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Neutral Endopeptidase Terminates Substance P-Induced Inflammation in Allergic Contact Dermatitis[1]

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Sensory nerve-derived neuropeptides such as substance P demonstrate a number of proinflammatory bioactivities, but less is known about their role in inflammatory skin disease. The cell surface metalloprotease neutral endopeptidase (NEP) is the principal proteolytic substance P-degrading enzyme. This study tests the hypothesis that the absence of NEP results in dysregulated inflammatory skin responses. The effector phase of allergic contact dermatitis (ACD) responses was examined in $NEP^{-/-}$ knockout and $NEP^{+/+}$ wild-type mice and compared with the irritant contact dermatitis response in these animals. NEP was found to be normally immunolocalized in epidermal keratinocytes and dermal blood vessels. The ACD ear swelling response was 2.5-fold higher in animals lacking NEP and was accompanied by a significant increase in plasma extravasation and infiltration of inflammatory leukocytes. The augmented ACD response in $NEP^{-/-}$ animals was abrogated by either administration of a neurokinin receptor 1 antagonist or by repeated pretreatment with topical capsaicin. Similar to $NEP^{-/-}$ mice, the acute inhibition of NEP in $NEP^{+/+}$ animals resulted in an augmented ACD response. In contrast to the ACD responses, little differences were observed in the irritant contact dermatitis response of $NEP^{-/-}$ compared with $NEP^{+/+}$ animals after epicutaneous application of the skin irritants croton oil or SDS. Thus, these results indicate that NEP and cutaneous neuropeptides have a significant role in the pathogenesis of ACD. *The Journal of Immunology,* **2001, 166: 1285–1291.**

Sensory nerves in the skin respond to noxious stimuli such as chemical, electrical, thermal, mechanical injury, and UV irradiation by releasing neuropeptides that are capable of mediating a wide range of inflammatory cutaneous responses (1). Substance P (SP)[3] is one such neuropeptide that is widely distributed in the central and peripheral nervous system including the skin (2, 3). SP is capable of inducing a number of inflammatory responses including vasodilatation, plasma extravasation, leukocyte activation, endothelial cell adhesion molecule expression, cellular cytokine production, and mast cell activation (4). These SP proinflammatory effects are mediated by the neurokinin receptor 1 (NK1R) (5–7).

The biological actions of SP are terminated by neutral endopeptidase (NEP). NEP is a member of a family of cell surface zinc metalloproteinases that also includes endothelin-converting enzyme and the Kell blood group Ag (8, 9). NEP is expressed in several tissues including the kidney, small intestine, brain, airway epithelium, vascular endothelium, and skin (8). The administration of NEP inhibitors augments inflammation of the trachea (10, 11). NEP expression is markedly down-regulated during intestinal and respiratory infections, which may contribute to the tissue inflammation response in these diseases (12, 13). Deletion of NEP expression by targeted disruption of the NEP gene locus in mice results in an increased susceptibility to endotoxin shock and intestinal inflammation as well as to the induction of hypotension and a widespread plasma extravasation from postcapillary venules that is mediated by the NK1R and the bradykinin 2 receptor (14, 15).

Little is known about the relative contributions of the neurological system, SP, NK1R, and NEP in inflammatory skin disease. In this study, the role of NEP in modulating cutaneous inflammation in expression of allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) responses was examined. We tested the hypothesis that the absence or inhibition of NEP results in a dysregulated inflammatory skin response in these skin diseases. To address this possibility, we measured the relative contribution of cutaneous NEP in an experimental model of ACD compared with ICD using $NEP^{-/-}$ mice and corresponding wild-type animals. Our results indicate that the lack of NEP results in an increased cutaneous inflammatory response to challenge by allergens, but not by irritants. These results implicate the SP proteolytic enzyme NEP as an important modulator of ACD inflammatory responses in the skin.

Materials and Methods

Animals

$NEP^{-/-}$ mice were a gift from Dr. Craig Gerard (Harvard University, Boston, MA) (14). These mice were derived from the C57BL/6 strain with seven backcrosses. $NEP^{+/+}$ C57/BL6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and used as the control strain. Mice were housed in a barrier facility with free access to water and food. Males and

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[3] Abbreviations used in this paper: SP, substance P; NEP, neutral endopeptidase; ACD, allergic contact dermatitis; ICD, irritant contact dermatitis; NK1R, neurokinin receptor 1; DNFB, 2,4-dinitro-1-fluorobenzene.

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females were studied at 8–12 wk with five to seven mice per experimental condition.

Experimentally induced ACD and administration of neuromodulating agents

ACD was induced as previously described (16). Mice were sensitized on day 0 by painting 25 μl of 0.5% 2,4-dinitro-1-fluorobenzene (DNFB; Sigma, St. Louis, MO) in acetone/olive oil (4:1) on the shaved abdomen. Mice were challenged on day 5 by epicutaneous application of 10 μl 0.2% DNFB in acetone/olive oil (4:1) on the dorsal surface of the right ear. The left ears were treated with vehicle alone (acetone/olive oil, 4:1) and served as an internal control for these studies. The ear swelling induced by the carrier alone was negligible. To detect any potential irritant effects of the hapten, some mice were challenged with 10 μl 0.2% DNFB on the right ear without prior sensitization. The ACD response was determined by the degree of ear swelling of the hapten-exposed ear compared with that of the vehicle-treated contralateral ear before DNFB challenge and at 0–72 h after challenge, as measured with a micrometer.

