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The neuropeptide neuromedin U activates eosinophils and is involved in allergen-induced eosinophilia

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asthma models; knockout mouse; airway inflammation; cell adhesion; chemotaxis

NEUROMEDIN U (NMU) is a neuropeptide originally isolated from the spinal cord (15). The first biological activity ascribed to NMU was smooth muscle contraction of the uterus, but it is now known to reduce food intake and body weight (9), regulate circadian rhythm, and promote pronociceptive effects (18). NMU has two receptors (NMU-R1 and NMU-R2) that belong to the G protein-coupled receptor family that mobilizes intracellular Ca^{2+} stores in response to NMU binding, suggesting that they couple to members of the G_{q-11} subfamily of G proteins (3). Although NMU-R2 is localized specifically in nerve cells, NMU-R1 is abundantly expressed in various peripheral tissues, including various immune cells (8). Recently, NMU was shown to promote intracellular Ca^{2+} release and the

secretion of various cytokines in a mouse Th2 cell clone (11). Furthermore, we recently reported that NMU induces mast cell degranulation, which leads to early phase inflammation, such as vasodilation, extravasation, and neutrophil infiltration in inflamed sites (17), suggesting a role of NMU in immunoregulation.

A number of neuropeptides, such as substance P (SP) and neuropeptide Y (NPY), are expressed in central and peripheral nerve cells and are known to directly activate mast cells, triggering neurogenic inflammation and promoting further anaphylactic responses (5, 19, 23). Moreover, neuropeptides such as SP, NPY, calcitonin gene-related peptide (CGRP), and somatostatin (SOM) are known to regulate T cell adhesion to fibronectin via activation of β_1 -integrin (14). These data suggest the involvement of neuropeptides in inflammation. It has also been reported that airway hyperresponsiveness (AHR) is attenuated significantly in CGRP knockout (KO) mice in an antigen-induced asthma model (1). In this study, we hypothesize that NMU might also have pathophysiological roles in chronic airway inflammation, including airway eosinophilia. Because NMU activates Th2 cells in vitro (11) and mast cells in vivo and in vitro (17), the involvement of NMU in Th2-type diseases like AHR has been suggested. Using NMU-deficient mice and eosinophils, we demonstrate that NMU plays an important role in eosinophil infiltration rather than Th2 functions in vivo.

MATERIALS AND METHODS

Experimental animals. Mice deficient in the NMU gene (NMU-KO) were generated by gene targeting, as described previously (7). NMU-KO mice were backcrossed more than 10 times into a C57BL/6J background and compared with wild-type (WT) C57BL/6J mice purchased from Japan CLEA (Tokyo, Japan). All animal procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

Sensitization and challenge. Mice (10–12 wk old) were sensitized with intraperitoneal injections of 20 μg ovalbumin (OVA; grade V; Sigma Aldrich) plus 2.25 mg aluminium hydroxide (Pierce Chemical) on days 1 and 14. On days 26–28, mice underwent aerosol challenge with saline or 1% OVA for 20 min/day.

Measurement of eosinophil infiltration and cytokine release in bronchoalveolar lavage fluid and serum IgE. Total and differential bronchoalveolar lavage (BAL) cell counts were performed as described previously (13). Mouse IL-5, IL-13, and eotaxin were quan-

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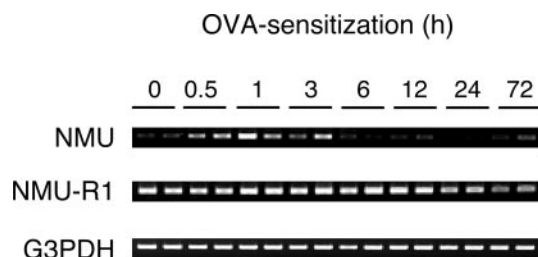


Fig. 1. Time course of the changes in neuromedin U (NMU) and NMU-receptor (R) 1 expression after ovalbumin (OVA) sensitization and challenge. Two different mice were examined at each point. G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

tified using ELISA kits (Biosource International), and serum levels of total IgE were analyzed by ELISA with rat antibody (Ab) to mouse IgE (Serotec).

Immunohistochemistry. Lungs of the mice were immersed in 4% paraformaldehyde overnight at 4°C and then tissues were embedded in paraffin. The sections were cut 5 µm by microtome and put on the Matsunami adhesive slide-coated slide (Matsunami, Osaka, Japan). The sections were stained with toluidine blue (pH 7.0; Muto, Tokyo, Japan). After photos were taken, sections were washed with 70% ethanol to be achromatized. Using the same sections, we performed immunohistochemical staining of NMU-R1 with a VECTASTAIN ABC-AP kit (Vector Laboratories). Briefly, sections were incubated with 3% normal goat serum for 1 h. After incubation in polyclonal rabbit anti-NMU-R1 Ab (10 µg/ml; Alpha Diagnostic) for 16 h at 4°C, sections were incubated with biotinylated anti-rabbit IgG for 30 min. After being washed in PBS, sections were treated with VECTASTAIN ABC-AP Reagent for 30 min. Samples were visualized in a Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories). The sections were counterstained with Mayer's hematoxylin for 3 min.

