRNA expression in AP (30). Therefore, in this study, we investigated the effect of NEP on mRNA expression of PPTA, NK1R, and NEP in the pancreas. Expectedly, we observed upregulation of PPTA and NK1R mRNA expression after induction of AP (Fig. 7C–F, Table I). However, we found that only NK1R expression was downregulated when mice with severe AP received exogenous NEP treatment (Fig. 7F). Inhibition of NEP by phosphoramidon or thiorphan did not affect caerulein-induced PPTA/NK1R upregulation (Fig. 7C, 7E). Interestingly, NEP mRNA expression was significantly decreased in moderate AP, but recovered to normal levels in severe AP (Fig. 7A, 7B).

Discussion

A great deal of preclinical data and some early clinical studies highlighted the importance of tachykinin interaction with their receptors for promoting inflammatory disorders (18, 19, 25, 31, 32). Thus, identifying the molecular mechanisms that modulate their expression is of crucial importance. Our earlier work has identified the mechanisms that lead to upregulation of SP in the pancreatic acinar cells following caerulein stimulation (11, 15). In the present study, we report that caerulein-induced AP suppressed NEP activity in the pancreas and lungs, which could contribute to elevated SP levels in the system and promote subsequent inflammatory responses. We also report that inhibition of NEP with phosphoramidon or thiorphan exacerbated inflammation and, for the first time to our knowledge, that treatment of AP-induced mice with exogenous NEP protected mice against severe AP.

Our findings identified that NEP was downregulated by caerulein in mouse and isolated mouse pancreatic acinar cells. Time course studies revealed an early and rapid decrease of NEP activity after induction of AP, suggesting that NEP might be involved in early inflammatory responses. Besides, sustained downregulation of NEP activity throughout the course of AP could contribute to uncontrolled inflammation. Interestingly, whereas pancreatic NEP mRNA expression remained downregulated during moderate AP, its expression during severe AP recovered to a level comparable to normal, healthy mice. The effects of longer exposure to caerulein on pancreatic NEP expression might suggest activation of recovery mechanisms that help to reverse the ongoing inflammation.

Although we showed a downregulation of NEP activity in caerulein-induced AP, the mechanisms that regulate its expression...
remains unclear. Caerulein might directly inactivate transcription factors that regulate NEP mRNA expression. However, this did not adequately address why downregulation of NEP activity occurred before downregulation of NEP mRNA in the pancreatic acinar cells. Activation of proteases, necrosis of pancreatic tissue, or generation of reactive oxygen species might also indirectly reduce NEP activity in caerulein-induced models (33). Oxidative stress was shown to inactivate NEP in the lungs (34, 35). Downregulation of NEP was also observed in inflammatory diseases independent of caerulein stimulation, such as inflamed rat intestine and

### Table II. Effect of NEP inhibition on expression of cytokines, chemokines, and adhesion molecules

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<th>Control</th>
<th>Cael6h</th>
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<td>48.49 ± 5.49</td>
<td>35.49 ± 2.46</td>
<td>38.07 ± 4.48</td>
</tr>
<tr>
<td>Expression in lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IL-1</td>
<td>13.52 ± 1.12</td>
<td>21.54 ± 2.25*</td>
<td>20.04 ± 2.52</td>
<td>18.21 ± 1.52</td>
<td>12.81 ± 1.65</td>
<td>13.42 ± 1.31</td>
</tr>
<tr>
<td>IL-6</td>
<td>11.91 ± 0.53</td>
<td>13.57 ± 2.05</td>
<td>14.07 ± 1.40</td>
<td>12.34 ± 1.36</td>
<td>9.78 ± 1.44</td>
<td>11.21 ± 1.49</td>
</tr>
<tr>
<td>TNF-α</td>
<td>10.00 ± 0.94</td>
<td>16.34 ± 2.49</td>
<td>20.94 ± 1.84*</td>
<td>18.69 ± 2.28*</td>
<td>13.17 ± 2.28</td>
<td>15.50 ± 2.75</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>6.87 ± 0.44</td>
<td>9.76 ± 0.76</td>
<td>12.56 ± 1.75*</td>
<td>10.57 ± 1.44*</td>
<td>6.85 ± 0.39</td>
<td>7.78 ± 0.65</td>
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<tr>
<td>MIP-2</td>
<td>11.16 ± 0.42</td>
<td>14.35 ± 1.36</td>
<td>17.22 ± 2.11*</td>
<td>15.77 ± 1.45*</td>
<td>11.69 ± 1.30</td>
<td>12.72 ± 0.82</td>
</tr>
<tr>
<td>E-selectin</td>
<td>21.92 ± 1.35</td>
<td>26.39 ± 2.88</td>
<td>29.60 ± 4.63</td>
<td>25.02 ± 2.94</td>
<td>18.43 ± 1.93</td>
<td>21.78 ± 2.02</td>
</tr>
<tr>
<td>P-selectin</td>
<td>140.4 ± 13.9</td>
<td>168.1 ± 15.7</td>
<td>193.9 ± 22.7</td>
<td>154.0 ± 18.8</td>
<td>142.4 ± 11.9</td>
<td>163.5 ± 10.6</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>218.3 ± 12.5</td>
<td>366.0 ± 21.6*</td>
<td>416.3 ± 55.9*</td>
<td>360.7 ± 44.5*</td>
<td>279.9 ± 38.6</td>
<td>259.7 ± 26.7</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>199.0 ± 16.3</td>
<td>220.0 ± 21.7</td>
<td>249.0 ± 32.6</td>
<td>184.2 ± 14.3</td>
<td>199.7 ± 28.6</td>
<td>177.8 ± 13.7</td>
</tr>
</tbody>
</table>

