Original Article

Inducible nitric oxide synthase plays important roles in allergic reactions of pollinosis in mice sensitized with pollen allergy

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To elucidate the possible involvement of nitric oxide (NO) derived from inducible NO-synthase (iNOS) in the pathogenesis of patients with allergic rhinitis, we analyzed changes in the frequency of sneezing, plasma levels of NO metabolites, α-melanocyte-stimulating hormone (MSH) and immunoglobulin E and tracheal expression of IgA and mast cell tryptase in control and iNOS$^{-/-}$ mice. Eight-week-old control and iNOS$^{-/-}$ male C57BL/6j mice were sensitized with Cry j I antigen. After the last intranasal challenge of antigen, changes in the frequency of sneezing and plasma levels of IgE, α-MSH and NO metabolites and tracheal expression of iNOS, IgA and mast cell tryptase were analyzed by ELISA and immunohistochemistry using specific antibodies. The sensitization of mice with Cry j I antigen increased plasma levels of NO metabolites, α-MSH and IgE and tracheal expression of iNOS, IgA and mast cell tryptase in control not but in iNOS$^{-/-}$ mice. Administration of N^G-nitro-L-arginine methyl ester strongly inhibited all these changes occurred in control mice. These results indicate that the symptom of pollinosis including sneezing is enhanced by iNOS derived NO through activation of α-MSH-receptor containing mast cells enriched with tryptase.

Key Words: **α-melanocyte stimulating hormone, inducible nitric oxide synthase, immunoglobulin A, mast cells, pollinosis**

Nitric oxide (NO) is synthesized by three forms of NO synthases (NOS) using L-arginine as a substrate.[1] Among three isoforms of NOS isozymes, inducible NOS (iNOS) has been known to play important roles in the regulation of inflammatory reactions and bactericidal actions of granulocytes.[2] NO derived from iNOS also play important roles in the modulation of symptoms of patients with inflammatory diseases including pollinosis and rhinitis.[3]

Ciliary expression of xenobiolsis and pathogens is an important defense mechanism of the respiratory mucosa.[4] Previous studies reported that NO synthesized in the nasal mucosa was detectable in exhaled air.[5,6] Nasal generation of NO have been reported with patients with inflammatory diseases, such as asthma[7] and viral infection of the upper airways.[8] Thus, elevated levels of nasal NO have been proposed to be an indirect marker for the inflammation in the airway. Histological studies on NOS suggested the enhanced production of NO in the nasal mucosa of patients with allergic rhinitis.[9,10] Furthermore, our previous study[11] reported that α-melanocyte-stimulating hormone (α-MSH) indicates the important role to the onset of pollinosis and may have some relation to α-MSH and NO.

However, the role of NO in the nasal response to allergens in patient rhinitis with remains unknown. Recent studies developed a model mouse whose symptoms are similar to those in patients with pollinosis.[12–14] These mice show increased frequency of sneezing and marked elevation of immunoglobulin E (IgE) in plasma particularly when they were challenged with pollen allergens.

To elucidate a role of NO in the etiology of pollinosis, we analyzed changes in the number of sneezing, plasma IgE and expression of immunoglobulin A (IgA) in the trachea of mice showing symptoms of pollen allergy[13] using N^G-nitro-L-arginine methyl ester (L-NAME), an inhibition for NOS. In addition, we manufactured a pollen allergy model with iNOS knockout mice and observed influence on symptom of the pollinosis. In addition, we pay attention to α-MSH and mast cell tryptase in this study. Both α-MSH and tryptase have been reported to play important roles in the modulation of immunological reactions. α-MSH is a potent suppressor of bacterial endotoxin-mediated inflammation and the inflammatory activity of macrophages and neutrophils induced by IL-1, TNF-α and IFN-β.[15,16] Tryptase, the most abundant protein product of human mast cells is emerging as an important mediator and target for therapeutic intervention in allergic disease.[17]

Materials and Methods

Animals. Specific pathogen-free, 9-week-old male C57BL/6j mice (SLC, Hamamatsu, Japan) and their iNOS-knockout (iNOS$^{-/-}$) mice (Jackson Laboratories, BarHarbor, ME) were subjected to experiments according to the Animal Care Regulations of Osaka City University Medical School.

