# Modulation of basophil activity: A novel function of the neuropeptide α-melanocyte–stimulating hormone

Markus Böhm, MD,[a] Mara Apel,[a] Koji Sugawara, MD,[b,c] Randolf Brehler, MD,[a] Kerstin Jurk, PhD,[d] Thomas A. Luger, MD,[a] Helmut Haas, PhD,[e] Ralf Paus, MD,[b,f] Britta Eiz-Vesper, MD,[g] Andrew F. Walls, PhD,[h] Evgeni Ponimaskin, PhD,[i] Manuela Gehring,[j] Alexander Kapp, MD,[j] and Ulrike Raap, MD[j] *Münster, Lübeck, Borstel, and Hannover, Germany, Osaka, Japan, and Manchester and Southampton, United Kingdom*

**Background: Little is known about the effect of neuropeptides on basophils, which are important effector cells in immune and allergic responses.**
**Objective: This study aimed at revealing the role of α-melanocyte–stimulating hormone (α-MSH) on basophil function.**
**Methods: Expression of melanocortin receptors and proopiomelanocortin (POMC) was analyzed by means of RT-PCR, Western immunoblotting, fluorescence-activated cell sorting, and double-immunofluorescence analysis. Signal transduction studies included cyclic AMP and $Ca^{2+}$ mobilization assays. Basophil activity was assessed based on CD63 surface expression and cytokine release.**
**Results: MC-1R expression was detectable in basophils isolated from human peripheral blood, as well as in basophils within nasal tissue. In isolated basophils from human blood, truncated POMC transcripts were present, but there was no POMC protein. Treatment of basophils with α-MSH increased intracellular $Ca^{2+}$ but not cyclic AMP levels. α-MSH at physiologic doses potently suppressed basophil activation induced by N-formyl-methionyl-leucyl-phenylalanine, phorbol 12-myristate 13-acetate, or grass pollen allergen in whole blood of healthy or allergic subjects, respectively. The effect of α-MSH on basophil activation was MC-1R mediated (as shown by blockade with a peptide analogue of agouti-signaling protein) and imitated by adrenocorticotropic hormone but not elicited by the tripeptides KPV and KdPT, both of which lack the central pharmacophore of α-MSH. Moreover, α-MSH at physiologic doses significantly suppressed secretion of 3 proallergic cytokines, IL-4, IL-6, and IL-13, in basophils stimulated with anti-IgE, N-formyl-methionyl-leucyl-phenylalanine, or phorbol 12-myristate 13-acetate.**
**Conclusion: Our findings highlight a novel functional activity of α-MSH, which acts as a natural antiallergic basophil-response modifier. These findings might point to novel therapeutic strategies in treating allergic diseases. (J Allergy Clin Immunol 2012;129:1085-93.)**

***Key words:*** *Basophils, melanocyte-stimulating hormone, melanocortin receptors, neuropeptides*

Although basophils represent only a diminutive percentage of all peripheral blood leukocytes (<1%), it is now established that these cells are key effectors in immune and allergic responses.[1,2] Like mast cells, they express the high-affinity IgE receptor FcεRI on their surface and thus release histamine and leukotriene $C_4$ on engagement of receptor-bound IgE with corresponding allergens. The involvement of basophils in allergic reactions is highlighted by the presence of these cells in affected tissues of patients with allergic asthma,[3] allergic rhinitis,[4] and atopic dermatitis.[3,5]

One regulatory system of the body influencing the function of various immune cells is the neuroendocrine system. Substantial evidence has been accumulated over the last few years that neuromediators and neurohormones are not only released by neuronal and neuroendocrine cells but also by resident cell types of peripheral organs, as well as by cells of the immune system itself. These mediators are capable of modulating the immune and neuroendocrine systems in a bidirectional manner.[6-8] However, with regard to basophils, little progress has been made in our understanding of their role within the neuro-immune-endocrine axis. Previously, it was reported that the $\beta_2$-adrenergic agonist fenoterol and epinephrine suppress antigen-mediated or stimulation-dependent histamine release of basophils.[9,10] Moreover, nerve growth factor enhanced IgE-dependent histamine release and IL-13 secretion.[11]

In this report we investigated whether basophils are targets for α-melanocyte–stimulating hormone (α-MSH). α-MSH is a melanocortin peptide derived from proopiomelanocortin (POMC) and, like adrenocorticotropic hormone (ACTH) and β-endorphin, is part of the classical neuroendocrine hypothalamic-pituitary-adrenal stress axis. Although β-endorphin binds to opioid receptors, α-MSH and ACTH elicit their biological effects through melanocortin receptors (MC-Rs), which belong to the superfamily of G protein–coupled receptors