RT-PCR analysis. For RT-PCR, total RNA was extracted using Trizol reagent (GIBCO-BRL). Synthesis of first-strand cDNA was performed using SuperScript II (Invitrogen Life Technologies) according to the manufacturer's instructions. The specific primers are described in online supplemental Table 1. (Supplemental data for this article may be found at <http://ajplung.physiology.org/cgi/content/full/00345.2005/DC1>.)

Cell differentiation and culture. For in vitro T cell differentiation, CD4⁺ T cells (1 × 10⁶ cells/ml) purified from splenocytes using MACS columns (Miltenyi Biotechnology) were cultured in RPMI 1640 and stimulated with plate-bound anti-mCD3ε Ab, anti-mCD28 Ab (10 µg/ml), and mIL-2 (1 ng/ml; Peprotech) in the presence of anti-interferon (IFN)-γ Ab (10 µg/ml), anti-IL-12 Ab (10 µg/ml), and anti-IL-4 Ab (10 µg/ml) for Th0, mIL-12 (10 ng/ml), and anti-IL-4 Ab (10 µg/ml) for Th1 or mIL-4 (10 ng/ml), anti-IFN-γ Ab (10 µg/ml), and anti-IL-12 Ab (10 µg/ml) for Th2. Cells were collected after 7 days, and then mRNA was extracted. Expression levels of IFN-γ, IL-4, GATA-3, and T-bet were detected by RT-PCR as described above. Bone marrow-derived mast cells (BMMCs) were obtained from bone marrow cells cultured in RPMI 1640 supplemented with 5 ng/ml murine IL-3 (Peprotech), 8% FCS, nonessential amino acids (GIBCO-BRL), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10 µM 2-mercaptoethanol for 4–6 wk, as described previously (20).

The IL-5-dependent murine cell line Y-16 was cultured in RPMI 1640 medium containing 5% FBS, 50 µM 2-mercaptoethanol, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 5 U/ml IL-5, as described previously (10).

Isolation of human peripheral blood eosinophils. Eosinophils were purified from the peripheral blood of individuals mildly allergic to pollen. Written informed consent was obtained from all

volunteers according to the guidelines established by the Kurume University Human Experimentation Committee. Briefly, venous blood was collected in syringes containing EDTA-Na. The isolation of peripheral blood eosinophils was performed using an eosinophil isolation kit (Chemicon) according to the manufacturer's instructions.

Ca²⁺ mobilization assay. The Ca²⁺ mobilization assay was performed as described previously (6). Y-16 cells were seeded in black-walled, clear-base, 96-well tissue culture plates (Costar) at 3 × 10⁵ cells/well. The cells were then incubated at 37°C for 1 h in HEPES-buffered Hanks' balanced salt solution (pH 7.4) containing 2.5 mM probenecid and 4 µM fluo 4-AM (Molecular Probes). The cells were washed four times in solution without fluo 4-AM, and then changes in intracellular Ca²⁺ concentrations were measured with FLEXstation (Molecular Devices) before and after the addition of samples.

Immunocytochemical analysis. Y-16 cells were stimulated with 50 IU/ml IL-5 or indicated concentrations of eotaxin or NMU for indicated periods. Cell extracts were immunoblotted with anti-extracellular/signal-regulated kinase (ERK) 1/2 and anti-phospho-ERK1/2 (Cell Signaling), as described previously (22).

