Deletion of neutral endopeptidase exacerbates intestinal inflammation induced by Clostridium difficile toxin A

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ONE OF THE MOST COMMON FORMS of iatrogenic infection is diarrhea and intestinal inflammation induced by the bacterium Clostridium difficile (20). Toxin A (TxA) is a large molecular weight protein released from C. difficile that is responsible for diarrhea and acute intestinal inflammation (20). The mechanism of TxA-induced enteritis involves toxin binding to enterocyte receptors, leading to activation of sensory and enteric nerves that results in enhanced intestinal secretion and motility, degranulation of mast cells, and infiltration of the mucosa by neutrophils (19, 35). Substance P (SP) released from the endings of sensory neurons and its neurokinin-1 receptor (NK1R) are critical mediators of TxA-induced inflammation in experimental animals. Thus chemical and surgical ablation of sensory neurons (7, 26, 27), pharmacological NK1R antagonism (36), or genetic deletion of the NK1R (8) markedly abrogate TxA-induced enteritis. SP activates the NK1R on a variety of intestinal cell types, including enteric nerves, endothelial cells, lamina propria macrophages, and leukocytes to stimulate plasma extravasation, fluid secretion, mast cell degranulation, and generation of cytokines (6, 13, 27). Although it is well established that SP and NK1R play an essential role in the initiation and progress of the secretory and inflammatory responses to TxA, there is no information on the mechanisms that potentially terminate these TxA-induced inflammatory responses.

Cellular responses to SP are tightly regulated. Termination of responses to SP are usually considered from the viewpoint of the NK1R. SP binding triggers translocation of G protein receptor kinases and protein kinase C to the plasma membrane, where they phosphorylate the NK1R to promote its interaction with β-arrestins (1, 22, 30, 31, 38, 43). β-arrestins disrupt association of the NK1R with heterotrimeric G proteins and serve as adaptors for clathrin-dependent endocytosis of the NK1R, both of which contribute to desensitization of signal transduction. However, mechanisms that limit the concentration of SP in the extracellular fluid represent the earliest step in restricting its proinflammatory effects before receptor activation can proceed.

The concentration of SP in interstitial fluid is a balance between release from nerves and removal by dilution and degradation. The cell-surface enzyme neutral endopeptidase (NEP; EC 3.4.24.11) degrades SP by phosphatidylinositol-dependent phosphorylation of the NK1R, both of which contribute to desensitization of signal transduction. However, mechanisms that limit the concentration of SP in the extracellular fluid represent the earliest step in restricting its proinflammatory effects before receptor activation can proceed.

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hydrolyzing Gln\textsuperscript{6}-Phe\textsuperscript{7}, Phe\textsuperscript{7}-Phe\textsuperscript{8}, and Gly\textsuperscript{9}-Leu\textsuperscript{10} bonds and plays a major role in terminating the biological effects of SP (28, 29). NEP is widely distributed throughout the intestine, where it is expressed by many cells that are regulated by SP, including enterocytes, endothelial cells, neurons, and myocytes (5). Coexpression of NEP with the NK1R markedly diminishes responses to SP (33), and NEP inhibitors magnify the effects of SP in cell lines, tissues, and intact animals (10, 23, 25, 33). Although SP release and subsequent activation of the NK1R initiate TxA-induced enteritis, the role of NEP in terminating this inflammation is unknown. An understanding of the role of NEP in TxA-induced enteritis is of considerable interest, because NEP is downregulated in the inflamed intestine, which could exacerbate inflammation (15).

The purpose of this investigation was to determine the role of NEP in terminating TxA-induced enteritis. To do so, we used the TxA animal model of intestinal secretion and inflammation, which closely resembles the secretory and inflammatory changes seen in human \textit{C. difficile} infection (20). Our aims were to 1) compare the inflammatory responses to TxA in NEP\textsuperscript{+/+} and congenic NEP\textsuperscript{−/−} animals, 2) determine the effectiveness of recombinant human NEP (rhNEP) as an anti-inflammatory agent, and 3) examine whether NEP inhibitors that are under development as therapeutic agents have proinflammatory effects.

**METHODS**

**Animals.** All experimental protocols were approved by the Animal Care and Use Committee of the University of California at San Francisco. NEP\textsuperscript{−/−} mice were back-crossed for seven generations into C57BL6 mice (Taconic, Germantown, NY) (24). Deletion of NEP was confirmed by Southern blotting, as described previously (24). For NEP\textsuperscript{+/+} mice, C57/BL6 mice of the same weight, age, and sex as the knockout animals were used. Colonies were maintained under climate-controlled (12:12-h light-dark cycle) conditions in a barrier facility.