Ears were obtained from euthanized mice and processed for histologic examination. In some experiments, the effect of neuropeptide depletion on ACD was determined by application of the sensory neurotoxin capsaicin (Zostrix HP cream; GenDerm, Lincolnshire, IL) containing 0.075% *trans*-8-methyl-*N*-vanillyl-6-nonenamide (capsaicin) to the right ears of sensitized $NEP^{-/-}$ mice twice a day for 3 days before Ag challenge and then at multiple times after challenge (15 min, and 7, 14, and 24 h). As a control, the left ears of these mice were treated at each time point with vehicle alone. In a separate study, mice were treated with Zostrix HP cream vehicle only, which was prepared according to the formula provided by GenDerm. In the capsaicin studies, the ear thickness of both the right and left ear of each mouse was measured at 24, 48, and 72 h after challenge. To determine the effect of acute NEP inhibition on ACD in wild-type animals, the NEP inhibitor phosphoramidon (Sigma) was injected into the tail veins of anesthetized sensitized $NEP^{+/+}$ mice at a dose of 2.5 mg/kg at 1, 4, and 8 h after Ag challenge. The effect of SP receptor blockage on ACD was measured by administration of the NK1R antagonist SR 140333 (1.2 μmol/kg, i.v.) (Sanofi Recherche; Montpellier, France) to anesthetized sensitized $NEP^{-/-}$ mice at 2 and 6 h after Ag challenge. All studies were conducted two to three times to verify reproducibility of results.

Induction of ICD

After measurement of the ear thickness of untreated animals, mice were treated with 10 μl of the cutaneous irritants croton oil in acetone (0.25, 0.8, or 1.6%; Sigma) or SDS in distilled water (2 or 10%) on the dorsal surface of the right ear to induce ICD as previously described (17). The left ear was treated with vehicle only. The ICD response was determined by the degree of ear swelling of the irritant-exposed ear compared with that of the vehicle-treated contralateral ear before irritant challenge and at 0–48 h after challenge as measured with a micrometer. From some mice of each group ears were harvested after 24 h and processed for histologic evaluation. Experiments were performed twice to verify reproducibility of the results.

Measurement of microvascular permeability

ACD was induced as previously described (16) in $NEP^{-/-}$ and $NEP^{+/+}$ mice. Microvascular permeability was determined by cutaneous Evans blue extravasation. To measure extravasation of Evans blue, anesthetized mice were injected with 30 mg/kg Evans blue (Sigma) in 0.9% NaCl into a femoral vein (18). Seven minutes after Evans blue injection, mice were transcardially perfused with 50 ml PBS containing 100 U/ml heparin and 200 ml 1% paraformaldehyde in a 0.05% citrate buffer, pH 3.5. Ears were removed, rinsed in saline, blotted, and weighed. Half of each tissue was dried at 60°C for 48 h and reweighed; the other half was incubated in 1 ml formamide (Sigma) for 48 h at room temperature to extract the Evans blue. Evans blue was quantified by spectrophotometry (18). Tissue extravasation is expressed as nanograms Evans blue per milligram of dry weight of tissue. This study was conducted twice with similar results.

Histology

Ears were fixed in 10% PBS-buffered formalin, and 3- to 5-μm paraffin sections were stained with hematoxylin and eosin and examined by light microscopy to assess histologic changes and immune cell infiltration.

Immunofluorescence

Ears were fixed in 4% paraformaldehyde for 24 h at room temperature and embedded in paraffin or snap frozen, embedded in OCT compound (Miles, Elkhart, IN), sectioned, and fixed in 4% paraformaldehyde for 20 min. Sections were processed for immunofluorescence (19). Slides were incubated with a rabbit antiserum to recombinant human NEP (1:250, overnight, 4°C) (20). Endothelial cells were identified using an Ab to von Willebrand factor (1:100; Dako, Carpinteria, CA).

Statistical analysis

Results are expressed as mean ± SE. Differences between multiple groups were examined using an ANOVA and Bonferroni *t* test. Mean differences with $p < 0.05$ were considered to be significant.

Results

Localization of NEP in the skin

Immunofluorescence studies were conducted to determine the distribution of NEP in normal skin and confirm that NEP expression was absent from $NEP^{-/-}$ mice. In $NEP^{+/+}$ animals, NEP was immunolocalized in the keratinocytes of the epidermis (Fig. 1*B*), in dermal microvascular endothelial cells (Fig. 1*D*), and in hair follicles (data not shown). As expected, NEP was not detected in the skin of $NEP^{-/-}$ mice (Fig. 1, *A* and *C*).