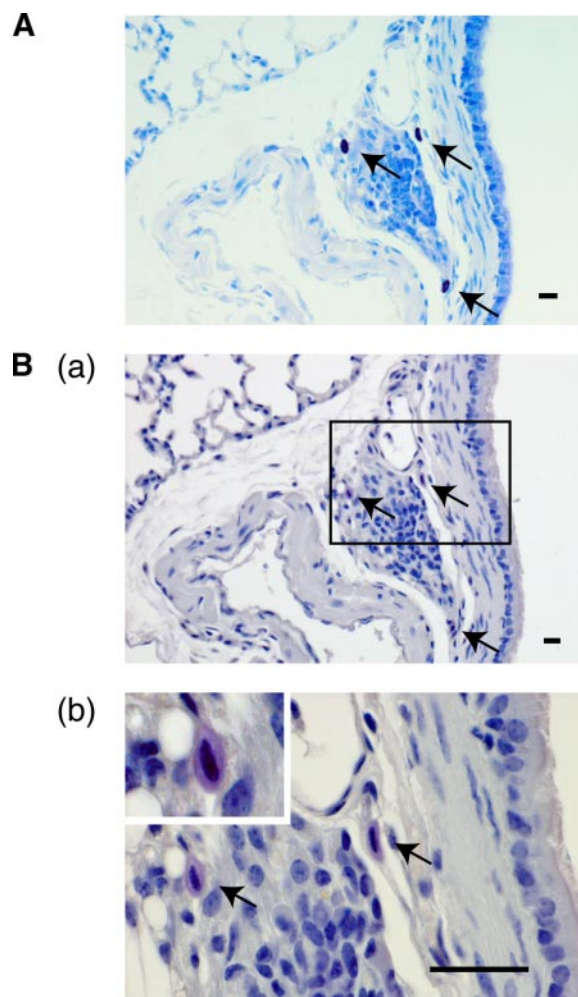


Fig. 2. Photomicrographs of NMU-R1 immunohistochemical staining in the lungs of nonsensitized wild-type (WT) mice. A: localization of mast cells stained with toluidine blue. B: mast cells (arrows) were immunostained with anti-NMU-R1 antibody. Bb is a magnified view of the rectangular region in Ba. Scale bars, 100 µm.

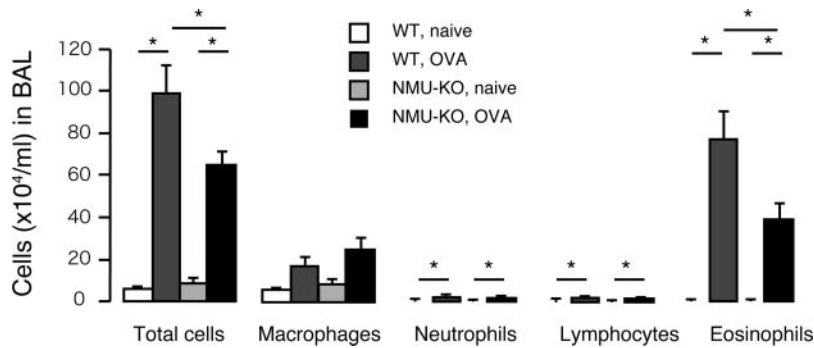


Fig. 3. Effects of NMU depletion on cell counts in bronchoalveolar lavage (BAL) fluid after sensitization and aerosol challenge with 1% OVA ($n = 9-10$ for each group). $*P < 0.05$.

Cell adhesion assay. Y-16 cells (2×10^6 cells/well) or human peripheral blood eosinophils (2×10^4 cells/well) were labeled with PKH26 dye (Sigma) according to the manufacturer's instructions. Cells were then incubated in the presence of NMU or eotaxin in fibronectin- or collagen type 1-coated 24-well plates for 2 h at 37°C. Nonadherent cells were removed by washing with PBS three times, and fluorescence of adherent cells was measured after washing with fluoroscan (Fluoroskan Ascent FL; Thermoelectron).

Chemotaxis assay. A chemotaxis assay was performed in a 96-well disposal chemotaxis plate (5 μ m pore size; Neuro Probe). Briefly, NMU or eotaxin was diluted in RPMI 1640 with 0.1% BSA and placed in the bottom wells (27 μ l). Next, 25 μ l of cell suspension (2×10^6 cells) was placed on the top wells of the chamber, which were separated from the bottom wells by a polycarbonate filter. The plate was incubated for 60 min at 37°C in a humidified incubator with 5% CO₂. The cells remaining on top of the filter were absorbed off, the filter tops were washed carefully, and the plate was centrifuged to

pellet all cells on the underside of the filter. The filter was then removed, and cells in the bottom wells were counted by light microscopy. Data are shown as a migration index (the no. of cells migrating to the chemoattractant/no. of cells migrating to the vehicle).

Statistics. All data are presented as means $\pm$ SE. Differences among groups were analyzed using one-way ANOVA together with post hoc Bonferroni analysis. $P < 0.05$ was considered significant.

RESULTS

Decreased eosinophil infiltration in an OVA-induced asthma model in NMU-KO mice. Because NMU-R1 is highly expressed in the lungs (17 and Fig. 1), we first confirmed the localization of NMU-R1 in the lungs of mice. In lungs of nonsensitized WT mice, NMU-R1 was expressed specifically

