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Human Mast Cells in the Neurohormonal Network: Expression of POMC, Detection of Precursor Proteases, and Evidence for IgE-Dependent Secretion of α-MSH

Metin Artuc[1], Markus Böhm[2], Andreas Grützkau[1], Alina Smorodchenko[1], Torsten Zuberbier[1], Thomas Luger[2] and Beate M. Henz[1]

Human mast cells have been shown to release histamine in response to the neuropeptide α-melanocyte-stimulating hormone (α-MSH), but it is unknown whether these cells express proopiomelanocortin (POMC) or POMC-derived peptides. We therefore examined highly purified human skin mast cells and a leukemic mast cell line-1 (HMC-1) for their ability to express POMC and members of the prohormone convertase (PC) family known to process POMC. Furthermore, we investigated whether these cells store and secrete α-MSH. Reverse transcriptase-PCR (RT-PCR) analysis revealed that both skin mast cells and HMC-1 cells express POMC mRNA and protein. Expression of the POMC gene at the RNA level in HMC-1 cells could be confirmed by Northern blotting. Transcripts for both PC1 and furin convertase were detectable in skin-derived mast cells and HMC-1 cells, as shown by RT-PCR. In contrast, PC2 transcripts were detected only in skin mast cells, whereas transcripts for paired basic amino acid converting enzyme 4 (PACE4) were present only in HMC-1 cells. Radioimmunoassays performed on cell lysates and cell culture supernatants from human skin-derived mast cells disclosed immunoreactive amounts of α-MSH in both fractions. Stimulation with an anti-IgE antibody significantly reduced intracellular α-MSH and increased extracellular levels, indicating IgE-mediated secretion of this neuropeptide. Our findings show that human mast cells are active players in the cutaneous POMC system. Mast cell-derived α-MSH may contribute to cutaneous hyperpigmentation as seen in patients with urticaria pigmentosa. Moreover, IgE-dependent release of α-MSH suggests an immunomodulatory role of this neurohormone during inflammatory and allergic reactions of the skin.

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INTRODUCTION

Mast cells are connective tissue-resident cells that are best known for their histamine- and leukotriene-mediated primary effector function during anaphylactic reactions. In recent years, there is however increasing evidence that these cells participate in various other immunological and inflammatory reactions, particularly through the generation and release of a broad spectrum of chemokines and peptide growth factors (reviewed by Henz *et al.*, 2001). Since many decades, mast cells have also been considered as regulators of neurohormonal reactions, but the molecular mechanisms underlying these phenomena are poorly clarified (see Metcalfe *et al.*, 1997 for a review). An important regulatory module of the immune-neurohormonal axis is the proopiomelanocortin (POMC) system. POMC-derived peptides such as adrenocorticotropin (ACTH), α-melanocyte-stimulating hormone (α-MSH), and β-endorphin are released in response to stress-related stimuli not only from the anterior pituitary but also from various cells within peripheral tissue like the skin. These molecules are of particular interest in cutaneous biology and pathology as they are not only involved in thermoregulation, melanin synthesis, and hair growth, but also in diverse immunological and inflammatory reactions in response to UV radiation and inflammatory stimuli (reviewed by Slominski *et al.*, 2000).

A possible role for POMC-derived peptides in mast cell-dependent allergic reactions has recently been substantiated

[1]*Department of Dermatology, Charité, Humboldt University, Berlin, Germany and* [2]*Department of Dermatology and Ludwig Boltzmann Institute of Cell Biology and Immunobiology of the Skin, University of Münster, Münster, Germany*

Correspondence: Dr Metin Artuc, Department of Dermatology, Charité, Campus Mitte, Humboldt University, Schumannstr., 20/21, Berlin D-10117, Germany. E-mail: metin.artuc@charite.de

Abbreviations: ACTH, adrenocorticotropin; FC, furin convertase; HMC-1, human mast cell line-1; α-MSH, α-melanocyte-stimulating hormone; NEP, neutral endopeptidase; PC, prohormone convertase; POMC, proopiomelanocortin; RT-PCR, reverse transcriptase-PCR; SCF, stem cell factor

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as cutaneous mast cells respond to α-MSH with the selective release of histamine (Grützkau *et al.*, 2000). Interestingly, more than one decade ago, immunohistochemical studies on skin sections from patients with urticaria pigmentosa demonstrated immunoreactivity for ACTH in cutaneous mast cells *in situ* (Akiyama *et al.*, 1991). Direct evidence for the expression of the POMC gene and/or precursor proteases that process POMC into POMC-derived peptides, that is, prohormone convertase (PC)1, PC2, furin convertase (FC), and paired basic amino acid converting enzyme 4 (PACE4) (reviewed by Seidah *et al.*, 1999), is however lacking in mast cells.