Mice were randomly given phosphoramidon (Phos, 5 mg/kg, i.v.) or thiorphan (Thior, 10 mg/kg, i.v.) before six hourly injections of caerulein (Cael6h, 50 µg/kg, i.p.) or normal saline. Tissue homogenates were subjected to ELISA assay and normalized with the DNA content of the homogenate and are expressed as picograms per microgram of DNA (n = 10 mice/group).

*p < 0.05 versus the control, #p < 0.05 versus caerulein-treated mice.

**FIGURE 5.** Effect of exogenous NEP on NEP activity and SP levels. Mice received a single dose of normal saline or exogenous NEP (1 mg/kg) along with 10 hourly caerulein injections. One hour after the last caerulein injection, mice were sacrificed and NEP activity and SP ELISA assays were performed as described in Materials and Methods. A, Pancreas NEP activity. B, Pancreas SP levels. C, Lung NEP activity. D, Lung SP levels. E, Plasma NEP activity. F, Plasma SP levels. Values are means ± SEM; n = 10 mice/time point. *p < 0.05 versus control, #p < 0.05 versus caerulein group.
Therefore, the regulatory mechanisms of NEP activity in vivo might also involve certain proinflammatory molecules, but they remain to be studied in greater detail.

SP is a major proinflammatory mediator in experimental AP (12, 14, 22). NEP is of critical importance in modulating SP-induced inflammatory responses, as SP is mainly degraded and inactivated by NEP.

Table III. Effect of NEP treatment on expression of cytokines, chemokines, and adhesion molecules

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cae10h</th>
<th>NEP + Cae10h</th>
</tr>
</thead>
<tbody>
<tr>
<td>In the pancreas</td>
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<tr>
<td>IL-1</td>
<td>3.15 ± 0.22</td>
<td>7.93 ± 0.69*</td>
<td>5.37 ± 0.68*</td>
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<tr>
<td>IL-6</td>
<td>4.08 ± 0.27</td>
<td>8.30 ± 0.83*</td>
<td>5.30 ± 0.58*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>6.62 ± 0.40</td>
<td>19.02 ± 2.08*</td>
<td>8.93 ± 1.48*</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>2.21 ± 0.29</td>
<td>5.48 ± 0.52*</td>
<td>3.56 ± 0.46*</td>
</tr>
<tr>
<td>MIP-2</td>
<td>4.11 ± 0.24</td>
<td>8.86 ± 0.64*</td>
<td>6.46 ± 0.53*</td>
</tr>
<tr>
<td>E-selectin</td>
<td>8.10 ± 0.57</td>
<td>12.77 ± 0.96*</td>
<td>9.35 ± 0.91*</td>
</tr>
<tr>
<td>P-selectin</td>
<td>26.36 ± 1.99</td>
<td>40.74 ± 2.79*</td>
<td>27.96 ± 2.58*</td>
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<tr>
<td>ICAM-1</td>
<td>33.96 ± 3.11</td>
<td>55.71 ± 5.97*</td>
<td>41.31 ± 3.14</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>35.63 ± 2.19</td>
<td>57.99 ± 5.80*</td>
<td>42.82 ± 4.86</td>
</tr>
<tr>
<td>In the lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1</td>
<td>13.52 ± 1.12</td>
<td>24.75 ± 1.88*</td>
<td>19.03 ± 1.69*</td>
</tr>
<tr>
<td>IL-6</td>
<td>11.91 ± 0.53</td>
<td>13.57 ± 2.05</td>
<td>12.35 ± 0.97</td>
</tr>
<tr>
<td>TNF-α</td>
<td>10.00 ± 0.94</td>
<td>31.82 ± 3.99*</td>
<td>18.07 ± 1.94*</td>
</tr>
<tr>
<td>MIP-1α</td>
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<td>9.11 ± 0.69*</td>
</tr>
<tr>
<td>MIP-2</td>
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<tr>
<td>E-selectin</td>
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</tr>
<tr>
<td>P-selectin</td>
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<td>173.5 ± 16.4</td>
<td>135.3 ± 16.4</td>
</tr>
<tr>
<td>ICAM-1</td>
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<td>473.5 ± 30.8*</td>
<td>316.5 ± 21.9*</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>199.0 ± 16.3</td>
<td>235.0 ± 20.5</td>
<td>195.8 ± 27.5</td>
</tr>
</tbody>
</table>