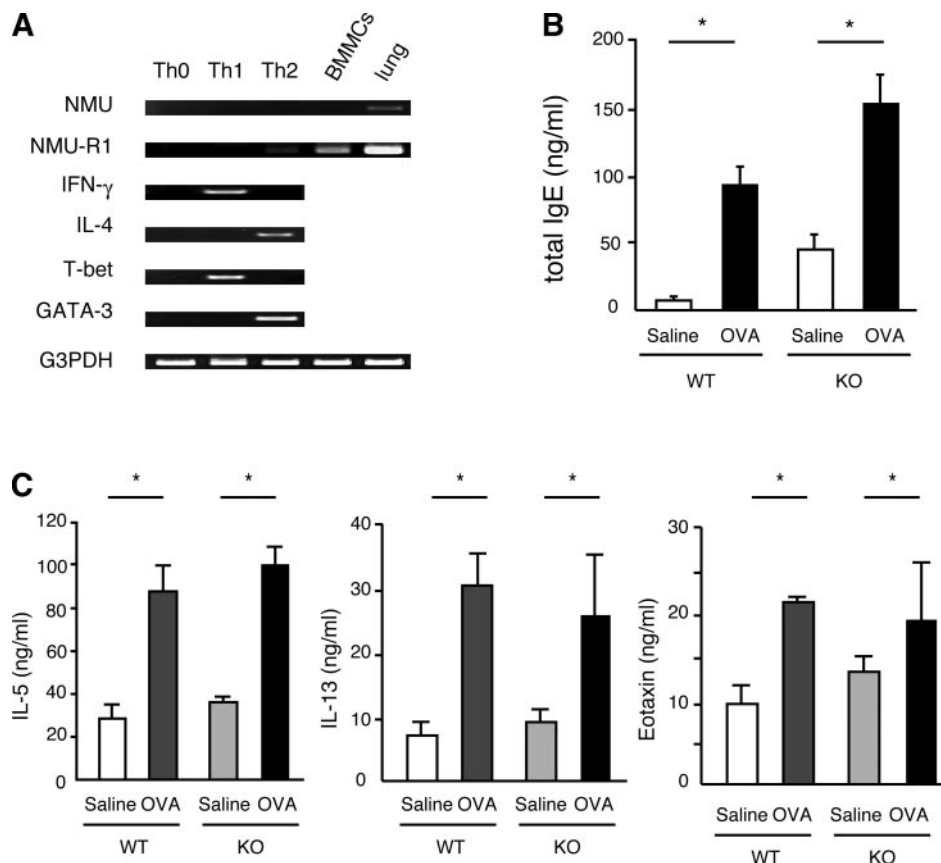


Fig. 4. Th2 cell- and B cell-mediated responses in WT and NMU-knockout (KO) mice. A: expression of NMU and NMU-R1 mRNA in Th0, Th1, and Th2 cells and lungs derived from WT mice as determined by RT-PCR. B: serum concentrations of total IgE in WT and NMU-KO mice ($n = 9-10$ for each group). C: levels of IL-5, IL-13, and eotaxin in BAL fluid in our OVA-induced asthma model ($n = 5$ for each group). BMMCs, bone marrow-derived mast cells; IFN- γ , interferon- γ . Data are means $\pm$ SE. $*P < 0.05$.