**NEP localization.** To localize immunoreactive NEP, NEP\textsuperscript{+/+} and NEP\textsuperscript{−/−} mice were anesthetized with pentobarbital sodium (60 mg/kg ip) and transcardially perfused with 4% paraformaldehyde in 100 mM PBS, pH 7.4. Segments of small intestine and kidney were processed to obtain frozen tissue sections for immunohistochemistry using antibody 20, as described previously (5).

**NEP enzymatic assays.** NEP enzymatic activity was measured in extracts of tissues from NEP\textsuperscript{+/+} and NEP\textsuperscript{−/−} mice by a fluorometric assay using glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamine as a substrate, as described previously (5). Only activity that was inhibited by 1 μM thorphan or phosphoramidon (selective NEP inhibitors) was attributed to NEP. Activity was expressed as picomoles of 4-methoxy-2-naphthylamine (MNA) generated per micrograms of protein per hour.

**Induction of intestinal inflammation.** TxA from \textit{C. difficile} was purified to homogeneity as previously described (37). Male and female mice (20–35 g) were fasted overnight but allowed access to water. Mice were anesthetized with ketamine. Through a midline laparotomy, two 4-cm ileal loops were ligated and injected with either 0.1 mL of 50 μM Tris-HCl buffer pH 7.4 (control) or buffer containing TxA (0.5–5 μg) (8, 36). The abdomen was closed, and the animals were allowed to regain consciousness. Mice became ambulatory within 60–90 min of completion of the surgery. Three hours after administration of TxA, mice were killed with pentobarbital sodium (200 mg/kg ip), and the intestinal loops were removed. The loop length, weight, and fluid volume were recorded. A portion of the loop was frozen in 50 mM KH\textsubscript{2}PO\textsubscript{4} buffer, pH 6, containing 0.5% hexadecyltrimethyl ammonium bromide (Sigma Chemical, St. Louis, MO) for measurement of myeloperoxidase (MPO) activity (19). The remaining tissue was fixed in 10% formalin and embedded in paraffin, and sections were stained with hematoxylin and eosin for histological grading of ileal inflammation (8, 36). Some NEP\textsuperscript{−/−} mice were injected with rhNEP, BSA (DAKO, Carpenteria, CA), or boiled rhNEP (controls) (all 3 mg/kg) in the tail vein 5 min before surgery. rhNEP was a gift from Dr. Donald Payan (Rigel, South San Francisco, CA). Similarly, some NEP\textsuperscript{+/+} mice received an injection of the NEP inhibitor phosphoramidon (3 mg/kg, Sigma Chemical) or saline vehicle.

**MPO assay.** Ileal tissue was homogenized and sonicated, and the homogenate was centrifuged (12,000 g, 15 min). MPO activity in the supernatant was quantified with a microtitr plate assay using 5-O-dianiside (Aldrich, Milwaukee, WI) as the substrate (19). Human neutrophil MPO (Calbiochem, San Diego, CA) was used to generate a standard curve. Supernatants were assayed in duplicate at three dilutions, and the values falling within the linear portion of the standard curve were used. Results are expressed as units of MPO per gram of wet tissue.

**Histology.** The severity of inflammation was scored in coded slides by a pathologist on a scale of 1 (mild) to 3 (severe) for epithelial damage, edema, and neutrophil infiltration as previously described (8, 36).

**Statistics.** Results are reported as means ± SE. Data were compared using ANOVA and multiple comparison testing with the Student-Newman-Keuls t-test or Student’s t-test with \( P < 0.05 \) considered to be significant.

**RESULTS**

**Characterization of NEP\textsuperscript{−/−} mice.** To confirm the absence of NEP in NEP\textsuperscript{−/−} mice, we examined the expression of NEP protein by immunofluorescence and of NEP activity using an enzymatic assay. As previously reported (5), immunoreactive NEP was most abundant in the brush border of the renal proximal tubule and the small intestine in NEP\textsuperscript{+/+} mice (Fig. 1A). NEP was also detected in other tissues, such as the muscularis externa of NEP\textsuperscript{+/+} mice (not shown). There was no specific staining in the kidney and small intestine of NEP\textsuperscript{−/−} animals (Fig. 1A). NEP activity was very high in extracts of the kidney and small intestine, followed by the trachea and lungs, skin, urinary bladder, stomach, pancreas, and colon of NEP\textsuperscript{+/+} mice (Fig. 1B). Activity was at background levels in NEP\textsuperscript{−/−} animals (Fig. 1B). These results confirm the absence of NEP protein and enzymatic activity in NEP\textsuperscript{−/−} mice.

