Terminating the Stress: Peripheral Peptidolysis of Proopiomelanocortin-Derived Regulatory Hormones by the Dermal Microvascular Endothelial Cell Extracellular Peptidases Neprilysin and Angiotensin-Converting Enzyme

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The skin including the microvascular endothelium is an established peripheral source and target of the immunomodulatory proopiomelanocortin (POMC) peptides ACTH and α-MSH. Whereas intracellular POMC peptide generation is well characterized, less is known on their extracellular processing in peripheral tissues by the neuropeptide-specific zinc metalloproteases neprilysin (NEP) and angiotensin-converting enzyme (ACE). This may locally control POMC peptide bioavailability and activation of ACTH/α-MSH-specific melanocortin receptors (MCs). In a cell-free system, endothelial cell (EC) membranes prepared from ACE\textsuperscript{high}/NEP\textsuperscript{low}-expressing primary human dermal microvascular ECs and the ACE\textsuperscript{low}/NEP\textsuperscript{high}-expressing EC line HMEC-1 degraded ACTH\textsubscript{1–39} over time, resulting in temporary increased α-MSH immunoreactivity. Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy peptide mapping and electrospray ionization-mass-spectroscopy sequencing identified several stable fragments generated from ACTH\textsubscript{1–39}, ACTH\textsubscript{1–24} and α-MSH by EC membranes or recombinant NEP and ACE. Whereas some fragments could be assigned to a cell-specific NEP or ACE activity, other degradation products require additional enzyme activity. Pharmacological NEP inhibition enhanced the ACTH and α-MSH-mediated activation of EC ec-topically expressing MC\textsubscript{1}. Likewise, selected peptides such as α-MSH\textsubscript{1–12} generated from ACTH\textsubscript{1–39} and α-MSH by recombinant NEP displayed equipotent MC\textsubscript{1}-activating properties in vitro and antiinflammatory activity in murine allergic contact dermatitis in vivo as compared with the parental peptides. Thus, NEP and ACE significantly contribute to the EC processing of stress hormones (ACTH) and antiinflammatory peptides (α-MSH), which modulates MC\textsubscript{1} activation but does not completely inactivate the peptide ligand. Because NEP and ACE are regulated by inflammatory mediators and UV light, this may be important for ACTH/MSH-modulated skin inflammation. (Endocrinology 148: 2793–2805, 2007)
receptor (MC1) (17). Leakage of larger POMC fragments from the pituitary cells into the circulation has been reported (18), and ACTH and α-MSH are potentially released from the anterior pituitary cells into the circulation (19). Subsequently additional substrates such as bradykinin (BK) or the proinflammatory peptide Ang II. ACE also degrades SP and Ang II, which contains a single catalytic site identical with the C-terminal domain of somatic ACE (15).

Previous studies suggest that thermolysin-like peptidases participate in the processing and/or degradation of POMC peptides (reviewed in Ref. 16). This may control the bioavailability of ACTH and α-MSH (16), which are high-affinity ligands of specific G protein-coupled melanocortin 1 receptors (MC1) (17). Leakage of larger POMC fragments from anterior pituitary cells into the circulation has been reported (18), and ACTH and α-MSH are potentially released from extravillous cells including dermal microvascular ECs (19). Likewise, in human and murine skin, ACTH/MSH-related peptides of variable length have been described (20–22). The purpose of this study was to determine the capability of the EC-derived peptidases NEP and ACE to process POMC peptides by using a bioanalytical approach and to address potential functional consequences with respect to MC1 activation in vitro and murine allergic inflammation in vivo. As outlined below, NEP and ACE are fundamentally involved in processing of POMC peptides by EC.

Materials and Methods

Cell culture

Primary human dermal microvascular ECs (HMECs) were obtained from PromoCell (Heidelberg, Germany). HMECs and the cell line HMEC-1 were grown in a supplemented microvascular EC basal media (EBM-MV kit system; PromoCell, as supplied by the manufacturer) in a humidified atmosphere at 37 °C and 5% CO2. Experiments were conducted with cells in passages 3–6. HMEC cultures were characterized by their typical cobblestone morphology using light microscopy and by analysis for their capacity to express factor VIII-like antigen or CD31 (platelet endothelial cell adhesion molecule-1). Before stimulation, HMECs or HMEC-1 were plated at a density of 25,000 cells/cm2. In some experiments (assessment of intracellular cAMP and luciferase activity), cells were deprived from growth factors and fetal bovine serum by culturing in stimulation medium for 24 h before stimulation with POMC peptides (EBM-MV, no supplements except 0.5% fetal bovine serum and antibiotics).