In this study, we have therefore investigated whether human cutaneous mast cells as well as a human leukemic mast cell line (HMC-1) express POMC and precursor proteases. Furthermore, we have studied the release of α-MSH by FcεRI-dependent pathways. Our data provide first evidence that human mast cells express the POMC gene and possess the enzymatic machinery to process this precursor hormone. We also demonstrate that human mast cells generate and secrete immunoreactive amounts of α-MSH, a process augmented by stimulation of the cells with anti-IgE antibodies. These findings provide novel clues to our understanding of pigmentary changes in patients with cutaneous mastocytosis and furthermore suggest mast cell-derived α-MSH as a novel modulator of inflammatory/allergic skin reactions.

RESULTS

Human skin mast cells and HMC-1 cells express POMC mRNA and protein

Reverse transcriptase-PCR (RT-PCR) analysis of RNA derived from freshly isolated, unstimulated human mast cells as well as from HMC-1 cells (data not shown) revealed positive signals for POMC (Figure 1a, lane 1). The amplification product was of the expected size of 260 bp, corresponding to exon 3 of the POMC gene (Slominski *et al.*, 1995). The POMC-related amplification products of human skin mast cells and HMC-1 cells comigrated exactly with an amplicon generated by RT-PCR from RNA derived from human keratinocytes serving as positive control (data not shown). To substantiate the expression of POMC in skin-derived human mast cells and HMC-1 cells, Western immunoblotting was performed using an antibody directed against ACTH 18–33 that cross-reacts with POMC (Schiller *et al.*, 2001; Böhm *et al.*, 2005). In normal human melanocytes used as a positive control, two immunoreactive bands at 33 and 31 kDa were detected, possibly representing differentially glycosylated POMC isoforms (Figure 1b). Skin-derived human mast cells expressed only the 33 kDa form, whereas HMC-1 cells expressed only the 31 kDa form. In contrast, cell extracts from the neuroblastoma cell line SK-N-MC lacking POMC expression (Verbeeck et al., 1992) did not show any POMC-related immunoreactive band. Stripping of the original blot and reprobing with an antibody against the ubiquitously expressed extracellular kinase-2 indicate that the absence of a POMC-related band in SK-N-MC cells was not owing to a lack of protein in the sample (Figure 1b).

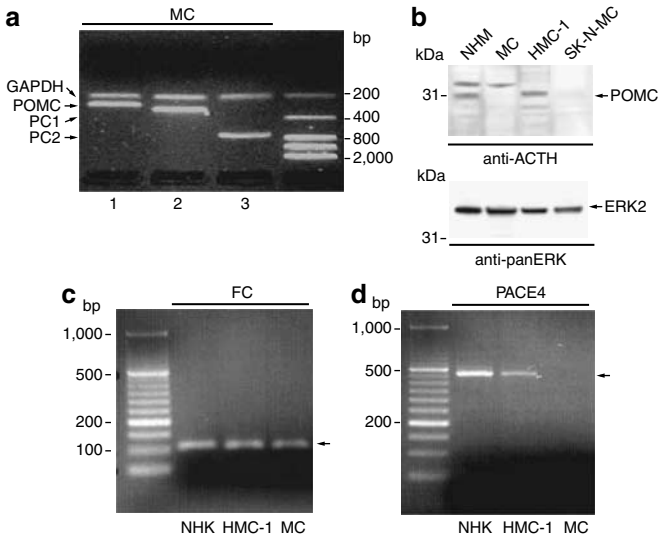

Figure 1. Detection of POMC and precursor proteases in purified human skin MC and HMC-1 cells. (**a**) Total RNA was extracted from the above cells, reverse-transcribed, and amplified with specific primers against POMC (lane 1, 260 bp), PC2 (lane 2, 299 bp), and PC1 (lane 3, 674 bp) followed by agarose gel electrophoresis. FC and PACE were amplified with specific primers yielding amplicons of 111 and 456 bp. (**c**, **d**) cDNA from normal human keratinocytes (NHK) served as positive control for FC and PACE4. For detection of POMC protein, identical amounts of protein (50 μg/lane) were subjected to SDS-PAGE and Western immunoblotting using an antibody against ACTH that cross-reacts with POMC. (**b**, upper panel) As a positive control, lysates from normal human melanocytes (NHM) and as a negative control lysates from SK-N-MC cells were used. (**b**, lower panel) For control of identical protein loading, the membrane was reprobed with an antibody that detects the ubiquitously expressed extracellular kinase 2.