**Deletion of NEP exacerbates TxA-induced fluid secretion and granulocyte infiltration.** We compared enteritis to graded doses of TxA (0.5–5 μg) in NEP\textsuperscript{+/+} and NEP\textsuperscript{−/−} mice using accumulation of fluid in the intestinal lumen and MPO activity in the intestinal wall as endpoints. Basal fluid secretion (expressed as loop weight-to-length ratio, in mg/cm) in control loops
(no TxA) was similar in NEP +/+ and NEP −/− animals (33 ± 2 and 36 ± 2 mg/cm, respectively) (Fig. 2A). In NEP +/+ mice, TxA induced a dose-dependent increase in secretion that was statistically different from basal to 1 µg and maximal to 5 µg TxA (Fig. 2A). In NEP −/− mice, the secretory response was more robust for all doses of TxA. A significant increase in secretion above basal was observed to 0.5 µg TxA. Compared with NEP +/+ mice, secretion in NEP −/− animals was increased by 200% at 0.5 µg and 135% at 5 µg TxA (Fig. 2A). Basal granulocyte infiltration (assessed by MPO activity) in control loops was similar in NEP +/+ and NEP −/− animals (3.6 ± 2 and 1 ± 0.2 U/g, respectively) (Fig. 2B). Compared with NEP +/+ mice, MPO activity in NEP −/− animals was increased by 270% at 0.5 µg and 150% at 1 µg TxA (Fig. 2B). However, MPO activity was the same in both groups at 2 µg TxA and was higher in NEP +/+ mice for 5 µg TxA. Thus deletion of NEP reduced the threshold and increased the magnitude of TxA-induced fluid accumulation and granulocyte infiltration. This effect was especially pronounced when submaximal doses of TxA were used. Therefore, we used the half-maximal dose of TxA in NEP −/− mice (0.5 µg) for all subsequent experiments. This dose of TxA induced reliable inflammation in NEP −/− mice but had a minor effect in wild-type animals.

**Pretreatment with rhNEP attenuates exacerbated TxA-induced enteritis in NEP −/− mice.** To confirm that the exacerbated enteritis observed in NEP −/− mice was due to lack of NEP, we pretreated animals with rhNEP. Pretreatment with BSA or boiled, inactive rhNEP (3 mg/kg iv, controls) had no effect on basal secretion (Fig. 3A), e.g., basal secretion was 33 ± 6 mg/cm (n = 3) with boiled rhNEP and 36 ± 2 mg/cm (n = 23) with saline. When rhNEP (3 mg/kg iv) was administered, basal secretion was 54 ± 4 mg/cm (n = 7). Pretreatment with BSA or boiled rhNEP had no effect on TxA-induced intestinal secretion (Fig. 3A). In marked contrast, pretreatment with rhNEP diminished TxA-induced intestinal secretion. In BSA-treated mice TxA stimulated secretion by 300%, whereas in NEP-treated mice TxA stimulated secretion by only 120% over basal (Fig. 3A). This reduction was observed despite the increase in basal fluid secretion in mice treated with rhNEP. In a similar manner, MPO activity induced by TxA was not affected by pretreatment with BSA, but was markedly inhibited by pretreatment with rhNEP (Fig. 3B). Thus administration of rhNEP
reduces the exacerbated fluid secretion and granulocyte infiltration observed in NEP \(^2/2\) mice, confirming the role of NEP in terminating TxA-induced enteritis. Pretreatment with NEP inhibitor phosphoramidon exacerbates TxA-induced enteritis in NEP \(^1/1\) mice. NEP inhibitors are under development as therapeutic agents, and therefore it is important to understand their proinflammatory effects. Because NEP deletion exacerbated TxA-induced enteritis, we reasoned that NEP inhibition would have a similar effect in NEP \(^+/+\) mice. Pretreatment of NEP \(^+/+\) mice with phosphoramidon (3 mg/kg iv) increased TxA-induced secretion by 180% and MPO activity by 260% compared with TxA-treated control animals (Fig. 4). Phosphoramidon had no effect on basal secretion or MPO activity in loops filled with buffer. Thus inhibition of NEP exacerbated TxA-induced enteritis.