Flow cytometry

For the analysis of HMECs or HMEC-1 cell surface expression, detached cells were stained with a mouse antihuman CD10 monoclonal antibody conjugated to fluorescein isothiocyanate (clone B-E3; Acris GmbH, Hidenhausen, Germany). For the detection of ACE, an antihuman CD143 mAb (clone 9B9; Acros) followed by fluorescein isothiocyanate (FITC)-conjugated antimouse IgG antibody (Sigma-Aldrich, Taufkirchen, Germany) was used. Flow cytometry was performed on a FACSCalibur cytometer using CellQuest Pro Software (BD Biosciences, Heidelberg, Germany).

Membrane preparation

For preparation of crude cell membranes, HMEC-1 or HMECs were grown to subconfluence (80–90%), washed with PBS, and detached with Accutase (PAA Laboratories, Pasching, Austria). Cells collected by centrifugation (300 × g) were washed with PBS and resuspended in 0.05 M Tris/HCl (pH 7.4). Subsequently cells were lysed by sonication (10 × 5 sec on ice) and subjected to 100,000 × g centrifugation (30 min, 4 °C). Membrane pellets were washed twice with ice-cold 0.05 M Tris-HCl (pH 7.4), resuspended in 0.05 M Tris-HCl (pH 7.4), and the protein content was adjusted to 1 mg/ml.

Determination of NEP and ACE enzymatic activity

NEP activity of cells or cell membranes was determined using the artificial substrate glutaryl-Ala-Ala-Phe-4-methoxy-2-naphtylamide (MNA) in the presence of aminopeptidase M (APM) or APMLX (23). Fluorescence of MNA was detected at 425 nm emission wavelength in a Fluoromax-2 fluorometer with DataMax 2.2 software (Instruments S.A. Inc., Edison, NJ) and an integrated GRAMS/386 data processing and database software (Galactic Industries Corp., Salem, NH) using an excitation wavelength set to 340 nm. NEP enzyme activity was calculated from a standard curve of 0–3000 pmol MNA, which was linear in the range of 0–3000 pmol MNA and expressed in microunits with 1 μU = 1 pmol MNA per hour per microgram protein. ACE activity was determined using a protocol modified from one previously published (24). Accordingly, plasma or cell lysates were mixed in a 1:8 sample to reagent volume ratio with a solution containing 1 mM of the substrate N-(3-[2-fluorophenyl]propyl)-Phe-Cys-Gly and 300 mM NaCl in 80 mM boric acid (pH 8.2 at 37 °C). After incubation (5 min/37 °C), the reaction mixture was transferred to a semimicro glass cuvette, and the absorbance change at 345 nm was recorded after a lag phase of 2 min for 5 min every 30 sec with a Beckman spectrophotometer. ACE activity was determined from a known standard containing human recombinant ACE (Sigma Diagnostics).

Determination of ACTH and α-MSH

HMECs and HMEC-1 cells or cell membranes, respectively, were incubated with ACTH1–24 or for various time points. Cell or membrane supernatants were harvested and ACTH and α-MSH IR was determined using commercially available RIA (EuroDiagnostics, Malmo, Sweden) as described (19).

POMC peptide processing by EC membranes or recombinant proteases

ACTH/MSH-related peptides were obtained from Bachem AG (Bubendorf, Switzerland), except α-MSH3–13, which was synthesized by IRIS Biotech GmbH (Marktredwitz, Germany). Peptide processing was performed as described (25). Briefly, peptides were incubated with defined amounts of HMECs or HMEC-1 membrane protein in 0.05 M Tris-HCl (pH 7.4) at 37 °C for various time points (1–240 min). Specific degradation by NEP or ACE was determined by the addition of NEP and ACE.
ACE inhibitors [phosphoramidon (PA), thiophan (TP), and captopril, respectively; Sigma-Aldrich]. To evaluate influences of other proteases, incubations were performed in the presence of 10 mm phenylmethylsulfonylfluoride (serine proteases), 1 mm pepstatin A (aspartic proteases), 1 mm α-iodoacetamide (cysteine proteases), 10 pm of the metalloprotease inhibitor 1,10 phenanthroline (all from Sigma-Aldrich), or 10 mm Na-EDTA in the presence or absence of divergent cations such as Ca2+ (10 mm; 1.5 M KCl) or Mg2+ (10 mM, 1% DMSO). The pH dependency of peptide fragmentation was analyzed in a 100 mM sodium acetate buffer at pH 5.0. For incubation with rhuNEP (generously provided by Nigel W. Bunnett, University of California, San Francisco, San Francisco, CA), peptides were incubated for 30 and 120 min at 37 C with 0.1–10 pmol (–0.01–1.0 μg) rhuNEP in 0.05 M Tris-HCl (pH 7.4) in the presence or absence of NEP and ACE inhibitors or 10 μM ZnCl2, respectively. Incubations of α-MSH and ACTH1–39 with 1.7 and 0.17 pmol rhuACE (0.2 and 0.02 μg protein, respectively; R&D Systems GmbH, Wiesbaden, Germany) were performed at 37 C in 0.1 M Tris/HCl (pH 8.3), 0.3 mM NaCl, and 10 μM ZnCl2. The reactions were terminated by the addition of 10 μM NEP or ACE inhibitors, respectively.