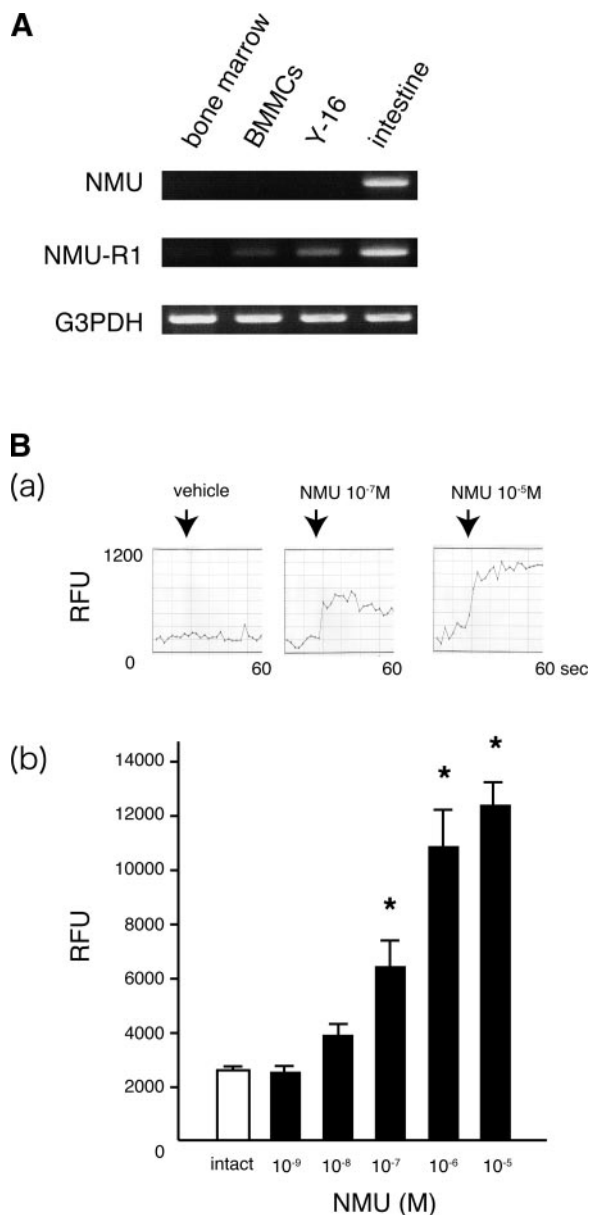


Fig. 5. Expression of NMU-R1 and the effects of NMU on Ca^{2+} mobilization in Y-16 cells. **A**: levels of NMU and NMU-R1 mRNA in Y-16 cells and indicated organs as determined by RT-PCR. **B**: intracellular Ca^{2+} mobilization after stimulation of Y-16 cells with NMU. **Ba**: representative time course of intracellular Ca^{2+} mobilization in fluo 4-loaded Y-16 cells. **Bb**: summary of maximum intracellular Ca^{2+} levels after stimulation with the indicated concentrations of NMU ($n = 3$ for each point). RFU, relative fluorescence units. $*P < 0.05$.

in mast cells (Fig. 2). We did not detect such signals using normal rabbit serum (data not shown).

Next, we investigated the role of NMU in bronchial asthma using an OVA-induced asthma model. NMU mRNA levels at 0.5–3 h after OVA sensitization were upregulated in the lungs of WT mice (Fig. 1). On the other hand, OVA sensitization and challenge resulted in an increased number of eosinophils in the BAL fluid of WT mice. However, eosinophil infiltration in the BAL fluid after allergen exposure was significantly attenuated in NMU-KO mice compared with WT mice ($P < 0.05$),

although there was no significant difference in the number of lymphocytes and neutrophils (Fig. 3).

NMU involvement in Th2 responses in the airway inflammation model. NMU-R1 mRNA levels in Th2 cells differentiated from the spleen of WT mice in vitro were much lower than those in BMMCs (Fig. 4A). Serum IgE levels in NMU-KO mice after OVA sensitization were not significantly different or were even higher than those in WT mice (Fig. 4B). Furthermore, there was no difference in levels of Th2-derived cytokines such as IL-5, IL-13, and eotaxin in BAL fluid after OVA challenge between WT and KO mice (Fig. 4C).

Direct NMU induction of Ca^{2+} elevation and ERK activation in eosinophilic cells. Next, we examined the direct effects of NMU on eosinophils using an IL-5-dependent cell line, Y-16 cells (10). Y-16 cells were isolated from bone marrow cells in the presence of IL-5 and characterized as an immature B cell line. However, we found that Y-16 cells responded to eotaxin (see Fig. 7) and had a property similar to eosinophils. We first confirmed the expression of NMU-R1 in Y-16 cells. Levels of NMU-R1 expression in Y-16 cells were comparable to those in BMMCs (Fig. 5A). As shown in Fig. 5B, NMU stimulation of Y-16 cells resulted in a dose-dependent increase in intracellular Ca^{2+} ($P < 0.05$). We also examined the activation of ERK1/2 with NMU in Y-16 cells. Western blotting with anti-phospho-ERK1/2 revealed weak but significant phosphorylation of ERK2 with 10^{-5} M NMU, reaching maximum levels at 5 min and returning to baseline levels by 15 min (Fig. 6). Levels of ERK2 phosphorylation at 5 min with 10^{-5} M NMU were higher than those with 10 ng/ml eotaxin at 5 min.

NMU induction of eosinophil adhesion to ECM components and promotion of chemotaxis. Next, we investigated whether NMU induces eosinophil adhesion to the components of the ECM (fibronectin and collagen type I) using Y-16 cells. NMU induced marked levels of eosinophil adhesion to fibronectin and to collagen type I, with a maximum level of 10^{-7} M NMU ($P < 0.05$). The adhesive effect of eosinophils to fibronectin induced by NMU was much higher than that induced by eotaxin (Fig. 7). An eosinophil chemotaxis assay was also performed using Boyden microchambers. Migration of eosinophils with NMU was observed only at 10^{-4} M ($P < 0.05$) and was almost comparable to that with 10 or 100 ng/ml eotaxin (Fig. 8). We also examined whether NMU activates human primary eosinophils. NMU-R1 was expressed in human peripheral blood eosinophils, with the level of NMU-R1 expression in eosinophils being higher than in total peripheral blood cells (Fig. 9A). Furthermore, NMU induced human eosinophil

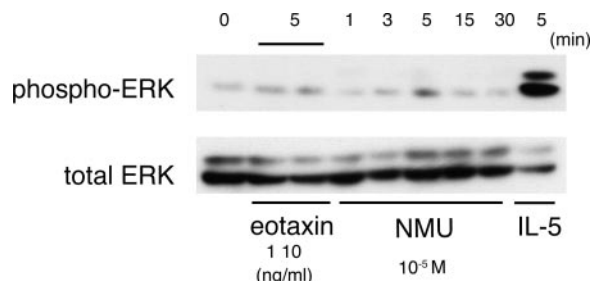


Fig. 6. Effects of NMU on extracellular/signal-regulated kinase (ERK) activation in Y-16 cells.