**Deletion and inhibition of NEP exacerbates TxA-induced histological damage of intestinal mucosa.** Tissue sections were scored for epithelial damage, edema, and neutrophil infiltration. In NEP \(^+/+\) mice, TxA induced a dose-dependent increase in histological damage of the mucosa (results not shown). Whereas 0.5 \(\mu g\) TxA did not induce microscopically detectable epithelial damage in NEP \(^+/+\) mice, there was noticeable epithelial damage in NEP \(^-/\) mice (Table 1). Similarly, TxA-induced edema and neutrophil infiltration were markedly higher in NEP \(^-/\) than in NEP \(^+/+\) mice. The total histological score in NEP \(^-/\) mice was approximately twofold greater than in NEP \(^+/+\) mice. Whereas pretreatment of NEP \(^-/\) mice with rhNEP decreased the microscopic damage to that of NEP \(^+/+\) animals, pretreatment of NEP \(^+/+\) mice with phosphoramidon exacerbated the damage to near NEP \(^-/\) levels (Table 1).

Representative photomicrographs of tissues are shown in Fig. 5. The appearance of the control ileum under basal conditions was identical in NEP \(^+/+\) and NEP \(^-/\) mice (Fig. 5, A and D, respectively). In NEP

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**Fig. 2.** Toxin A (TxA)-induced intestinal secretion (A) and myeloperoxidase (MPO) activity (B) in NEP \(^+/+\) and NEP \(^-/\) mice. Ileal loops were injected with 0–5 \(\mu g\) of TxA. After 3 h, weight (in mg) and length (in cm) were recorded and the ratio determined to quantify intestinal secretion. Tissue was also taken for MPO assays to quantify granulocyte infiltration (\(n = 6–25\) mice/group). At all doses of TxA, increased secretion was observed in NEP \(^-/\) mice compared with NEP \(^+/+\) animals. Increased MPO activity was observed in NEP \(^-/\) mice treated with submaximal doses of TxA. *\(P < 0.05\) compared with NEP \(^+/+\).

**Fig. 3.** The effect of recombinant human NEP (rhNEP) on intestinal secretion (A) and MPO activity (B) in NEP \(^-/\) mice treated with 0.5 \(\mu g\) TxA. Mice were injected with saline vehicle (control) or 3 mg BSA or rhNEP in the tail vein 5 min before formation and injection into the ileal loop (\(n = 6\) mice/group). Pretreatment with rhNEP markedly reduced TxA-induced intestinal secretion and granulocyte infiltration. *\(P < 0.05\) compared with basal; #\(P < 0.05\) compared with TxA-treated controls or BSA groups.
Our results show that TxA-induced enteritis is dramatically enhanced in mice lacking NEP and in wild-type animals treated with NEP inhibitors. To our knowledge, this is the first study to directly demonstrate the importance of NEP in the pathophysiology of intestinal secretion and inflammation in response to a bacterial toxin. Because SP is a principal mediator of TxA-induced enteritis (8, 36) and it is the most favorable substrate for NEP (28, 29), the exacerbated response in NEP-deficient animals is likely due to diminished degradation of SP. Thus degradation of SP by NEP serves to restrict the proinflammatory effects of SP and to terminate TxA-induced enteritis. The observation that NEP deficiency exacerbates inflammation may be of importance because NEP is downregulated in the inflamed intestine (15), which may contribute to the inflammatory response. The finding that rhNEP prevents inflammation in NEP−/− mice suggests that rhNEP could be considered as a novel form of treatment for SP-mediated inflammation in the gut. In contrast, the marked proinflammatory effect caused by NEP inhibitors suggests that these drugs may have important side effects.

NEP terminates neurogenic inflammation. Our results show that genetic deletion or pharmacological inhibition of NEP exacerbates TxA-induced enteritis and support an important role for neuropeptides such as SP as proinflammatory agents in the intestine (32, 34). In view of the major role of SP in TxA-induced enteritis and the finding that SP is one of the most kinetically favorable NEP substrates (assessed by turnover rate) (28, 29), it is highly likely that disruption of NEP exacerbates inflammation due to the diminished degradation of SP. In support of our results, deletion of NEP or administration of NEP inhibitors results in spontaneous plasma extravasation in multiple tissues, including the ileum (23), and exacerbates trinitrobenzene sulfonic acid-induced colitis (41) and allergic dermatitis by SP/NK1R-dependent mechanisms (39). NEP inhibitors also magnify other effects of SP in many systems, including the gastrointestinal tract, airway, and skin (10, 16, 25, 42). Surprisingly, MPO activity was higher in NEP+/+ mice than NEP−/− mice receiving high doses of TxA. This result suggests that NEP may play a protective role against excessive granulocyte infiltration and raises the possibility that