Mice were randomly given 10 hourly caerulein (Cae10h; 50 μg/kg, i.p.) injections, and recombinant mouse NEP (1 mg/kg, i.v.) was given during the second caerulein injection. Tissue homogenates were subjected to ELISA assay and normalized with the DNA content of the homogenate and are expressed as picograms per microgram of DNA (n = 10 mice/group).

*p < 0.05 versus the control, #p < 0.05 versus caerulein-treated mice.
In this study, we ascertained the role of NEP in two opposite ways: inhibition of NEP with phosphoramidon or thiorphan, or administration of exogenous recombinant mouse NEP to increase NEP activity. Treatment of mice with exogenous NEP significantly decreased SP levels in the mice, occurring in parallel with increased NEP activity. In contrast, NEP inhibitor-treated mice showed markedly increased SP levels when compared with vehicle-treated mice. Interestingly, exogenous NEP may be reasonably resilient to metabolism, as markedly elevated activity in the pancreas, lungs, and plasma was still present 10 h after treatment. A previous study also suggested that the stability of exogenous NEP was not compromised in the presence of oxidants or inflammatory fluids (38). Additionally, the inhibitory effect of phosphoramidon and thiorphan decreased over time, showing evidence of drug metabolism. In normal healthy mice, NEP inhibitor treatment still decreased basal NEP activity at the end of the 8-h treatment protocol. On the contrary, in AP-induced mice groups, pretreatment of phosphoramidon or thiorphan showed no difference in NEP activity levels with mice that did not receive the treatment. Hence, it appears that the effects of phosphoramidon and thiorphan were neutralized at a faster rate in AP-induced mice than in normal, healthy mice.

The present study demonstrates that NEP is protective against the damaging effects of caerulein-induced AP by modulating physiological SP levels. Pancreatic edema, increased plasma amylase activity, and infiltration of neutrophils into the inflamed tissue are well-known characteristics of caerulein-induced AP (7, 27, 33, 39). In AP, plasma amylase level is markedly increased due to this enzyme escaping into the blood from damaged pancreatic tissues (40). Exogenous NEP protected mice against pancreatic damage and edema, as demonstrated by significantly lowered plasma amylase activity and tissue water content. On the contrary, mice pretreated with NEP inhibitors exacerbated pancreatic damage. These results agreed with previous reports showing the protective role of NEP in diet-induced hemorrhagic pancreatitis in mice (22). Our findings were also in line with previous reports showing potentiated pancreatitis when animals were challenged with exogenous SP. Exogenous SP elevated pancreatic microvascular permeability and interstitial space in caerulein-treated mice, which contribute to pancreatic microcirculatory dysfunction (41). Exogenous SP also increased caerulein-stimulated amylase output (42). Despite this, the effects of exogenous SP administration on cytokine response in AP-induced animals remain to be explored in detail.

A major feature of AP is the infiltration of neutrophils into the pancreas. Induction of AP significantly increased MPO activity in the pancreas, indicating massive neutrophil infiltration. In AP-induced mice, MPO activity was abolished by exogenous NEP treatment but further increased by NEP inhibitor treatment. Various molecules are responsible for recruitment of inflammatory cells and propagation of inflammation. Clinically, the presence of endotoxins is linked to more severe cases of AP (43). Exogenous administration of endotoxins also strongly propagated inflammation and caused a much more severe form of hemorrhagic AP in mice (44). However, the absence of detectable endotoxins in the plasma precludes its role as a proinflammatory mediator in our model of acute pancreatitis (45). Despite this, a tilt of the balance toward upregulation of proinflammatory mediators, which include cytokines (IL-1, IL-6, and TNF-α), chemokines (MIP-1α, MIP-2), and adhesion molecules (ICAM-1, VCAM-1, E-selectin, P-selectin), was responsible for propagating inflammation in caerulein-induced AP (46–48). In our study, a significant reduction of