Sensitization and nasal antigen challenge. The experiments were curried out according to the method of Nomiya *et al.*[13] Mice were subcutaneously injected with 10 μg of Cry j I (Hayashibara, Okayama, Japan) (Two major allergens, Cry j I and Cry j II, have been isolated from *C. japonica pollen*, and an effective animal model used Cry j I has been developed.) mixed with 4 mg of aluminum hydroxide gel (Sigma, St. Louis, MO) and sensitized by repeated administration three times every other day. One week after the final sensitization to Cry j I, they were challenged intranasally with the allergen for seven consecutive days. Five days after the last challenge, the frequency of sneezing was counted for 5 min as described previously.[18]

Treatment of animals with L-NAME. Male mice were intraperitoneally injected with 20 mg/kg of L-NAME (N^G-nitro-L-arginine-methyl ester HCl, antagonist of nitric oxide syntase; Sigma, St. Louis, MO) dissolved in 0.1 M phosphate buffer (pH 7.2) 15 min before or 3 h after the 4th challenge of animals.[19] The same volume of phosphate buffer was administered to the

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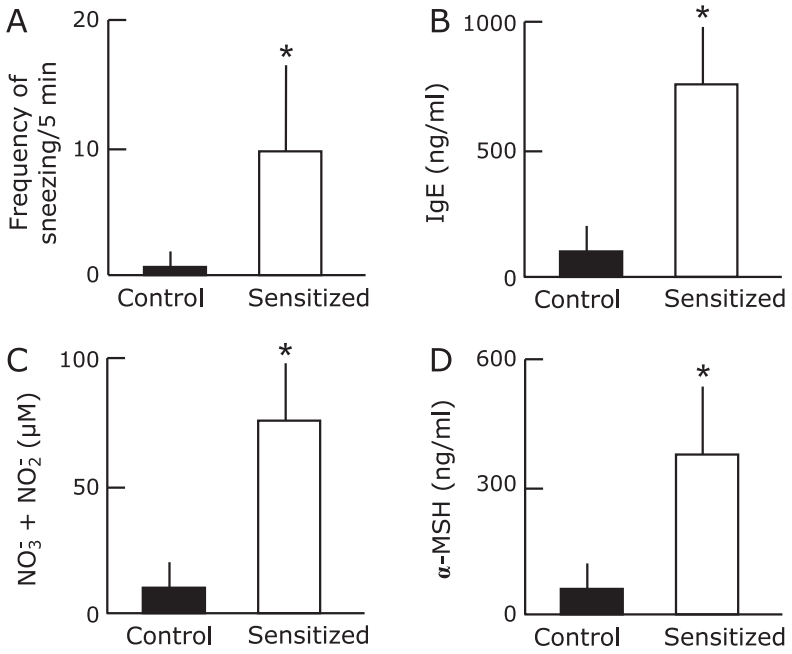

Fig. 1. The frequency of sneezing (A) and plasma levels of IgE (B), NO metabolites (NO_3^- + NO_2^-) (C) and α-MSH (D) in the control and the sensitized mice. The frequency of sneezing and plasma level of various factors are determined as described in the text. Values are presented as the mean ± SD of the values from 10 animals. $p<0.05$ in comparison to the control mice.