Enhanced expression of ACD in $NEP^{-/-}$ mice

To assess the role of NEP in modulating cutaneous inflammation, we compared the ACD response in $NEP^{-/-}$ to $NEP^{+/+}$ mice. In DNFB-sensitized mice, application of DNFB to the right ear induced marked swelling in both the $NEP^{+/+}$ and $NEP^{-/-}$ animals (Fig. 2). Notably, the response was significantly higher (80–120%) in $NEP^{-/-}$ compared with $NEP^{+/+}$ mice at all time points. DNFB challenge in nonsensitized animals failed to induce ear swelling in either the $NEP^{-/-}$ or $NEP^{+/+}$ mice, indicating that the reaction to DNFB was allergic and not due to an irritant effect of the DNFB vehicle solution.

Increased plasma extravasation in $NEP^{-/-}$ mice

We have previously demonstrated that $NEP^{-/-}$ mice have increased permeability of postcapillary venules under basal conditions (18). To determine whether this lack of NEP causes increased microvascular permeability during the expression of ACD, we challenged sensitized and nonsensitized $NEP^{-/-}$ and $NEP^{+/+}$ mice with DNFB and measured extravasation of plasma proteins labeled with Evans blue. Without prior sensitization, DNFB has little effect on extravasation of Evans blue in $NEP^{-/-}$ and $NEP^{+/+}$ mice (Fig. 3, *a* and *b*). In sensitized animals, DNFB induced marked plasma extravasation in $NEP^{-/-}$ and $NEP^{+/+}$ compared with carrier-treated control ears (Fig. 3, *c* and *d*). Extravasation at 6 h after Ag challenge was significantly higher in $NEP^{-/-}$ mice compared with the $NEP^{+/+}$ animals, indicating that this critical component of the ACD response acts early in this cutaneous inflammatory response (Fig. 3, *c* and *d*). These results indicate that cutaneous plasma extravasation regulated by NEP is an important early component of the efferent phase of ACD.

Modulation of ACD responses in mice by the neuropeptide modulators capsaicin, SR140333, and phosphoramidon

We tested the possibility that augmented efferent ACD responses and extravasation of plasma proteins during ACD in $NEP^{-/-}$ mice was due to

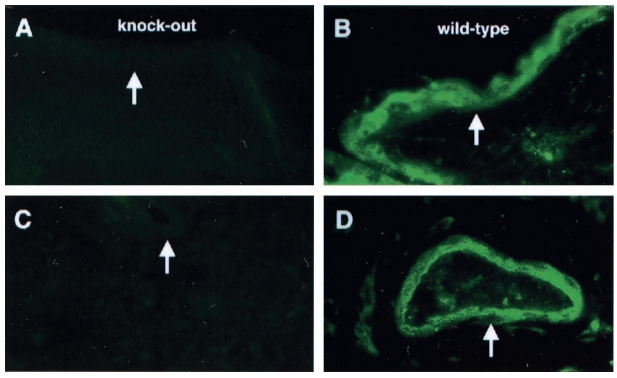

FIGURE 1. Expression of NEP in the skin of $NEP^{-/-}$ and $NEP^{+/+}$ mice. Ears of $NEP^{-/-}$ and $NEP^{+/+}$ mice were examined for NEP expression by indirect immunofluorescence using NEP-specific Abs. Arrows designates location of epidermis (*A* and *B*) and dermal vessel walls (*C* and *D*). Magnification: ×400.

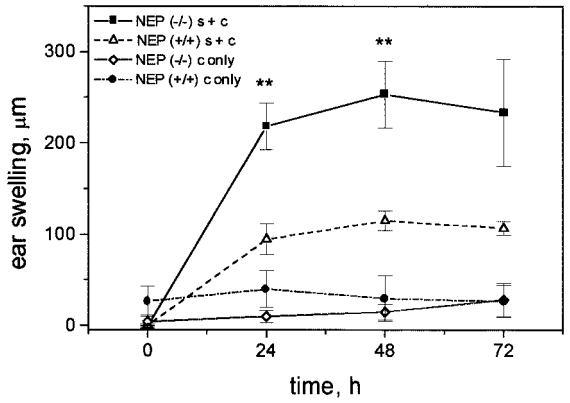

FIGURE 2. Enhanced ACD in NEP$^{-/-}$ mice. NEP$^{-/-}$ and NEP$^{+/+}$ mice were sensitized (s) with 0.5% DNFB on the shaved abdomen at day 0 and challenged (c) with 0.2% DNFB on the right ear at day 5. Other animals were challenged without prior sensitization (c only). The ACD response was determined by the degree of ear swelling of the hapten-exposed ear compared with that of the vehicle-treated contralateral ear before DNFB challenge and at 24, 48, and 72 h after challenge. Ear swelling values are given in micrometers as mean ± SEM, $n = 15$. *, $p < 0.05$; **, $p < 0.01$ for NEP$^{-/-}$ vs NEP$^{+/+}$ mice.