From the Departments of [a]Dermatology and [d]Anaesthesiology and Intensive Care, Experimental and Clinical Haemostasis, University of Münster; [b]the Department of Dermatology, University of Lübeck; [c]the Department of Dermatology, Osaka City University Graduate School of Medicine; [e]the Division of Cellular Allergology, Research Center Borstel; [f]the School of Translational Medicine, University of Manchester; the Departments of [g]Transfusion Medicine, [i]Cellular Neurophysiology, and [j]Dermatology and Allergy, Hannover Medical School; and [h]the Immunopharmacology Group, University of Southampton, Southampton General Hospital.

Supported by a research grant from the Innovative Medizinische Forschung (IMF) to M.B. (BÖ 110532).
Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.
Received for publication September 14, 2010; revised November 9, 2011; accepted for publication November 9, 2011.
Available online December 17, 2011.

Corresponding author: Markus Böhm, MD, Department of Dermatology, University of Münster, Von Esmarch-Str. 58, D-48149 Münster, Germany. E-mail: bohmm@uni-muenster.de.

0091-6749/$36.00

doi:10.1016/j.jaci.2011.11.012

*Abbreviations used*
ACTH: Adrenocorticotropic hormone
ASIP: Agouti-signaling protein
BAT: Basophil activation test
cAMP: Cyclic AMP
FACS: Fluorescence-activated cell sorting
FITC: Fluorescein isothiocyanate
fMLP: N-formyl-methionyl-leucyl-phenylalanine
MC-R: Melanocortin receptor
MSH: Melanocyte-stimulating hormone
PMA: Phorbol 12-myristate 13-acetate
POMC: Proopiomelanocortin

with 7 transmembrane domains.[12] The 5 cloned MC-Rs, MC-1R to MC-5R, differ in their relative binding affinity for the naturally occurring melanocortin peptides. Accordingly, α-MSH and ACTH bind to MC-1R with similar affinity, whereas MC-2R, which is expressed primarily in the adrenal cortex, exclusively binds ACTH. Initially characterized as a melanotropic (pigment-inducing) peptide, α-MSH has been demonstrated to have biological effects far beyond pigmentation, such as regulation of exocrine glands,[13] collagen metabolism,[14] immunomodulation,[8,15] or cytoprotection.[15] In this report we investigated the role of α-MSH on basophil function.

## METHODS

### Reagents

α-MSH was purchased from Calbiochem (Schwalbach, Germany). ACTH, KPV, and KdPT were from Bachem (Bubendorf, Switzerland). Phorbol 12-myristate 13-acetate (PMA), N-formyl-methionyl-leucyl-phenylalanine (fMLP), anti-IgE, and complement C5a were obtained from Sigma-Aldrich (Munich, Germany). IL-3 was purchased from R&D Systems (Minneapolis, Minn).

### Isolation of human basophils

Isolation of human basophils was performed from 120 mL of peripheral blood from healthy subjects or from patients with intermittent allergic rhinitis to grass pollen confirmed by positive skin prick test reactions to grass pollen extract only and not to a panel of 12 common inhalant allergens (ALK-Abelló, Wedel, Germany) or from donors with unknown phenotype of 500 mL from leukocyte reduction filters of thrombocyte apheresis (transfusion rings). Basophils were isolated with a magnetic cell-sorting kit (Miltenyi Biotec, Bergisch Gladbach, Germany, and Stem Cell Technologies, Grenoble, France) by using established protocols.[16-18] The cumulative purity of the isolated basophils ranged from 99% to 100%. All procedures were approved by the Local Ethical Committees of the Universities of Lübeck (for Borstel), Hannover, and Münster.

### RT-PCR

Total RNA was prepared from 100% pure human basophils derived from healthy donors by using the RNeasy kit (Qiagen, Santa Clarita, Calif). After DNA digestion with DNAse (Promega, Madison, Wis), 1 μg of total RNA was reverse transcribed with 15 U of AMV-RT (Promega) and amplified with established primer sets against all known MC-Rs.[14] All amplification products were of the expected size. Positive controls consisted of cDNA from normal human melanocytes (for MC-1R) and of genomic DNA from diploid fibroblasts (for MC-2R to MC-5R). For amplification of POMC transcripts related to exon 3 of the *POMC* gene, primers and conditions were identical to those described by Slominski et al.[19] Transcripts related to exons 2 to 3 of *POMC* were amplified by using primers and conditions described by others.[20] Negative controls consisted of $H_2O$ (contamination control) and RNA (genomic DNA contamination control) instead of the template.