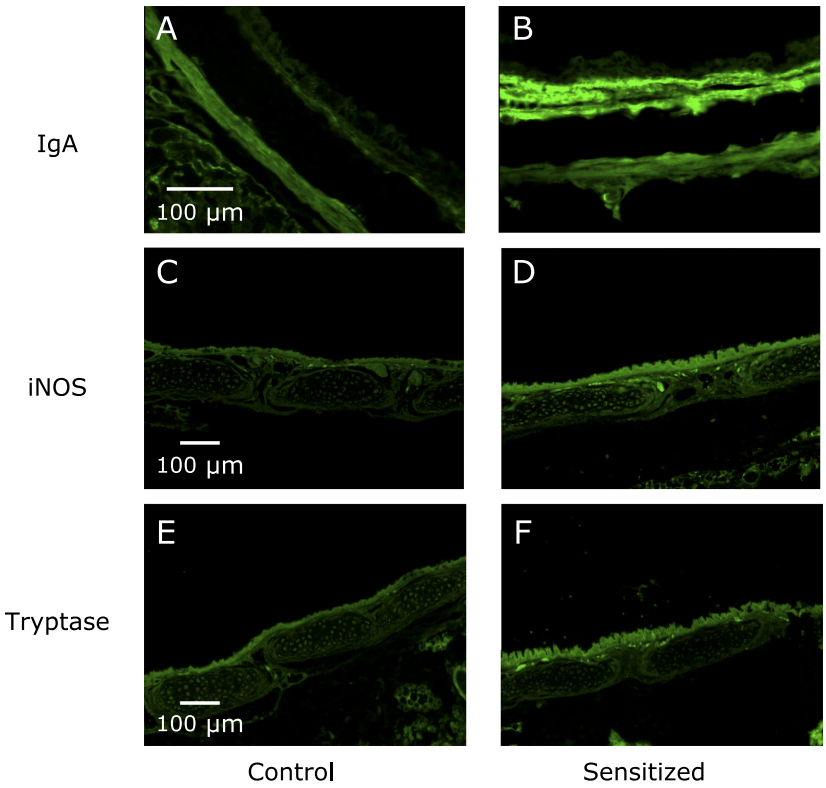

Fig. 2. IgA (A, B), iNOS (C, D) and mast cell tryptase (E, F) expression in the trachea of the control and the sensitized mice. IgA, iNOS and mast cell tryptase are significantly increased in the sensitized mice in comparison to the control mice. Histological data show one typical experiments from 10 animals. Scale bars = 100 μm.

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control group.

Preparation and staining of the trachea. Two days after the last challenge, the tracheal specimens were fixed in phosphate-buffered paraformaldehyde (4%), embedded in frozen Tissue Tek, OCT compound, and cut into 5 μm thick sections. The expression of immunoglobulin A (IgA) in tissues was evaluated immunohistochemically under a fluorescent microscope as described previously.[20] To investigate the expression of iNOS and mast cell tryptase, the sections of the trachea were washed in PBS, and subsequently incubated with rabbit anti-iNOS and goat anti-mast cell tryptase (1:100) polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C for overnight. The incubated specimens were washed in PBS and reacted incubated at room temperature for 2 h with FITC-conjugated (iNOS) swine anti-rabbit immunoglobulin and FITC-conjugated (mast cell tryptase) rabbit anti-goat immunoglobulin (1:30; Dako Cytomation, Glostrup, Denmark). The expression of iNOS and mast cell tryptase were evaluated immunohistochemically under a fluorescent microscope.

Quantification of neuronal hormones, IgE and NOx by enzyme-linked immunosorbent assay (ELISA). Blood samples were taken from the heart 5 days after the last challenge, and the plasma samples were fractionated. The plasma levels of α-MSH and IgE were determined using a commercial ELISA kits (α-MSH; Phenix Pharmaceuticals Inc., CA; IgE; YAMASA Co., Chiba, Japan). Plasma levels of nitrate + nitrite (NOx) were determined by an assay kit for NOx (DOJINDO, Kumamoto, Japan) according to the manufacturer's instructions.

Statistical analysis. All data were expressed as the mean ± SD derived from 10 animals. The results obtained from the animal groups were analyzed using either Student's *t* test or ANOVA using a computer software program. Differences were considered to be significant when $p<0.05$.

Results

Effect on Cry j I antigen on sneezing, IgE, NOx and α-MSH. The frequency of sneezing significantly increased after sensitization of mice with Cry j I antigen (Fig. 1). When sensitized with the antigen, plasma levels of NO metabolites ($NO_3^- + NO_2^-$) increased markedly. Plasma levels of α-MSH and IgE also increased significantly in Cry j I-sensitized mice.

iNOS, IgA and mast cell tryptase in the trachea in the pollen allergy model mice. Since changes observed with the sensitized mice suggested the occurrence of allergic inflammation, we observed histological changes in the trachea using specific antibodies to iNOS, IgA and mast cell tryptase. As shown in Fig. 2, expression of iNOS, IgA and mast cell tryptase in the trachea of the Cry j I-sensitized mice increased markedly. In the increase part of the expression, IgA was respiratory epithelium, lamina propria and eprichondrium, and iNOS and mast cell tryptase were lamina propria and perichondrium.