diminished degradation of cutaneous SP. First we tested the potential of the neurotoxin capsaicin to inhibit the augmented ACD response in NEP$^{-/-}$ animals by depleting the skin of neuropeptides in sensory nerves. Zostrix HP cream (capsaicin) was applied to the skin multiple times before and after Ag challenge. Pretreatment of the skin with capsaicin before DNFB challenge slightly increased the swelling, which was likely due to the initial proinflammatory effects of capsaicin-induced neuropeptide release (data not shown). After Ag challenge, we observed a profound reduction of the cutaneous inflammatory response at 24 and 48 h in NEP$^{-/-}$ mice treated with capsaicin compared with NEP$^{-/-}$ animals not treated with capsaicin (Fig. 4, *a* and *b*). Animals treated with Zostrix vehicle alone demonstrated no inflammatory response (data not shown). Therefore, the elevated ACD response observed in NEP$^{-/-}$ mice can be inhibited by the neurotoxin capsaicin, thus supporting the role of cutaneous neuropeptides and an intact functional peripheral sensory nervous system in ACD.

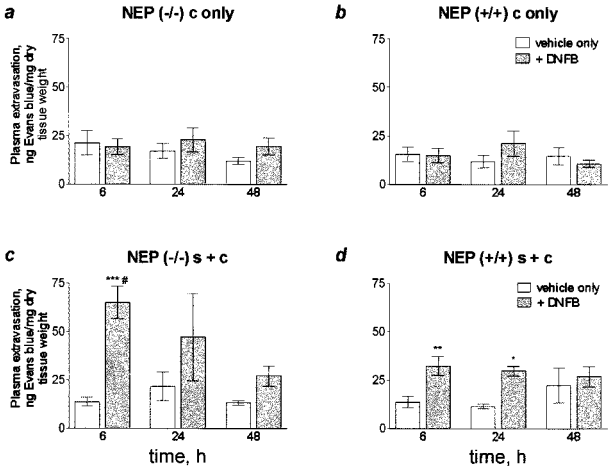

FIGURE 3. Increased plasma extravasation in ears of NEP$^{-/-}$ mice. NEP$^{-/-}$ and NEP$^{+/+}$ mice were sensitized and challenged (s+c) on the right ear with DNFB (*c* and *d*) or challenged only (c) on the right ear (*a* and *b*). Evans blue was injected into a femoral vein at the time points indicated, mice were transcardially perfused, and quantification of Evans blue leakage in the ears was performed. All extravasation values are expressed as mean ± SEM, $n = 8$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ comparing basal levels of NEP$^{-/-}$ or NEP$^{+/+}$ mice; #, $p < 0.05$ for NEP$^{-/-}$ mice compared with NEP$^{+/+}$ mice.

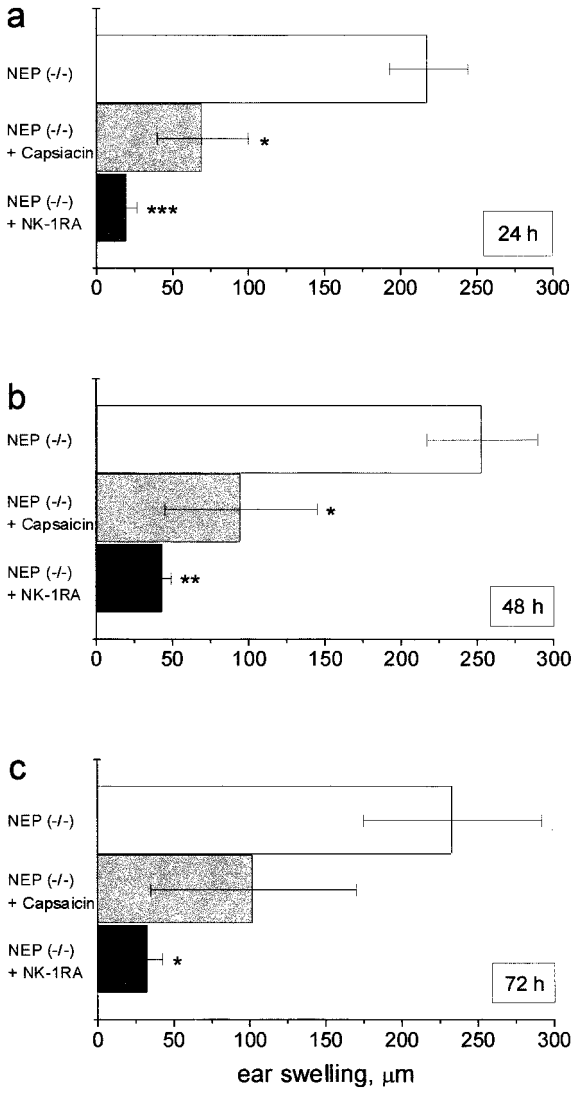

FIGURE 4. Effect of topical capsaicin and systemically delivered NK1R antagonist (SR 140333) on ACD in NEP$^{-/-}$ mice. Ear swelling responses were measured in NEP$^{-/-}$ mice sensitized with 0.5% DNFB with or without epicutaneous treatment with Zostrix HP cream on the right ear (0.075% capsaicin) twice a day over a period of 3 days before challenge and at 15 min, and 7, 14, and 24 h after challenge. Other groups of animals sensitized with DNFB were treated systemically with the NK1R antagonist SR 140333 (i.v., 1.2 μmol/kg) at 2 and 6 h after Ag challenge. The ACD response was determined by the degree of ear swelling of the hapten-exposed ear compared with that of the vehicle-treated contralateral ear before DNFB challenge and at 24, 48, and 72 h after challenge (*a–c*). All ear swelling values are represented as mean ± SEM, $n = 4$–5. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ for Zostrix- or SR 140333-treated NEP$^{-/-}$ mice vs untreated NEP$^{-/-}$ mice.