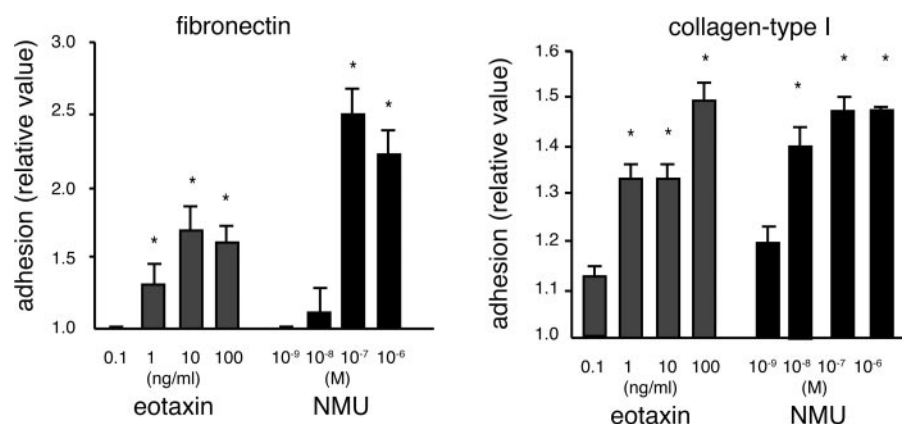


Fig. 7. Induction of eosinophil adhesion to fibronectin and collagen type I with NMU. Each bar is expressed relative to the control ($n = 3$ at each point). * $P < 0.05$.

cell adhesion to fibronectin and collagen type I-like mouse Y-16 cells ($P < 0.05$; Fig. 9B).

DISCUSSION

Our present study demonstrated that NMU-KO mice exhibited reduced eosinophilia in an antigen-mediated asthma model, suggesting that NMU exacerbates airway eosinophilia in bronchial asthma. Although AHR, as well as eosinophilia, is a major characteristic of bronchial asthma, AHR to ACh aerosol was not affected by disruption of the NMU gene (data not shown). Another neuropeptide, CGRP, is also known to be involved in the pathological condition of asthma (1). However, in CGRP-KO mice, antigen-induced AHR was remarkably reduced, whereas eosinophil infiltration was not different from that in WT mice (1). This might be because CGRP mainly stimulates smooth muscle cells or epithelial cells of the airway, whereas NMU mainly activates eosinophils. The expression of CGRP-R and NMU-R must therefore be defined to evaluate the functional differences between CGRP and NMU. Previously, NMU-R1 has been shown to be expressed in intestinal epithelial cells (9). However, our present immunohistochemical study demonstrated that NMU-R1 is highly expressed in mast cells, rather than epithelial cells, in the lungs of nonsensitized mice (Fig. 2). This result is consistent with our recent report that NMU-R1 is expressed in mast cells and that NMU promotes mast cell-mediated inflammation (17). Because the onset of OVA-induced airway inflammation is partly dependent on mast cells activated by IgE, which is produced by B cells with the help of Th2 cells, the activation of mast cells by NMU at an early stage might exacerbate the airway inflammation and contribute to the accumulation of activated eosinophils during allergen exposure, resulting in progressive inflammatory tissue damage.

We found that NMU mRNA levels in the lungs of WT mice were upregulated after the antigen sensitization. Our previous study revealed that NMU, like other neuropeptides such as SP and CGRP, is synthesized by sensory C-fibers (16), suggesting the possibility that NMU might also be released from sensory C-fibers in the respiratory tree. However, the upregulation of NMU mRNA in the lungs after antigen sensitization suggests that there is an alternative source of NMU. Because NMU has been shown to be expressed in various antigen-presenting cells, including monocytes, dendritic cells, and B cells, antigen

sensitization might induce the production of NMU from infiltrated inflammatory cells.

In this allergy model, the cytokine production of Th2 cells plays essential roles in regulating airway eosinophilia, AHR, and serum IgE levels (24). Recently, NMU-R1 expression was shown in a mouse Th2 cell clone, and NMU was shown to stimulate Th2 type cytokine production (11). However, relatively increased IgE levels were observed after antigen challenge in NMU-KO mice compared with WT mice. Furthermore, there were no differences in levels of Th2-derived inflammatory mediators such as IL-5, IL-13, and eotaxin between WT and NMU-KO mice, suggesting that NMU is not involved in Th2-dependent responses induced by antigen exposure *in vivo*. This is probably because the expression levels of NMU-R in physiological Th2 cells, unlike Th2 cell lines, are not sufficiently high enough to elicit responses to NMU, as shown in Fig. 4.