Detection of transcripts for precursor proteases in human skin-derived mast cells and HMC-1 cells

To further check whether human skin mast cells and HMC-1 cells possess the enzymatic equipment to cleave POMC into POMC-derived peptides, we performed RT-PCR analysis with primers against all members of the PC family of serine proteases that are capable of processing POMC (Seidah *et al.*, 1999). In human skin, mast cell expression of both PC1 and PC2 was detectable (Figure 1a, lanes 2 and 3), whereas in HMC-1 cells, only PC1 could be detected (data not shown). The PC1- and PC2-related amplification products in skin mast cells were of the expected size of 674 and 299 bp (Schiller *et al.*, 2001; Böhm *et al.*, 2005). FC, another member of the PC family, was also detectable in both human skin mast cells and HMC-1, cells as shown by RT-PCR and amplification of a specific product of 111 bp (Figure 1c). On the other hand, transcripts for PACE4 were detected only in HMC-1 cells but not in human skin mast cells (Figure 1d). In both of the latter RT-PCR analyses, RNA from human epidermal keratinocytes served as positive control (Pearton *et al.*, 2001), generating expected bands with a size of 111 bp (for FC) and of 456 bp (for PACE4), respectively. Negative controls with substitution of the template with H_2O were consistently negative (data not shown).

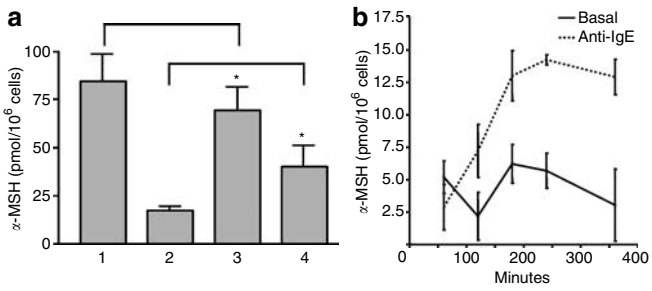

Figure 2. Detection of α-MSH in purified human skin mast cells. For measuring intracellular and secreted fractions pf, α-MSH cells were kept in culture medium alone (columns 1 and 2) or stimulated with anti-IgE (columns 3 and 4) for 24 hours. Amounts of α-MSH are expressed in absolute values (pmol). (**a**) Data show means ± SD of eight separate experiments. Columns 1 and 3: cell lysates; columns 2 and 4: cell supernatants. For analysis of the time-dependent release of α-MSH in response to anti-IgE, cells were kept in medium (basal) or in medium plus anti-IgE (anti-IgE). Amounts of α-MSH are expressed in absolute values (pmol). (**b**) Data show means ± SD of three separate experiments. *$P<0.05$, compared with non-stimulated mast cells.

Production and secretion of α-MSH by human dermal mast cells and HMC-1 cells

In order to assess the biological relevance of POMC, PC1, and PC2 expression in human mast cells, we investigated the production and secretion of α-MSH in human skin-derived mast cells under basal conditions and after treatment with an anti-IgE antibody. Cross-linking of mast cells bound IgE with antibodies against IgE or treatment with anti-FcεRI antibodies are well-established experimental procedures mimicking the IgE-dependent effector pathway *in vivo*. Figure 2a shows the detected amounts of α-MSH as measured by radioimmunoassay in skin mast cells under basal conditions (i.e. in cells kept in culture medium for 24 hours) and in cells treated with an anti-IgE antibody over the same period of time. Intracellular levels of α-MSH (as represented by cell lysates) were higher (mean: 84.2 ± 14.2 pmol/10^6 cells) than those amounts found in cell culture supernatants (mean: 17.75 ± 1.8 pmol/10^6 cells). In cells treated with the anti-IgE antibody for 24 hours, intracellular α-MSH levels slightly declined as compared to non-stimulated cells. Most interestingly, compared to cells kept in culture medium only, a robust increase ($P<0.05$) in the amount of extracellular α-MSH (40.5 ± 10.1 pmol/10^6 cells) was noted, suggesting together with the aforementioned observation release of α-MSH from intracellular stores. In HMC-1 cells, immunoreactive amounts of α-MSH were also detectable in intracellular and extracellular fractions (Figure 2a). Treatment of HMC-1 cells with phosphoramidon, a pharmacological inhibitor of neutral endopeptidase (NEP) 24.11, resulted in a marked decrease of α-MSH as compared to controls (data not shown).