To determine whether the augmented ACD response in NEP$^{-/-}$ mice was dependent on SP interaction with its high affinity receptor, NK1R, we treated NEP$^{-/-}$ animals with the specific NK1R antagonist SR140333. Intravenous injection of SR140333 at 2 and 6 h after DNFB challenge significantly inhibited cutaneous inflammation at 24-, 48-, and 72-h time points (Fig. 4, *a* and *b*). These results indicate that the augmented ACD response in NEP$^{-/-}$ mice is dependent on SP activation of NK1R.

To determine whether acute inhibition of NEP in NEP$^{+/+}$ mice also increased the ACD response, as in the NEP$^{-/-}$ animals, we treated NEP$^{+/+}$ mice with the selective NEP inhibitor phosphoramidon. Intravenous administration of phosphoramidon at 1, 4, and 8 h after DNFB challenge of NEP$^{+/+}$ mice caused a marked increase in the ACD response in a similar fashion as we previously observed in the NEP$^{-/-}$ animals (Fig. 5). Phosphoramidon did not effect cutaneous inflammation in nonsensitized

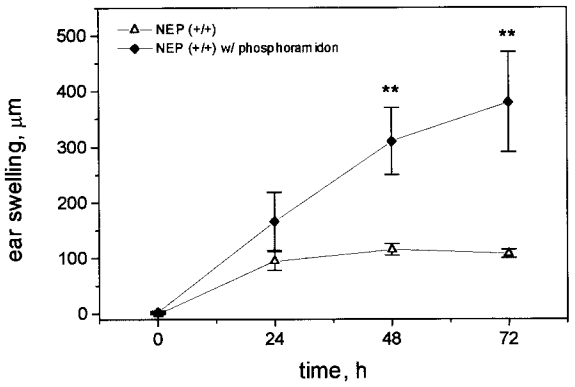

FIGURE 5. Effect of systemically delivered NEP inhibitor phosphoramidon on ACD in $NEP^{+/+}$ mice. $NEP^{+/+}$ mice were sensitized with DNFB on day 0 and challenged with DNFB on the right ear on day 5. Animals were treated with systemic phosphoramidon 1, 4, and 8 h after Ag challenge (i.v., 2.5 mg/kg). The ACD response was determined by the degree of ear swelling of the hapten-exposed ear compared with that of the vehicle-treated contralateral ear before DNFB challenge and at 24, 48, and 72 h after challenge. All ear swelling values are represented as mean ± SEM, $n = 5$. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ for phosphoramidon-treated $NEP^{+/+}$ mice vs untreated $NEP^{+/+}$ mice.

animals (data not shown). Thus, the acute inhibition of NEP in wild-type animals results in augmented expression of ACD responses, which further supports the role of this SP-degrading enzyme in Ag-mediated cutaneous inflammation.

Altered cutaneous histologic changes during ACD in $NEP^{-/-}$ mice

We further characterized the effector phase of ACD in $NEP^{-/-}$ and $NEP^{+/+}$ mice by cutaneous histological examination. No significant histological differences were observed in the skin of $NEP^{-/-}$ and $NEP^{+/+}$ animals treated with vehicle only (Fig. 6, *A* and *E*). Likewise, no differences in tissue histology were observed between DNFB-challenged ears of nonsensitized $NEP^{-/-}$ and $NEP^{+/+}$ mice (data not shown). In sensitized $NEP^{-/-}$ mice, we observed significant dermal edema, a massive inflammatory infiltrate consisting predominantly of neutrophils and mononuclear cells, and epidermal hyperplasia 16 and 72 h after Ag challenge (Fig. 6, *B* and *C*). Notably, edema, cellular infiltrate, and epidermal hyperplasia were clearly less prominent at these time points after Ag challenge in sensitized $NEP^{+/+}$ mice (Fig. 6, *F* and *G*). Treatment of sensitized $NEP^{-/-}$ mice with an NK1R antagonist after DNFB challenge resulted in a dramatic reduction in cutaneous edema, leukocyte infiltration, and hyperplasia (Fig. 6*D*) so that the histology of these ears was comparable to that of the control ears (Fig. 6*A*), whereas the systemic treatment of sensitized $NEP^{+/+}$ mice with the NEP inhibitor phosphoramidon after Ag challenge (Fig. 6*H*) markedly augmented the histologic parameters of the ACD inflammatory response compared with untreated animals (Fig. 6*E*). These results indicate that NEP, SP, and NK1R play a significant role in modulating the histologic inflammatory changes at sites of Ag challenge during the effector phase of ACD.