**Transfection**

ECs and cells of the embryonic kidney cell line HEK293 were transfected by electroporation (modified from Ref. 26) with a pcDNA3.1 expression vector-based cDNA expression construct (University of Missouri-Rolla cDNA Resource Center, Rolla, MO) containing either the full-length human MC1 cDNA (GenBank accession no. NM_002886) or the human MC4 cDNA with a 3′-N-terminal hemagglutinin (HA) tag (HA-MC4). In some experiments, ECs were transfected with a PathDe/H9262 and 0.02

**Sucrose density gradient centrifugation and Western blotting**

Untransfected or HA-MC1-transfected ECs were subjected to sucrose density gradient centrifugation as described with modifications (27).

**Statistical analysis**

Results are expressed as arithmetic mean ± SEM. The unpaired t test was used to calculate the statistical significance. Differences between multiple groups were examined using an ANOVA (Bonferroni t test). Mean differences with P < 0.05 were considered to be significant.

**Results**

**Expression of endothelial NEP and ACE**

To analyze the relative expression of neprilysin and ACE in ECs, untreated primary dermal microvascular ECs or HMEC-1 were stained with FITC-conjugated antibodies against human NEP or ACE, respectively. HDMECs express 40–55% ACE but only up to 15% NEP, whereas HMEC-1 display a reciprocal NEP and ACE expression profile corresponding to 40–50% NEP and up to 10% ACE, respectively (Fig. 1).

**Degradation of ACTH1–39 by ECs**

To determine, whether EC are capable of degrading or processing POMC peptides, cell membranes prepared from the EC

lysed, and assayed using reporter lysis buffer and luciferase assay buffer (Promega Corp., Madison, WI). Samples were read for 10 sec in a Gen-Probe single tube luminometer (MGM Instruments Inc., Hamden CT).
cell line HMEC-1 were incubated with ACTH1–39. When the ACTH content in membrane supernatants was monitored over time by using an ACTH-specific RIA, ACTH1–39 continuously decreased to the level of controls after 4 h (Fig. 2A). Interestingly, in the same supernatants, α-MSH immunoreactivity (IR) temporarily increased peaking at 60 min after the addition of ACTH (Fig. 2B). This generation of α-MSH IR could be prevented by the addition of an NEP inhibitor (PA), indicating that an NEP-like protease participates in this cleavage (Fig. 2C).

Fig. 1. NEP and ACE expression in microvascular ECs. Primary HDMEC (top) or cells of the EC line HMEC-1 (bottom) were stained with FITC-conjugated monoclonal Abs against human ACE (open histogram, bold line) or NEP (shaded histogram) and subjected to flow cytometry.

Next, the fate of ACTH1–39 after incubation with HDMECs (Fig. 3) and HMEC-1 membranes (Fig. 4) was investigated in

Fragmentation of POMC peptides by EC cell membranes

Next, the fate of ACTH1–39 after incubation with HDMECs (Fig. 3) and HMEC-1 membranes (Fig. 4) was investigated in
After incubation with HDMEC membranes, MALDI-TOF analysis revealed fragmentation of ACTH1–39 to a number of larger fragments that included ACTH2–17, corticotropin-like intermediate peptide (ACTH18–39), ACTH17–39, ACTH16–39, and ACTH15–39. Likewise, a number of smaller fragments 5–11 amino acids in length derived from the N terminus of the molecule, such as ACTH5–10, ACTH5–12, ACTH5–13, ACTH5–14, ACTH5–15, and ACTH5–16 could also be detected. Inhibition of NEP by PA/TP predominantly inhibited the generation of N-terminal ACTH fragments (Fig. 3B), whereas ACTH18–39, ACTH2–17, ACTH17–39, ACTH16–39, and ACTH15–39 were still detectable. In contrast, after incubation of ACTH1–39 with HMEC-1 membranes in the presence of NEP inhibitors, a significant change in the cleavage pattern of ACTH was observed (Fig. 4C). Rather than before V13, cleavage now occurred predominantly before K15, and the intensities of both ACTH5–12 and ACTH2–17 were clearly diminished. When HMEC-1 membranes were incubated with rhuACE-18–39, ACTH17–39, or ACTH16–39 were identical with those detected after incubation with HDMEC membranes in the absence of enzyme inhibitors. Therefore, ACE, which is abundantly expressed by HDMECs, may to a major degree be responsible for the generation of these peptides by HDMECs (Fig. 3D).