As the kinetics of mediator release from mast cells differs widely depending on the type of mediator and stimulus studied, a more detailed study of α-MSH release was conducted with human skin-derived mast cells (Figure 2b). After 1 hour of treatment with the anti-IgE antibody, α-MSH levels in the cell culture supernatants were about the same between non-stimulated and stimulated cells. Anti-IgE treatment resulted in a robust increase of α-MSH at later time points, with peak values at 3–4 hours. Values for non-stimulated cells dropped, on the other hand, slightly by 2 hours of incubation, and remained more or less at the 1-hour value over the entire time of incubation.

DISCUSSION

The present data provide first evidence for an active contribution of mast cells to peripheral tissue POMC-derived peptide levels by demonstrating expression of the POMC gene and generation of immunoreactive amounts of α-MSH. As an essential prerequisite for POMC peptide generation, both human skin-derived mast cells and HMC-1 cells were found to express members of the PC family of precursor proteases that are capable of processing POMC into α-MSH (Seidah *et al.*, 1999). Indeed, the skin mast cell used for our investigative studies derived from foreskin a specialized ''sexual'' tissue, which is densely innervated and contains many peptidergic nerves (Vaalasti *et al.*, 1988). However, it remains speculative whether mast cells from foreskin are different from mast derived from skin of other body sites with regard to expression of POMC, PCs, or α-MSH secretion. The foreskin-derived mast cells used in our studies expressed PC1 and PC2 similar to human epidermal melanocytes (Peters *et al.*, 2000) and dermal fibroblasts (Schiller *et al.*, 2001) and several cell types of murine skin (Mazurkiewicz *et al.*, 2000). However, in this study, we were unable to detect PC2 transcripts in HMC-1 cells, although this transformed cell line expressed FC. It has been reported that cellular transformation can be associated with loss of neuroendocrine differentiation markers such as PC1 and PC2 (Scopsi *et al.*, 1995). In light of the detected α-MSH in HMC-1 cells, it is possible that POMC processing is mediated via FC and PC1, or by an alternative mechanism. Accordingly, we could demonstrate that the amounts of α-MSH released by HMC-1 cells decline in cells treated with the NEP 24.11 inhibitor phosphoramidon. Previously, it had been shown that NEP 24.11 can convert ACTH into α-MSH (Smith *et al.*, 1992). Therefore, it will be interesting to investigate the expression of NEP 24.11 and its putative ACTH-converting activity in skin-derived mast cells and HMC-1 cells in more detail.

In recent years, it has become apparent that the rapid death of tissue mast cells during *in vitro* culture can be prevented by the addition of stem cell factor (SCF) and IL-4. This has been shown for intestinal mast cells (Bischoff *et al.*, 1999) and has been confirmed by us regarding skin mast cells (Babina *et al.*, 2004). These molecules act synergistically in that SCF alone has only minor effects on mature mast cells, but in the presence of IL-4, there was a strong increase of proliferation, an enhancement of IgE-dependent mediator release, and furthermore a switch to a Th-2 type cytokine pattern (Bischoff *et al.*, 1999; Lorentz *et al.*, 2000). SCF is also a well-known secretagogue for human skin mast cells (Frenz *et al.*, 1997; Gibbs *et al.*, 2001). We can therefore not exclude that priming with SCF and IL-4 might have induced the production and baseline secretion of α-MSH, or at least caused an enhancement of this process. A major direct secretory effect of SCF is, however, unlikely as this requires an