Similar ICD responses in $NEP^{-/-}$ and $NEP^{+/+}$ mice

When $NEP^{-/-}$ and $NEP^{+/+}$ mice were treated with various concentrations of the topically applied irritant croton oil (0.25, 0.8, and 1.6%), the ear swelling response was dose dependently increased in NEP knockout and wild-type animals, reaching a peak swelling 6–8 h after croton oil challenge (Fig. 7, *a–c*). However, in contrast to the results observed during ACD, the ICD response of $NEP^{-/-}$ animals to croton oil was not significantly different from that in wild-type $NEP^{+/+}$ animals (Fig. 7). Histologically, the cellular inflammatory response in ICD was also not visibly different in $NEP^{-/-}$ mice compared with $NEP^{+/+}$ animals (Fig. 8, *C* and *D*). Likewise, the ICD response to challenge with 2 or 10% SDS was not significantly different in $NEP^{-/-}$ and wild-type mice when measured from 0 to 48 h as indicated for the croton oil studies (data not shown). Thus, NEP is an important inflammatory modulator of ACD responses, but not ICD cutaneous inflammation.

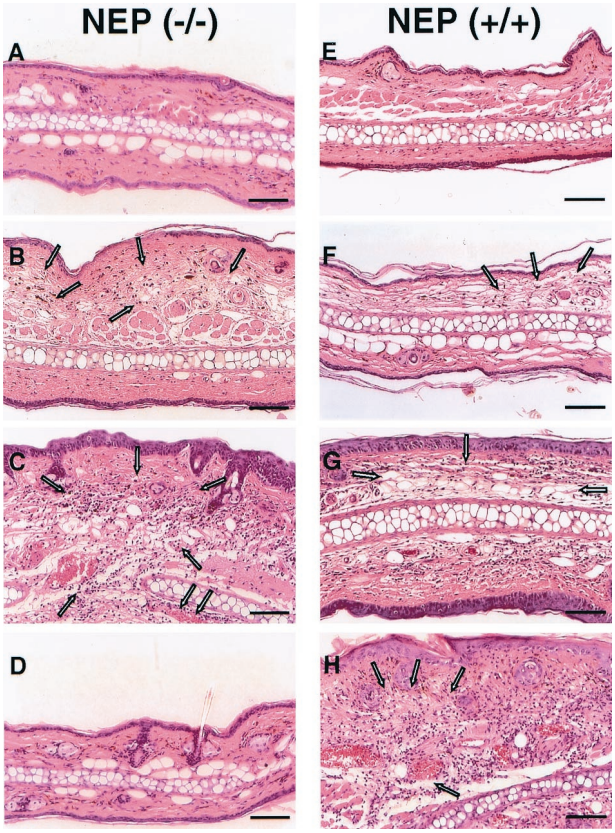

FIGURE 6. Altered histologic changes in $NEP^{-/-}$ animals during ACD. The cutaneous histologic changes in $NEP^{-/-}$ and $NEP^{+/+}$ mice was examined after DNFB exposure. Control ears of sensitized $NEP^{-/-}$ (*A*) and $NEP^{+/+}$ (*E*) mouse ears were treated with vehicle only. $NEP^{-/-}$ mice and $NEP^{+/+}$ mice were sensitized and challenged with DNFB and examined histologically at either 16 (*B* and *F*) or 72 h (*C* and *G*). Sensitized $NEP^{-/-}$ mice were challenged with DNFB and treated with the NK1R antagonist SR 140333 (1.2 μmol/kg), then ear tissue was examined at 72 h (*D*). Sensitized $NEP^{+/+}$ mice were challenged with DNFB and treated with the NEP inhibitor phosphoramidon (2.5 mg/kg), then ear tissue was examined at 72 h (*H*). Cutaneous histologic changes were examined in tissue stained with hematoxylin and eosin. Scale bars = 100 μm.

Discussion

There is increasing evidence that neuropeptides derived from sensory nerves are important mediators of inflammation in various tissues including the skin. SP, in particular, has been demonstrated to have a broad range of proinflammatory effects in vitro and in vivo by the activation of the NK1R on various immune and nonimmune cell types (5, 21). In previous studies, cutaneous neuropeptides including SP have been reported to modulate immediate and delayed-type skin hypersensitivity reactions (22–24). SP, neurokinin A, and calcitonin gene-related peptide are released in response to the epicutaneous application of the allergen oxazolone (25). SP agonists or SP antagonists are capable of augmenting (26, 27) or inhibiting (28, 29) cutaneous cellular immune responses, respectively. The epicutaneous application of SP has also been reported to increase ear-swelling responses during ACD expression in a murine model of contact dermatitis (30, 31). In contrast to SP, calcitonin gene-related peptide is capable of inhibiting cutaneous allergic responses (32, 33). None of these previous studies address the role of NEP in modulating cutaneous inflammation.