We recently reported that NMU-R1 is expressed in mast cells and that NMU promotes mast cell-mediated inflammation. Our present study demonstrated that NMU-R1 is also expressed in the mouse eosinophil cell line Y-16 and that NMU induces intracellular Ca^{2+} elevation and ERK phosphorylation and activation, which trigger cell adhesion to components of the ECM and chemotaxis of eosinophils. Ca^{2+} elevation in Y-16 cells was observed with 10^{-8} M NMU, which is much lower than that required for Ca^{2+} elevation in peritoneal mast cells (17). Thus Y-16 cells were

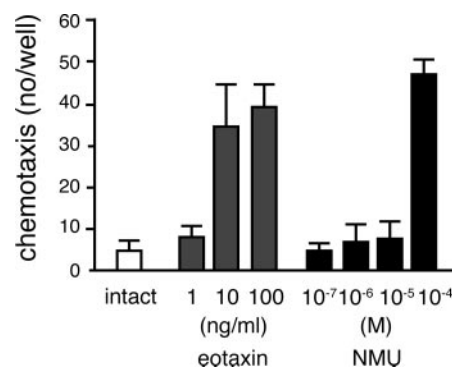


Fig. 8. Effect of NMU on eosinophil chemotaxis. Each experiment was performed in triplicate.

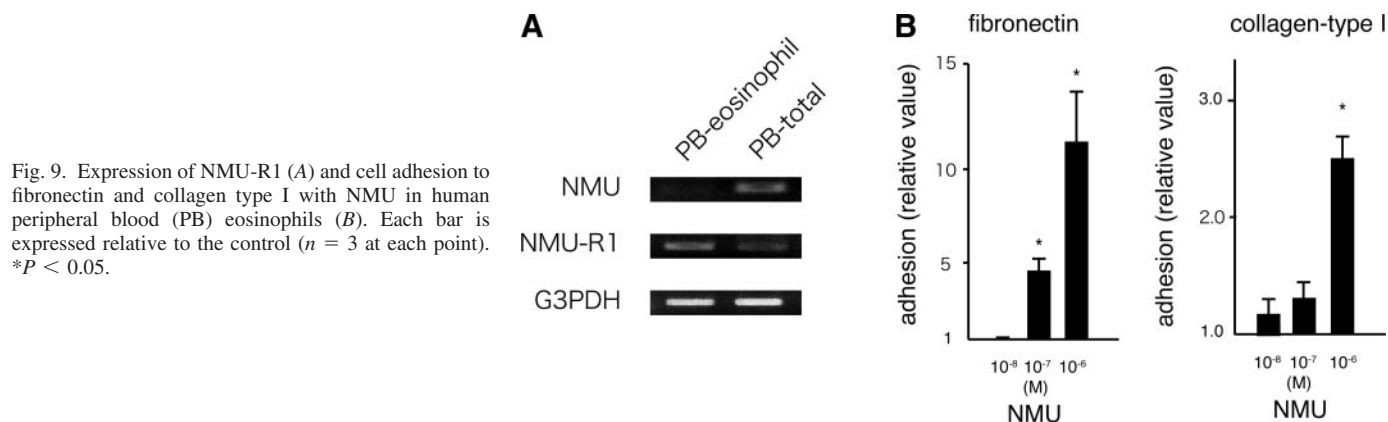


Fig. 9. Expression of NMU-R1 (A) and cell adhesion to fibronectin and collagen type I with NMU in human peripheral blood (PB) eosinophils (B). Each bar is expressed relative to the control ($n = 3$ at each point). * $P < 0.05$.

much more sensitive to NMU in terms of Ca^{2+} mobilization than mast cells.

Eotaxin is the most important chemoattractant of eosinophils. Chemokines including eotaxin exert their effects through CC chemokine receptors (CCRs), which, like NMU-R, belong to the G protein-coupled receptor family. Whereas NMU-R specifically couples to the G_{q-11} subfamily of G proteins, inducing the mobilization of intracellular Ca^{2+} and activation of protein kinase C via phospholipase C/inositol trisphosphate (IP_3) pathways, CCRs couple to a wide spectrum of G proteins (19). It has been reported that the activation of CCR-3 with eotaxin induces Ca^{2+} transients via the IP_3 pathway in eosinophilic cell lines such as acute myelogenous leukemia cells (26). CCR-3-dependent Ca^{2+} transients are inhibited by pertussis toxin, suggesting that the receptor is coupled to G_i -type G proteins.

There are at least three subtypes of the mitogen-activated protein kinase family [ERK, c-Jun/ NH_2 -regulated kinase (JNK), and p38]. CCR-3 is known to mediate phosphorylation and activation of ERK1/2 and p38, but not JNK, in eosinophils and is required for actin polymerization and rapid shape changes associated with chemotactic responses (12). Our present studies show that NMU also induces ERK phosphorylation, but phosphorylation of p38 by NMU was not detected (data not shown).