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Values are means ± SE; n = 7 mice for all treatment groups, except n = 5 for neutral endopeptidase (NEP) +/+ buffer (control) mice. Loops were filled with buffer or 0.5 µg/ml toxin A (TxA). NEP +/+ mice were pretreated with 3 mg/kg (iv) phosphoramidon and NEP −/− with 3 mg/kg (iv) recombinant human NEP (rhNEP) immediately before loop formation. Tissue sections were scored for epithelial damage, edema, and neutrophil infiltration on a scale of 1 (mild) to 3 (severe). *P < 0.05 vs. same strain receiving TxA; †P < 0.05 vs. NEP +/+ receiving TxA.
peptides that are NEP substrates may have both proinflammatory and anti-inflammatory effects. Although diminished degradation of SP is the most likely mechanism of exacerbated enteritis, the reduced degradation of other peptide mediators may also contribute to enhanced response. Calcitonin gene-related peptide is also involved in the pathophysiology of TxA-induced enteritis (18). This peptide is coreleased with SP from primary spinal afferent neurons and acts on arterioles to stimulate vasodilatation and hyperemia, which contributes to SP-induced inflammation (2, 3). However, calcitonin gene-related peptide is a kinetically unfavorable substrate for NEP (17), and thus diminished degradation of this peptide is unlikely to account for the exacerbated inflammatory response.

Our results support the hypothesis that SP degradation by cell surface enzymes such as NEP is one of the earliest mechanisms for terminating the proinflammatory effects of tachykinins. Other mechanisms may include NK1R uncoupling and endocytosis (1, 22, 30, 31, 38, 43). Although disruption of these processes diminishes desensitization of certain G protein-coupled receptors in vivo (9, 21), the role of these mechanisms in terminating NK1R-mediated intestinal inflammation remains to be determined.

Importance of NEP in regulating inflammation. The observation that NEP terminates SP-mediated enteritis raises the possibility that diminished expression of NEP may exacerbate inflammation. NEP activity is diminished by 84-fold in the mucosa and circular muscle and by 12-fold in the longitudinal muscle layer in the inflamed intestine of rats infected with Trichinella spiralis, which results in a 2- to 6-fold reduction in the rate of SP degradation (15). These findings, together with the present observation that disruption of NEP exacerbates enteritis, suggest that downregulation of NEP contributes to intestinal inflammation in experimental animals. It remains to be determined whether downregulation of NEP contributes to inflammation in the human intestine.

Fig. 5. Tissue damage due to TxA. NEP +/+ (A–C) and NEP −/− mice (D–F) received buffer (A and D; control) or 0.5 μg TxA (B, C, E, and F) in ileal loops. Mice were pretreated with 3 mg/kg (iv) phosphoramidon (C) or rhNEP (F). Note that mucosal damage (arrowheads) and neutrophil infiltration (arrows) were exacerbated in NEP −/− mice compared with NEP +/+ animals. Inflammation in NEP +/+ mice was exacerbated by phosphoramidon, whereas inflammation in NEP −/− animals was diminished by rhNEP. Scale bar, 20 μm.
Administration of rhNEP markedly suppressed the exaggerated inflammatory and secretory responses to TxA in NEP −/− mice. In support of these findings, rhNEP diminishes experimental colitis (41). These observations raise the possibility of using recombinant NEP to treat inflammatory conditions. Because NEP degrades several proinflammatory peptides, such as SP and bradykinin, this approach may offer an advantage over specific antagonists that disrupt signaling by a single receptor. Recombinant NEP also inhibits proliferation of lung cancer cells in intact mice due to the degradation of peptide growth factors, which supports the therapeutic usefulness of recombinant proteases (4). Surprisingly, rhNEP, but not inactivated rhNEP, stimulated basal fluid secretion. Due to the extremely high purity of the preparation, this effect is unlikely to be due to contamination. However, it is possible that NEP degrades an antiserospective peptide.

Inhibitors of neuropeptide processing are widely used and effective drugs. Angiotensin-converting enzyme (ACE) inhibitors are effective for treatment of congestive heart failure and hypertension, and NEP inhibitors have been evaluated in patients with heart failure (12, 14, 40). Mixed ACE and NEP inhibitors are also under development. Our findings that administration of NEP inhibitors to experimental animals exacerbates TxA-induced enteritis suggests a potential side effect of such drugs, namely uncontrolled inflammation. We (11, 23) have previously reported that administration of ACE and NEP inhibitors to mice results in increased plasma extravasation in the airway and intestine due to diminished degradation of bradykinin and SP. Whether NEP and ACE inhibitors have similar proinflammatory effects in humans remains to be evaluated.

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