The local tissue bioactivity of SP appears to be tightly controlled by its release from sensory nerves, the presence of NK1R on target cells, and local expression of SP-degrading enzymes such as NEP

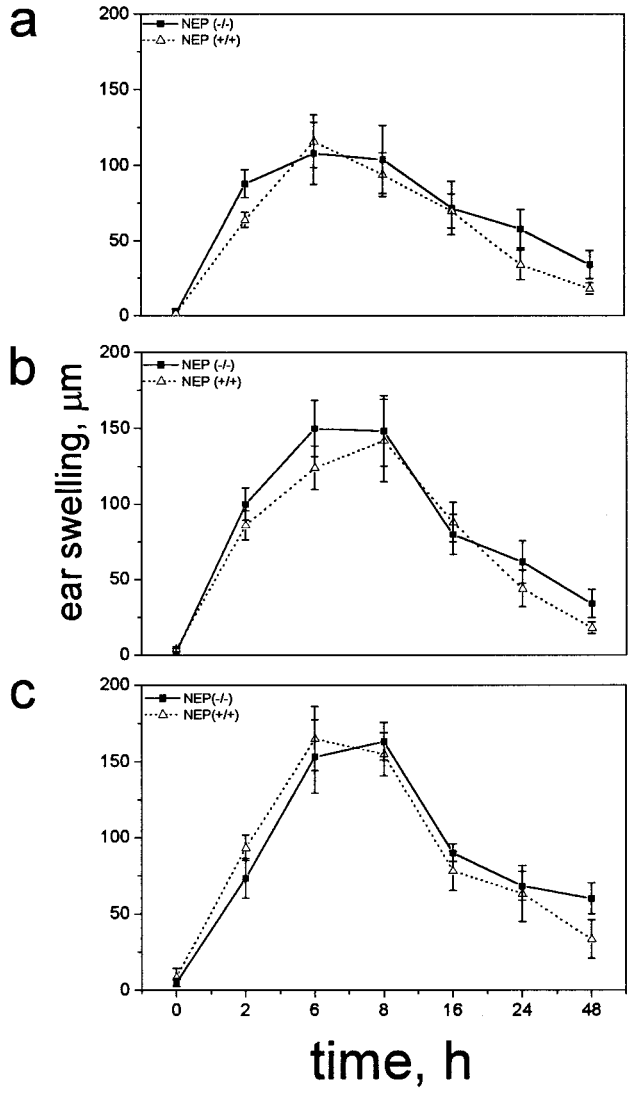

FIGURE 7. ICD responses in NEP$^{-/-}$ and NEP$^{+/+}$ mice. NEP$^{-/-}$ or NEP$^{+/+}$ mice were treated with 0.25% (*a*), 0.8% (*b*), or 1.6% croton oil (*c*) on the right ear to induce irritation. The left ear was treated with vehicle only. The ICD response was determined by the degree of ear swelling of the irritant-exposed ear compared with that of the vehicle-treated contralateral ear before croton oil challenge and at 2–48 h after challenge. All ear swelling values are given in micrometers as mean ± SEM, $n = 10$. Differences between the ear swelling responses in NEP$^{-/-}$ and wild-type mice were not statistically significant in this study.

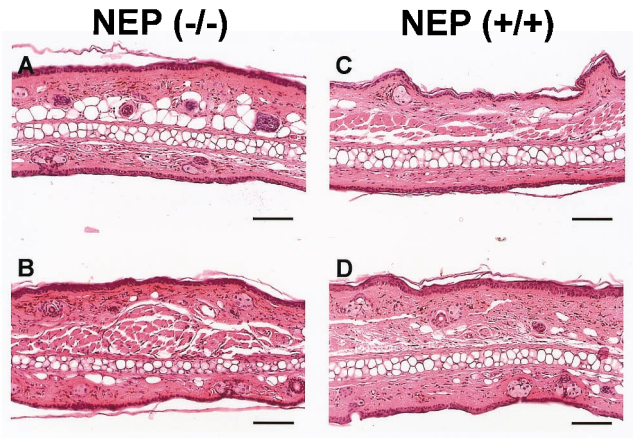

FIGURE 8. Histologic evaluation of NEP$^{-/-}$ and NEP$^{+/+}$ mouse ears during ICD. Cutaneous histologic changes were examined in NEP$^{-/-}$ and NEP$^{+/+}$ mice. Control studies used NEP$^{-/-}$ (*A*) and NEP$^{+/+}$ (*B*) mice in which ears were treated with vehicle alone. NEP$^{-/-}$ mice (*C*) and NEP$^{+/+}$ mice (*D*) were treated with 0.8% croton oil on the right ear to induce irritation. Cutaneous histologic changes were examined 24 h after croton oil treatment after staining tissue with hematoxylin and eosin as described. Scale bars = 100 μm.

(34). NEP is a potent regulator of in vivo neuropeptide biological responses (11, 35). The role of NEP in modulating systemic inflammatory response is supported by a recent study demonstrating that NEP$^{-/-}$ animals are highly sensitive to bacterial endotoxins and endotoxic shock (14). In addition to hypotension, NEP$^{-/-}$ mice demonstrated an increased basal level of plasma extravasation in various organs as compared with NEP$^{+/+}$ animals. This increased plasma leakage in NEP$^{-/-}$ mice could be abrogated by the injection of recombinant NEP, the NK1R antagonist SR140333, or a bradykinin B_2 receptor antagonist (18).