extended time period (Gibbs *et al.*, 2001). Furthermore, the fact that POMC, PC1, PC2, and FC are expressed in freshly isolated mast cells make a basic, constitutional ability of mast cells to produce this molecule more likely. Moreover, the detection of POMC and immunoreactive amounts of α-MSH in HMC-1 cells support a constitutive production of α-MSH by mast cells. Interestingly, HMC-1 cells are able to grow independently of externally added SCF owing to an activating SCF mutation (Furitsu *et al.*, 1993) so that an SCF effect is endogenously operative. This may also explain why HMC-1 cells express a full-length transcript of the POMC gene, whereas no or only a truncated form of the POMC gene has been found in other monocytic and T-lymphoblastic leukemic cell lines (Murao *et al.*, 1998). However, as HMC-1 cells respond only poorly or not at all to IgE receptor cross-linking (Butterfield *et al.*, 1988; Weber *et al.*, 1996), we could not examine whether the cells are also able to secrete α-MSH.

The fact that mast cells generate POMC-derived peptides such as α-MSH has several novel implications for the cutaneous neurohormonal network. Accordingly, α-MSH released by mast cells may be involved in the pathogenesis of cutaneous hyperpigmentation in patients with urticaria pigmentosa, a skin disease well characterized by interstitial accumulation of mast cells that may fill-out the whole papillary dermis. Typically, the dermatopathologic picture of lesional skin in these patients also includes increased epidermal melanization, a finding possibly related to α-MSH released by the increased numbers of dermal mast cells. It should, however, be noted that mast cell secrete a plethora of growth factors, cytokines, and mediators, many of which can stimulate melanogenesis (reviewed by Slominski *et al.*, 2004). Thus, future studies are needed to clarify whether α-MSH derived from mast cells is biologically active and to what extent it contributes to the overall pigmentary activity of mast cell mediators. Another novel aspect of neurohormonal regulation by mast cells as shown by our study is the IgE-dependent release of α-MSH. It is known that activation of mast cells by anti-IgE antibodies also leads to the release of the proinflammatory cytokines tumor necrosis factor-α and IL-8 within 2–4 hours (Gibbs *et al.*, 2001). Our data on the IgE-mediated release of α-MSH extend these findings and, moreover, show that the time course of α-MSH secretion by skin mast cells follows a similar pattern like that of tumor necrosis factor-α and IL-8. It is difficult to speculate on the immunoregulatory function of α-MSH released by mast cells. We have previously reported that human mast cells express receptors for α-MSH and that α-MSH treatment of these cells induces histamine release (Grützkau *et al.*, 2000). These findings would actually suggest an autocrine proallergic or proinflammatory role of α-MSH in mast cells. In light of the majority of reported modulatory actions of α-MSH (Lipton and Catania, 1997; Luger *et al.*, 1999), this neuropeptide may, however, rather act as a paracrine regulator limiting the extent of inflammatory, immune, or allergic reactions.

Besides the IgE-mediated activation pathway, mast cells can be stimulated to release histamine, leukotrienes, cytokines, and growth factors by polybasic molecules such as mastaropan, and Compound 40/80, by peptide-like substances such as substance P, calcitonin gene-related peptides, somatostatin, neurotensin, and vasoactive intestinal peptide or by the complement fragments C3a and C5a (reviewed by Metcalfe *et al.*, 1997; Henz *et al.*, 2001). In the past, some of these molecules, like histamine and tumor necrosis factor-α, have been implicated in the increase of α-MSH production in rat tissue and in monocytes/macrophages, respectively (Kjaer *et al.*, 1995; Knigge *et al.*, 1995; Taherzadeh *et al.*, 1999). A stimulatory effect of the above factors on POMC expression, POMC processing, or release of α-MSH by mast cells is thus well possible in view of the fact that they belong to the monocyte/macrophage lineage as well (Metcalfe *et al.*, 1997). In addition, UV light has been reported as another potent means by which α-MSH and ACTH are upregulated in several cutaneous cell types (Chakraborty *et al.*, 1996; Scholzen *et al.*, 2000). Recent findings demonstrating the modulatory role of UV radiation also in cutaneous mast cells, in view of these findings, and, with a clear reduction of mediator release from mast cells on their *in vitro* exposure to UV light (Horio, 2000; Guhl *et al.*, 2005), would underline a potential role of α-MSH in this process.