### Western immunoblotting

Basophils were lysed in buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 1 mmol/L ethylenglycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid, and 0.01% $NaN_2$ plus a freshly added protease inhibitor cocktail (Roche, Mannheim, Germany); sonicated; and centrifuged. Supernatants were analyzed for protein content by using the modified Bradford assay (Bio-Rad, Richmond, Calif), followed by addition of 2× SDS Laemmli sample buffer and heating. Equal amounts of protein (35-70 μg of protein per lane) were then separated by means of denaturing 12% Tris-glycine SDS-PAGE and immunoblotted onto polyvinylidene difluoride membranes. Membranes were either blocked overnight in 5% dry milk (for detection of MC-1R) or in 10% BSA (for detection of POMC). Membranes were then incubated overnight with a polyclonal anti–MC-1R antibody (1:500; Chemicon, Temecula, Calif) or an anti-POMC mAb (1:500; Abcam, Cambridge, United Kingdom). After washing, membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (Amersham Life Science, Freiburg, Germany), and antigen-antibody complexes were visualized by means of enhanced chemiluminescence (Amersham Life Science). Identical protein loading was ensured by stripping and reprobing with an anti–β-actin (1:5000, Sigma-Aldrich) or an α-tubulin antibody (1:1000; Oncogene Research Products, San Diego, Calif).

### Double-immunofluorescence analysis

Nasal polyp samples (n = 3) were obtained from collaborating ear, nose, and throat surgeons from excess tissue not needed for diagnostic purposes from patients undergoing elective therapeutic polypectomy after written patient consent, as approved by the Local Ethics Committee (University of Lübeck, Lübeck, Germany). Cryosections were incubated overnight with an anti-basophil mAb (anti-BB1;1:10)[21] and a rabbit polyclonal antibody against amino acids 2 to 18 of the extracellular domain of human MC-1R (1:50) in the presence of antibody dilution buffer (DCS Innovative Diagnostik-Systeme, Hamburg, Germany). The anti–MC-1R antibody is described elsewhere.[22,23] After washing, slides were incubated for 45 minutes at room temperature with a goat anti-mouse IgG coupled to Alexa Fluor 546 and a fluorescein isothiocyanate (FITC)–conjugated goat anti-rabbit IgG (1:200; Invitrogen GmbH, Karlsruhe, Germany). As negative controls, rabbit IgG (DakoCytomation, Denmark A/S, Glostrup, Denmark) and murine IgG were used instead of the primary antibodies. All staining sections were viewed with a laser scanning confocal microscope (Fluoview 300; Olympus, Tokyo, Japan) running Fluoview 2.1 software (Olympus).

### Fluorescence-activated cell-sorting analysis

MC-1R surface expression was analyzed with fluorescence-activated cell-sorting (FACS) analysis on isolated human basophils. Basophils were blocked in PBS containing human IgG (10 mg/mL; Aventis Behring, Marburg, Germany), 0.02 g of gelatin, and 0.01 mL of 2 mol/L $NaN_3$ for 30 minutes at 4°C. Thereafter, basophils ($1 \times 10^5$) were incubated with a polyclonal antibody (25 μg/mL) against amino acids 2 to 18 of the extracellular domain of human MC-1R[22,23] or the respective isotype control (25 μg/mL, rabbit IgG, R&D Systems) or preimmune serum (25 μg/mL) for 1 hour at 4°C. After 2 washing steps, basophils were incubated with FITC-conjugated goat anti-rabbit antibody (25 μg/mL; Beckmann Coulter, Krefeld Germany) for 20 minutes at room temperature. After a final washing step, basophils were resuspended in PBS and analyzed in the FACSCalibur flow cytometer (BD, San Jose, Calif).

### Determination of cyclic AMP

After isolation from human peripheral blood of healthy donors (n = 4), basophils were immediately divided into aliquots of 50,000 cells per treatment. Cells were preincubated for 5 minutes with a commercially available phosphodiesterase inhibitor set (Calbiochem), followed by stimulation with α-MSH at $10^{-6}$ to $10^{-10}$ mol/L for 20 minutes at 37°C. Forskolin served as a positive control. Cells were finally lysed, and cyclic AMP (cAMP) levels were determined in duplicates by using a specific enzyme immunoassay (Amersham Life Sciences).