In this study, we used experimental murine models for ACD and ICD to study the relative contribution of NEP in these common types of cutaneous inflammation. Our results provide substantive evidence for a major role for SP, the NK1R, and NEP in ACD. ACD elicitation inflammatory responses were markedly greater in animals lacking NEP or in wild-type mice treated with a NEP inhibitor, which supports the physiologic contribution of NEP in down-regulating the neuroinflammatory component of ACD. Enhanced expression of the augmented ACD elicitation response observed in NEP$^{-/-}$ mice was prevented by depletion of SP from cutaneous sensory nerves with capsaicin or by administration of an NK1R antagonist, which demonstrates that diminished degradation of SP released from cutaneous sensory nerves in NEP$^{-/-}$ animals contributes to the efferent phase of allergic inflammatory responses in the skin. In contrast, no differences were noted in the ICD inflammatory responses of NEP$^{-/-}$ mice compared with wild-type animals. Thus, our data demonstrate that the absence of NEP causes augmented and therefore dysregulated inflammatory effector responses to allergens, but not to irritants such as croton oil or SDS in this murine model. This is a novel and somewhat unexpected observation that is in contrast to a number of previous studies that demonstrated an involvement of sensory nerves and neuropeptides such as SP in both types of inflammation (1, 24, 26, 27, 29–31, 36).

The precise mechanisms responsible for these differences between acute irritant inflammation and the hapten-specific ACD responses in mice with nonfunctional NEP is not entirely clear. There is evidence that the individual properties of the particular irritants croton oil or SDS may account for some of the ICD inflammatory responses (17, 37). Another possible explanation for the observed differences between ACD vs ICD responses is that in ACD responses, in contrast to ICD, the released cutaneous neuropeptides may directly or indirectly act on infiltrating T lymphocytes, inducing them to proliferate and secrete additional inflammatory mediators that can initiate a cascade of subsequent inflammatory events in the skin. SP is capable of directly augmenting T cell-mediated immune responses (23, 24). SP stimulates the proliferation of T lymphocytes (38), enhanced IL-2 production (39), and expression of T lymphocyte activation Ags such as IL-2 receptor α-chain (CD25) and RT1B MHC class II molecule (40). Released SP may also modulate ACD responses by acting on macrophages and APCs (41–43). For example, SP is capable of inducing IL-12 production in LPS-treated murine macrophages (44). This is of particular importance because a number of studies have

implicated IL-12 as an important mediator of cellular immunity. IL-12 is a potent costimulator for the development of Th1 cells (45). This is accomplished by IFN-γ induction in Th1 cells (46, 47) and promotion of Ag-dependent proliferation of activated T lymphocytes (48). SP is also capable of directly inducing T cell IFN-γ that is known to suppress IL-4 and the development of a Th2 cell phenotype as well as of enhancing mitogen-induced IFN-γ in human PBMC (49, 50). IL-12 has been demonstrated to act as an important mediator and adjuvant for ACD induction (51). Neutralization of IL-12 by Ab injection prevented the induction or expression of ACD, but not the ICD response elicited by croton oil in mice, suggesting that IL-12 participates in ACD rather than ICD responses (52). Thus, excessive cutaneous SP in NEP$^{-/-}$ mice may favor a distinct cytokine environment involving IL-12 and IFN-γ that promotes a Th1-mediated cellular immune response typical of ACD. These important immunologic activities could explain in part why excessive SP may promote an expression of ACD but not an ICD response.

This study does not directly address the role of NEP on the induction of ACD, which will be the focus of ongoing studies in our laboratory. Nonetheless, it is noteworthy that preliminary studies in our laboratory revealed that acute inhibition of the angiotensin-converting enzyme (ACE), another peptidase that is capable of degrading SP and the SP-inducing bradykinin, not only enhances the ACD response to DNFB in mice when systemically applied before hapten challenge (elicitation), but also when applied before sensitization (53). Thus, the basis of the exaggerated ACD response observed in mice with inhibited or functionally deleted SP-degrading peptidases is active during the initial contact with the hapten (afferent phase), which strongly suggests the possibility that increased SP is capable of boosting both the sensitization and elicitation phase of ACD. In ACD induction, it appears to be conceivable that SP may directly enhance functions of epidermal Langerhans cells or dermal dendritic APCs stimulating Ag presentation and/or migration into the regional lymph nodes. In favor of this hypothesis is a recent report indicating that a SP agonist promoted ACD induction and prevented or reversed hapten-specific tolerance induced by low-dose UV B radiation in vivo (26).

In summary, our data indicate that the absence or reduced activity of NEP, the primary proteolytic peptidase for SP, leads to the development of augmented inflammatory effector phase responses to allergens in a murine ACD model system, which may be due to the prolonged tissue presence of released SP in the inflamed tissue. Our observations further support the hypothesis that the expression and regulation of NEP, SP, and NK1R play an important role in inflammatory skin disease such as ACD. Thus, neuroinflammation is a significant component of the effector phase of ACD. Modulation of the neurocutaneous system may lead to novel therapeutic agents for inflammatory disease of the skin.

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