Taken together, the data as presented in this report are a first demonstration of the ability of cutaneous mast cells to synthesize and release the POMC peptide α-MSH. α-MSH may be one of the mediators responsible for cutaneous hyperpigmentation in skin diseases with increased numbers of dermal mast cells. Furthermore, the identification of an IgE-mediated secretory stimulus for this molecule, the kinetics of its release, and the findings reported on other secretory stimuli in other cell types of the skin suggest that mast cell-derived α-MSH may play an important role during the so-called delayed allergic reactions. Our findings further emphasize the existence of an intricate interactive network of mediator interplay between immune regulators and neurohormones in the skin. Clarification of the role of mast cell-derived α-MSH may not only shed light on the ever-increasing spectrum of biological activities of this molecule but may also help to better understand the pathogenesis of inflammatory, immune, and allergic diseases of the skin.

MATERIALS AND METHODS

Mast cells

Tissue mast cells were isolated from human foreskins. All use of human skin was conducted according to the Declaration of Helsinki Principles and was approved by Institutional Review Boards of Universitätsmedizin Berlin Charite. Cells were enriched by a sequence of enzymatic and mechanical tissue dispersions, followed by affinity purification with magnetic beads and a monoclonal antibody mAB YB5.B8 (kindly provided by L. Ashman, Adelaide, SW Australia) directed against the Kit receptor (CD 117). Final purity of mast cells was always >90%, as measured by flow cytometry using the c-kit receptor and the α-chain of the high-affinity IgE receptor as markers. Viability of mast cells was always >95%, as determined by Trypan blue staining. For further details, see Grützkau *et al.* (2000) and Artuc *et al.* (2002). The human leukemic mast cell line (HMC-1) was kindly provided by J.H. Butterfield, Rochester, MN). Cells were routinely grown as described before (Butterfield *et al.*, 1988; Artuc *et al.*, 2002).

RT-PCR

For RT-PCR, total RNA was isolated from purified human mast cells using the RNAesy system (Qiagen, Hilden, Germany), treated with RNAse-free DNAse to digest genomic DNA and, after ethanol precipitation, reverse-transcribed, as detailed recently (Artuc *et al.*, 2002). Successful cDNA synthesis was checked by PCR using glyceraldehyde-3-phosphate dehydrogenase-specific primers (sense: 5′-GATGACATCAAGAAGGTGGTG-3′ and antisense: 5′-GCTGTAG CCAAATTCGTTGTC-3′; product size: 190 bp) and 20 cycles of PCR (95°C for 45 seconds, 58°C for 45 seconds, and 72°C for 45 seconds), followed by a 10-minute extension step at 72°C. Nucleotide sequences for PC-1 and PC-2 primers and the corresponding amplification program were previously described by Schiller *et al.* (2001). PC-1 was amplified with the sense primer 5′-AGCAAACCCA ATCTCACCTG-3′ and the antisense primer 5′-TCTCCACCCCTCCT CTGTCAT-3′, yielding a 674 bp cDNA product by one cycle at 94°C for 10 minutes, 53°C for 45 seconds, 72°C for 1 minute, followed by 39 cycles at 94°C for 45 seconds, 53°C for 45 seconds, 72°C for 1 minute, and a final cycle at 94°C for 45 seconds, 53°C for 45 seconds, 72°C for 10 minutes; PC-2 was amplified with the sense primer 5′-AACGCAACCAGAAGAGGAGA-3′ and the antisense primer 5′-ATGGCCAACTTGGACTGGTA-3′, yielding a 299 bp cDNA product by one cycle at 94°C for 10 minutes, 53°C for 45 seconds, 72°C for 1 minute, followed by 39 cycles at 94°C for 45 seconds, 53°C for 45 seconds, 72°C for 1 minute, and a final cycle at 94°C for 45 seconds, 53°C for 45 seconds, 72°C for 10 minutes. POMC was amplified by primer pairs established by Slominski *et al.* (1995) and a modified protocol, with one cycle at 94°C for 10 minutes, 68°C for 45 seconds, 72°C for 1 minute, followed by 39 cycles at 94°C for 45 seconds, 68°C for 45 seconds, 72°C for 1 minute, and a final cycle at 94°C for 45 seconds, 68°C for 45 seconds, 72°C for 10 minutes. PACE4 was detected with primer pairs established by Cheng *et al.* (1997) generating an amplification product of 456 bp. The nucleotide sequence of the sense primer was 5′-CTATGGATTTGGTTTGGTGGAC-3′ and that of the antisense primer 5′-AGGCTCCATTCTTTCAACTTCC-3′. The thermocycler program consisted of one cycle at 94°C for 5 minutes, 57°C for 45 seconds, 72°C for 1 minute, followed by 35 cycles at 94°C for 45 seconds, 57°C for 45 seconds, 72°C for 1 minute, and a final cycle at 94°C for 45 seconds, 57°C for 45 seconds, 72°C for 10 minutes. For the detection of FC, recently established primer sets and conditions were used (Böhm *et al.*, 2005). Sense primer sequence was 5′-TGC TGGTCTTCGTCACTGTC-3′, and antisense primer sequence was 5′-TTGTAGGAGATGAGGCCACGG-3′ yielding a 111 bp product. The amplification protocol was one cycle at 94°C for 5 minutes, 60°C for 1 minute, 72°C for 2 minutes; 35 cycles at 94°C for 45 seconds, 60°C for 2 minutes, 72°C for 1 minute, followed by a final cycle at 94°C for 45 seconds, 60°C for 1 minute, and 72°C for 10 minutes. PCR products were visualized after separation on 1.5–2% tris-acetate-ethylenediamine tetraacetic acid (TAE)–agarose gels by staining with ethidium bromide and detected by the gel analysis software Grab IT.