**FIGURE 7.** Effect of NEP on mRNA expression of NEP, NK1R, and PPTA in the pancreas. NEP, NK1R, and PPTA mRNA expression was determined with real-time PCR. A, Inhibition of NEP on NEP mRNA expression. B, Exogenous NEP on NEP mRNA expression. C, Inhibition of NEP on PPTA mRNA expression. D, Exogenous NEP on PPTA mRNA expression. E, Inhibition of NEP on NK1R expression. F, Exogenous NEP on NK1R expression. Values are means ± SEM; n = 8 mice/time point. *p < 0.05 versus control, †p < 0.05 versus caerulein group.
proinflammatory mediator expression was observed in NEP-treated mice compared with mice without treatment. SP is able to upregulate these mediators in leukocytes and pancreatic acinar cells mainly via activation of NF-κB (49, 50). Thus, NEP reduces the proinflammatory effects of SP and protects mice against AP.

Lung inflammation is commonly associated with more severe cases of AP (51). Uncontrolled inflammation in the pancreas elevates systemic cytokine levels and propagates damage to distant organs, causing systemic inflammatory response syndrome. In our model of caerulein-induced AP, the lungs showed similar inflammatory responses with the pancreas, which were protected by exogenous NEP. Notably, pretreatment of NEP inhibitors did not aggravate inflammation in the lung following moderate AP, despite an increase in lung SP levels. It is possible that in our model of moderate AP, inflammatory responses in the lungs are still in an early stage, and the effects of SP were still insignificant. Nonetheless, the exact role of NEP in the lungs after induction of AP requires further investigation.

Diminished degradation of SP, in itself, did not appear to cause inflammation. Mice pretreated with phosphoramide or thiophan without induction of AP raised systemic SP levels but did not cause changes in tissue MPO activity, water content, and histological appearance. These observations suggest that agents other than NEP substrates must first initiate pathological conditions, and then SP acts as a molecule to promote the progression of inflammation. In our case, caerulein acted as the initiating agent to cause AP. Additionally, slightly increased cytokine/chemokine/adhesion molecule levels were observed in the pancreas and lung after inhibition of NEP when compared with untreated controls. This is not surprising, as previous studies have shown that the expression of cytokines and chemokines can be directly upregulated by NK1R activation via a NF-κB–dependent mechanism (8, 52).

Previous studies have shown disruption of SP–NK1R interaction by a NK1R antagonist, CP96,345, significantly reversed AP-induced PPTA/NK1R mRNA upregulation in the pancreas (30). Therefore, it was necessary to test how NEP affects mRNA expression of PPTA and NK1R after induction of AP. We found that only NK1R mRNA expression was abolished on NEP-treated, AP-induced mice, whereas all other treatment groups showed no significant changes when compared with their respective vehicle-treated controls. Activation of NK1R may contribute, but not dictate, the expression of PPTA/NK1R in mice. Furthermore, in our experiments, exogenous NEP did not completely abolish physiological SP availability. An in-depth study of SP on the expression of PPTA/NK1R is necessary to elucidate the mechanisms involved.

Preclinical experiments that involve disruption of SP–NK1R interaction, which include NK1R antagonism and blockade of SP release from sensory nerve endings, have successfully protected animals against AP. Results in this study describe targeting NEP as a novel mechanism to disrupt SP–NK1R interaction during AP, chiefly via degradation of SP. Administration of exogenous NEP was previously suggested as a safe and feasible method to protect animals against various inflammatory disorders (18, 20, 21, 38). Glucocorticoids, such as dexamethasone, are anti-inflammatory drugs that were found to increase NEP expression, making it a potential therapeutic option to target NEP in SP-mediated inflammatory responses (53, 54). One major limitation of our study is that the effects of NEP on other NEP substrates, such as bradykinins and amyloid β, were not adequately addressed. Besides this, SP can also be degraded by angiotensin-converting enzyme (16). Future work can be done to address the role of angiotensin-converting enzyme in SP-mediated responses in AP.

On the basis of this series of experiments, we have concluded that a high persistent level of SP during AP could be contributed by a disruption of NEP activity, leading to a detrimental inflammatory condition that increases and perpetuates pancreatic and lung injury. Pharmacological inhibition of the SP-degrading enzyme NEP led to increased availability of SP and exacerbated AP. Conversely, treatment of mice with exogenous recombinant NEP protects mice against the detrimental effects of severe AP. Taken together, it is hoped that the results of these experiments and future studies will lead to new approaches for the prevention of inflammatory cascade in patients with AP.

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Disclosures

The authors have no financial conflicts of interest.

References