Effect of L-NAME on tracheal inflammation and related factors. The frequency of sneezing of the pollen allergy-sensitized mice was decreased significantly by administration of L-NAME, an inhibitor of NOS (Fig. 3). L-NAME also inhibited the increase in plasma levels of NO metabolites, IgE and α-MSH. Immunohistochemical observation revealed that the increased expression of IgA and mast cell tryptase in the trachea was also suggested by treated animals with L-NAME. In addition, the

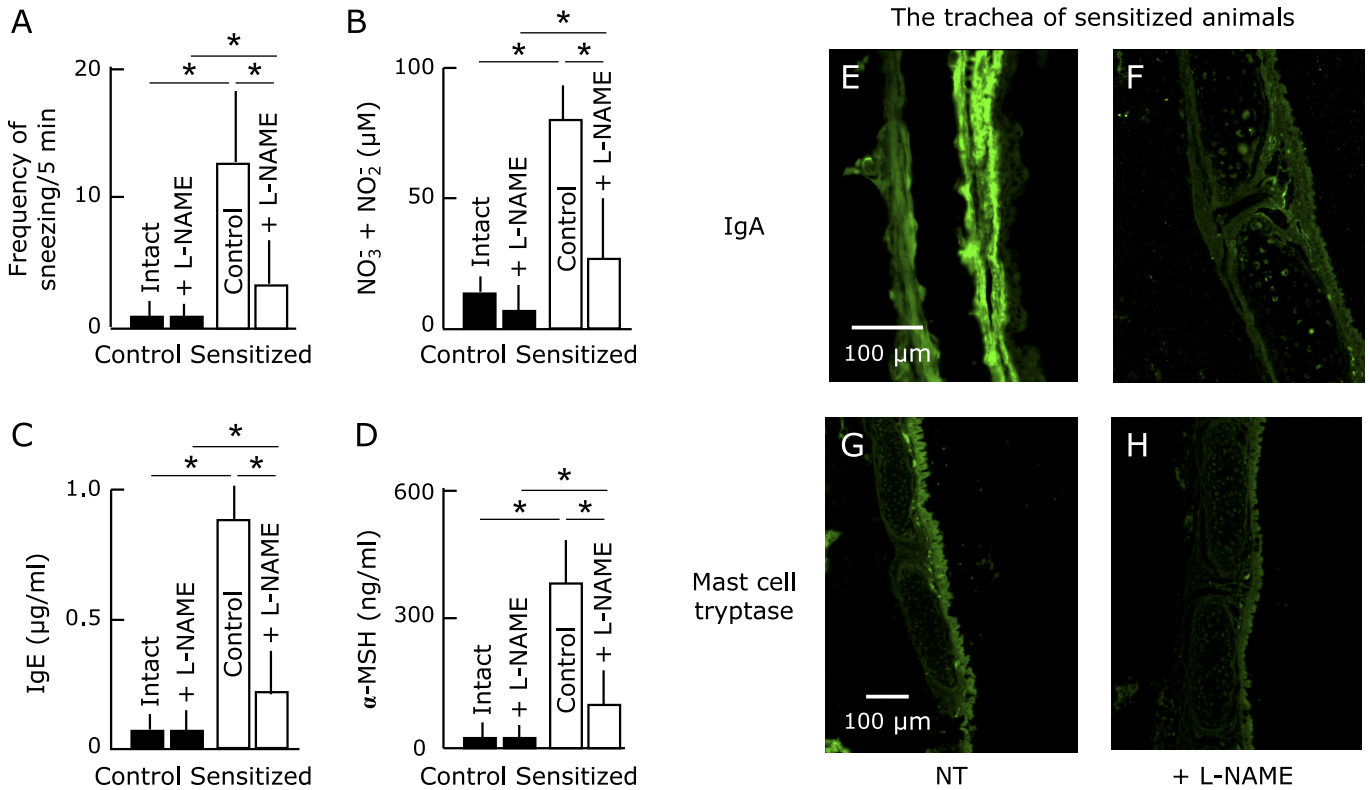

Fig. 3. The frequency of sneezing (A), plasma levels of NO metabolites ($NO_3^- + NO_2^-$) (B), IgE (C) and α-MSH (D), and expression of IgA (E, F) and mast cell tryptase (G, H) in the trachea. L-NAME was injected intraperitoneally in the sensitized mice. Values are increased in the sensitized mice in comparison to the control mice. However, injection of L-NAME reduced the increase in the frequency of sneezing, NO metabolites ($NO_3^- + NO_2^-$), IgE and α-MSH levels and expression of IgA and mast cell tryptase. Values are presented as the mean ± SD from 10 animals. *$p<0.05$. Histological data show one typical experiments from 10 animals. Scale bars = 100 μm.