The ability of inflammatory cells to adhere to and interact with components of the blood cell wall and ECM is essential for their extravasation and migration into inflamed sites. It has been reported that the adhesion of human T cells to fibronectin, a major glycoprotein component of the ECM, is induced by neuropeptides such as CGRP, NPY, and SOM (14). In agreement with this, NMU was shown to act directly on eosinophils to induce cell adhesion to fibronectin and collagen type I. Furthermore, a high concentration of NMU also induced eosinophil chemotaxis, which is comparable to that induced with eotaxin and which is also known to be involved in integrin activation and adhesion of eosinophils (25). However, because a very high concentration of NMU is required for eosinophil chemotaxis, the function of NMU as a chemoattractant might be small or limited to local regions where NMU levels are high. Although eotaxin has been shown to be an important chemoattractant for eosinophils, it is not the sole factor for eosinophil infiltration. Eotaxin-KO mice showed reduced but evident infiltration of eosinophils (21). We therefore hy-

pothesize that NMU is an additional factor attracting eosinophils into inflammation sites through activation of integrins. However, no synergic effects of NMU and eotaxin on cell adhesion and chemotaxis were observed (data not shown), suggesting that NMU-R and CCR-3 share an intracellular signal pathway.

Our studies provide evidence that NMU plays a critical role in airway eosinophilia in this antigen-induced asthma model, without influencing Th2 responses. Furthermore, NMU, as well as eotaxin, acts directly on eosinophils, playing a critical role in cell activation, adhesion, and migration. It has also been reported that eosinophils fail to accumulate in the lungs of G_q -deficient mice after allergy challenge in the absence of G_q signaling (2). These findings suggest that the activation of G_{q-11} -coupled NMU-R might play roles in airway eosinophilia in allergen-induced asthma models.

We therefore propose that NMU might be a useful therapeutic target for bronchial asthma and eosinophil-mediated inflammatory diseases.

In conclusion, using NMU-KO mice, we first demonstrated that NMU-R1 is expressed in the lungs and that NMU plays an important role in airway eosinophilia in an antigen-induced asthma model. In the sensitized NMU-KO mice, antigen-induced eosinophilia was significantly attenuated. Antigen challenge also induced IgE production from B cells and Th2-derived cytokine production, which were not significantly different between WT and NMU-KO mice. NMU-R1 was highly expressed in the mouse eosinophil cell line and in human peripheral blood eosinophils and directly induced Ca^{2+} mobilization and ERK2 phosphorylation, which resulted in cell adhesion to the components of the ECM and chemotaxis in vitro. These findings suggest that NMU induces eosinophil infiltration in allergic inflammatory states, directly activating eosinophils and remaining unaffected by Th2 responses. We propose that NMU is an important inflammatory mediator of eosinophil-mediated inflammation.

GRANTS

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Online supplemental table

Primers for PCR

mouse NMU:	5'-ATGGAATCCCCAAGTCAGCAT-3'	(forward)
	5'-ATCTGACCTATGACCTGGCAT-3'	(reverse)
mouse NMU-R1:	5'-TCTCTTCTCTCTGCCCAACACCAGCCT-3'	(forward)
	5'-CGAATACCACAACCAGTGCAAACAGCA-3'	(reverse)
human NMU:	5'-GCTTTGCTTTATGATTATGGGAAT-3'	(forward)
	5'-ACTTCTGTGTCTTCGAATAATGAAA-3'	(reverse)
human NMU-R1:	5'-CATCATGAGCGTGCTCTACCTGCTCAT-3'	(forward)
	5'-GGTAGAAGAAGATGCCGGAGATGACGT-3'	(reverse)
G3PDH:	5'-CAGAGCAGCGTCCCT GTGAAT-3'	(forward)
	5'-GGCTTACAGTGAACCACCTT-3'	(reverse)
T-bet:	5'-TGC CTG CAG TGC TTC TAA CA-3'	(forward)
	5'-TGC CCC GCTTCC TCTCCA ACC AA-3'	(reverse)
GATA-3:	5'-GAA GGC ATC CAG ACC CGA AAC-3'	(forward)
	5'-ACC CATGGCGGTGAC CATGC-3'	(reverse)
IFN- γ :	5'-AGC GGC TGA CTGAAC TCA GATTGTAG-3'	(forward)
	5'-GTC ACA GTT TTC AGC TGT ATA GGG-3'	(reverse)
IL-4:	5'-CGA AGA ACA CCA CAG AGA GTG AGC T-3'	(forward)
	5'-GAC TCA TTC ATG GTG CAG CTT ATC G-3'	(reverse)