Western immunoblotting

The cells were washed twice in phosphate-buffered saline, lysed in ice-cold lysis puffer (1 mM HEPES, 0.5% NP40, 0.5 mM $MgCl_2$, 0.1% SDS, 1 μg aprotonin/ml, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride), and then centrifuged at 4°C for 10 minutes at 10,000 *g*. Protein concentration was determined with the Coomassie Plus protein assay kit. Equal protein amounts (50 μg/lane) were separated by denaturing SDS-PAGE using 4–12% gradient gels (NuPAGE; Invitrogen, Carlsbad, CA). After Western blotting onto polyvinylidene difluoride membranes and blocking with 10% bovine serum albumin, membranes were incubated with an anti-ACTH antibody (Sigma, St Louis, MI) directed against the amino acids 18–39 of ACTH (1:1,000). As reported previously, this antibody cross-reacts with POMC (Schiller *et al.*, 2001; Böhm *et al.*, 2005), which also contains the amino acids 18–39 of ACTH. After washing, membranes were incubated for 20 minutes with a horseradish-peroxidase-conjugated secondary antibody (1:10,000; Amersham Life Science, Freiburg, Germany). Antigen–antibody complexes were visualized by enhanced chemiluminescence (Amersham). Membranes were stripped as detailed before (Böhm *et al.*, 1995), re-blocked, and incubated with an anti-extracellular signal-regulated kinase-1–4 antibody (1:5,000; BD Biosciences, Erembodegem, Belgium) to confirm identical loading of the lanes.

α-MSH radioimmunoassay

For quantitative determination of α-MSH, cell supernatants or cell lysates were harvested using the following protocol: purified mast cells were suspended in RPMI 1640 medium, supplemented with SCF, 50 ng (R&D Systems, Wiesbaden, Germany), and IL-4, 10 ng (R&D Systems, Wiesbaden, Germany) at a density of 10^{-6} cells/ml, and were kept as such or stimulated with an anti-IgE antibody (1/1000 dilution; Sigma) for up to 24 hours. Thereafter, supernatants were collected after centrifugation for 5 minutes at 5,000 r.p.m. and immediately stored at −80°C. Cells pellets were washed with phosphate-buffered saline, lysed with 1% Triton X-100, and stored as described above. Total protein content of cell lysates was determined using the Bio-Rad micro protein assay system (Bio-Rad Laboratories GmbH, München, Germany). Amounts of α-MSH in cell lysates and in cell culture supernatants were analyzed using a commercially available radioimmunoassay (EuroDiagnostica, Malmö, Sweden). The antiserum used in the α-MSH radioimmunoassay was directed against the C-terminal part of α-MSH recognizing α-MSH and Des-acetyl α-MSH, with no cross-reactivity against ACTH, β-MSH, or γ-MSH. To test for the involvement of NEP 24.11 in α-MSH synthesis, HMC-1 cells were incubated overnight with the endopeptidase inhibitor phosphoramidon (10^{-4}–10^{-7} M). The amount of α-MSH was subsequently determined in the medium by radioimmunoassay.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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