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expression of iNOS is uncommon by the administration of L-NAME (data not shown).

Effect of iNOS on the changes induced by sensitization with Cry j I antigen. It was been well documented that NO derived from iNOS plays important roles in inflammatory reactions,[21,22] we analyzed changes in the symptom and inflammatory factors in control and iNOS$^{-/-}$ mice before and after the sensitization (Fig. 4). The frequency of sneezing in the sensitized animals was suppressed significantly in iNOS$^{-/-}$ mice. Plasma levels of NO metabolites, IgE and α-MSH were also low in iNOS$^{-/-}$ mice. Expression of IgA and mast cell tryptase in the trachea of the sensitized animals was also suppressed in iNOS$^{-/-}$ mice.

Discussion

Nabe *et al.*[23] indicate that mepyramine strongly inhibits the occurrence of sneezing but not that of nasal blockage. Therefore, histamine derived from activated mast cells plays a major role in sneezing. One study suggests the mast cell functions to be regulated by NO since an NOS inhibitor, N^G-mono-methyl-L-arginine, enhances the release of lipopolysaccaride-induced histamines from rat peritoneal mast cells,[24] and sodium nitroprasside, a NO donor, inhibits the immunological and non-immunological release of histamines from the rat mast cell.[25,26]

This study indicates that sneezing does not occur in iNOS$^{-/-}$ mice. The consensus prior to this study is that the decreased frequency of sneezing is caused by a decrease of α-MSH through NO of the iNOS pathway.[27] We previously reported that the reduced frequency of sneezing in mice with pollinosis was significantly suppressed by the administration of an antagonist of the α-MSH receptor and therefore hypothesized that α-MSH may thus play an important role in the modulation of this allergic inflammation. [11]

In fact, the mast cells express MC1R and MC5R which are α-MSH receptors on the cell's surface, and α-MSH regulates the release of histamine from mast cells through MC1R and MC5R.[27] In these reports, the iNOS expression was observed to increase in pollinosis, and NO of the iNOS pathway induced α-MSH expression. α-MSH stimulated the release of histamine via MC1R and MC5R which are expressed on the surface of mast cells and it is therefore a possible mechanism for increasing the occurrence of sneezing. In fact, in iNOS$^{-/-}$ mice, which were sensitized in ovalbumin (OVA), have been reported to exhibit an increase of ear swelling and level of plasma OVA-IgE after the administration of α-MSH.[28] However, the frequency of sneezing after α-MSH administration in iNOS$^{-/-}$ sensitized mice is not predictably comparable to that of the sensitized control mice (data not shown). These observations are consistent with the hypothesis that the Cry j I-sensitized reactions underlie the mechanism for NO-dependent signaling in the cellular responses to activate the hypothalamo-pituitary pro-opiomelanocortin system, thereby enhancing the secretion of α-MSH. In addition, adrenocorticotropic hormone and β-endorphin showed and increase by sensitization like α-MSH, and this increase was suppressed in iNOS$^{-/-}$ mice.[28] Therefore, further examination is necessary to explore the possible contribution of other factors.

In addition, the mast cells synthesize and release a variety of cytokines and chemokines after stimulation.[29] α-MSH controls cytokines and chemokines through MC5R. We previously reported the expression of local MC5R (in the trachea) to be related to the

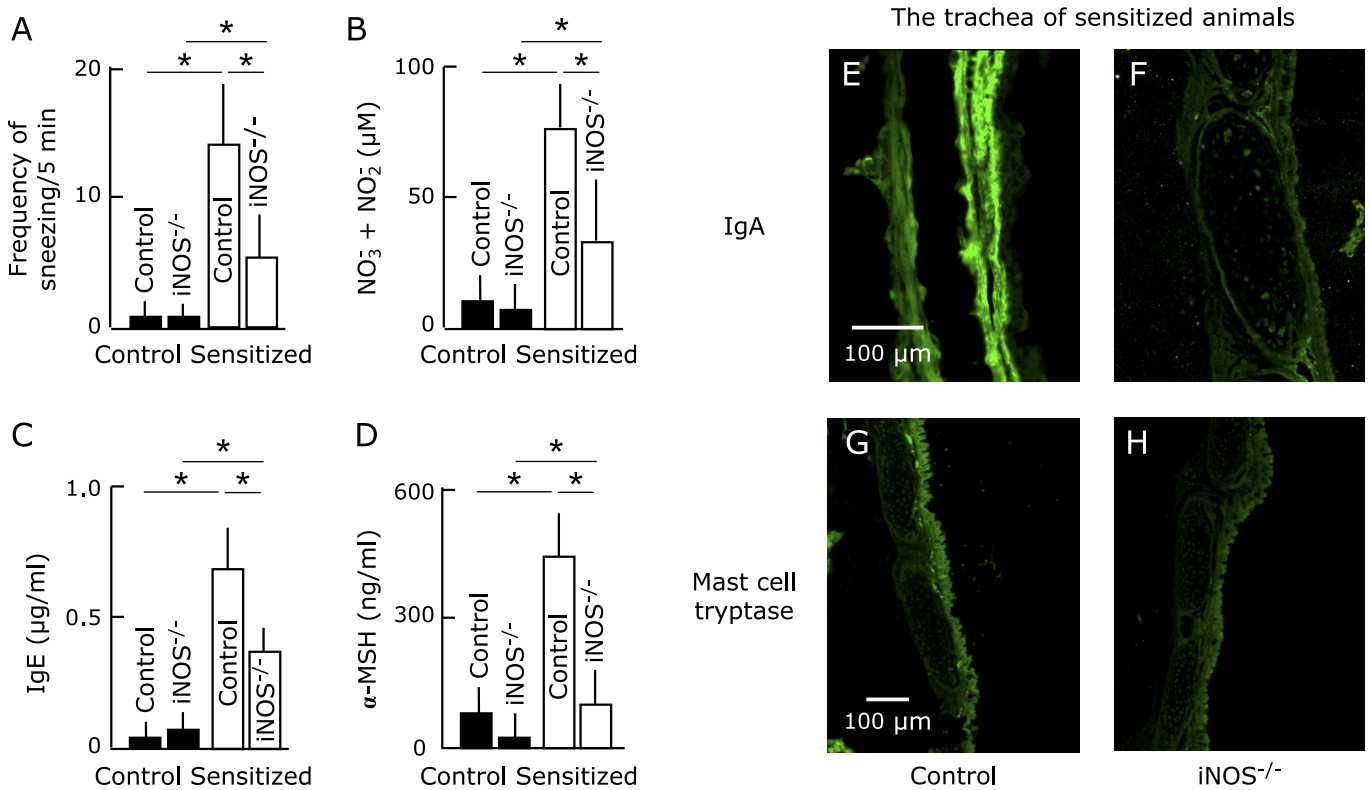

Fig. 4. The frequency of sneezing (A), plasma levels of NO metabolites (NO_3^- + NO_2^-) (B), IgE (C) and α-MSH (D), and expression of IgA (E, F) and mast cell tryptase (G, H) in the trachea. iNOS$^{-/-}$ mice were used for the sensitized mice. The values are increased in the sensitized mice in comparison to the control mice. The sensitized mice used for iNOS$^{-/-}$ mice showed are significantly decreased in comparison to the sensitized control mice. Values are the mean ± SD from 10 animals. $p<0.05$ in comparison to the control mice. Histological data show one typical experiments from 10 animals. Scale bars = 100 μm.

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increased frequency of sneezing and control of the immune system. In this regard, NO of the iNOS pathway may therefore be a regulatory factor regarding both sneezing and the immune system.

In summary, these finding suggest that α-MSH affects cytokine production and mediator release from mast cells through MC5R. Furthermore, the NO of the iNOS pathway may regulate the downstream effectors of α-MSH.

Abbreviations

α-MSH	α-melanocyte stimulating hormone
IgE	immunoglobulin E
iNOS	inducible nitric oxide synthase

Conflict of Interest

The authors have no conflict of interest.

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