**Fragmentation of POMC peptides by recombiant enzymes**

Next, ACTH1–39 and α-MSH were incubated with recombinant human NEP (rhuNEP) or rhuACE to identify POMC cleavage products that were specifically generated by these enzymes. Stable peptides identified after incubation of ACTH with rhuNEP were predominantly cleaved before hydrophobic amino acids (V, Y, F) (Table 1). Likewise, when α-MSH was incubated with rhuNEP, a characteristic cleavage pattern was observed (Fig. 4D and Table 3).
Fig. 4. Fragmentation of ACTH and α-MSH by HMEC-1 membranes. ACTH$_{1-39}$ (A and C) or α-MSH (B and D) was incubated for 60 min at 37°C with cell membranes from HMEC-1 without enzyme inhibition (A and B) or in the presence of the NEP inhibitors PA/TP (1 μM) each (C and D). The resulting fragments were purified, desalted, and subjected to MALDI-TOF analysis (A–D). Note that the fragmentation profile of ACTH is changed by the addition of the NEP inhibitors (C), and this addition also reduces the intensities of some of the detected α-MSH fragments or removes them (D).

age pattern could be observed (Fig. 5A and Table 2), which depended on the amount of protease used (Table 3). Notably, some cleavage products were identical with those obtained with HMEC-1 membranes (Fig. 4, A and B). This α-MSH proteolysis could be dramatically reduced by the addition of NEP inhibitors (PA/TP; Fig. 5C), whereas the addition of the ACE inhibitor captopril, ACE degraded α-MSH from the N terminus (Fig. 5D). All stable fragments obtained were verified by electrospray ionization (ESI)-MS/MS as exemplified for MSH$_{2-12}$ (Fig. 5F). Selected peptides such as α-MSH$_{2-12}$, which was generated from ACTH$_{1-39}$, ACTH$_{1-24}$, and ACTH$_{4-14}$ and α-MSH after H6, F7, and G10 (Fig. 5B). In the presence of the ACE inhibitor captopril, ACE degraded α-MSH from the N terminus, suggesting that products were not generated by these peptidases. In addition, incubation of ACTH with HMEC-1 membranes in the presence of serine protease inhibitors (phenylmethylsulfonylfluoride) resulted in a partially reduced generation of ACTH$_{2-17}$, suggesting that serine proteases may participate in the generation of this peptide. Likewise, the addition of EDTA resulted in an overall reduction in the amount of cleavage products observed (Fig. 5E and Table 3).

TABLE 1. Cleavage of ACTH$_{1-39}$ by rhuNEP after 30 min

<table>
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<th>Mass [M–H]$^-$</th>
<th>Fragment</th>
<th>Sequence</th>
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<td>2572.46</td>
<td>1-21</td>
<td>SYSMHFPWFGKPVGKKRRPYPK</td>
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<td>2346.24</td>
<td>1-19</td>
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<td>2006.05</td>
<td>2-17</td>
<td>YSMHFPWFGKPVGKKRR</td>
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<td>13-25</td>
<td>YGKRRPYPKVYPN</td>
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<tr>
<td>1524.70</td>
<td>1-12</td>
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<td>YSMHFPWFGKPVGKKRR</td>
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<td>1187.57</td>
<td>4-12</td>
<td>MEHPFGGKPVGKKRR</td>
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<tr>
<td>869.56</td>
<td>10-17</td>
<td>GTPGKVKKRR</td>
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</table>

α-MSH after H6, F7, and G10 (Fig. 5B). In the presence of the ACE inhibitor captopril, ACE degraded α-MSH from the N terminus, suggesting that products were not generated by these peptidases. In addition, incubation of ACTH with HMEC-1 membranes in the presence of serine protease inhibitors (phenylmethylsulfonylfluoride) resulted in a partially reduced generation of ACTH$_{2-17}$, suggesting that serine proteases may participate in the generation of this peptide. Likewise, the addition of EDTA resulted in an overall reduction in the amount of cleavage products observed (Fig. 5E and Table 3).
of peptide fragments, particularly of ACTH5–10, ACTH5–12, or ACTH5–14, indicative of a pivotal role of divalent cations in the processing of ACTH (data not shown).

**Functional aspects of POMC peptide fragmentation on MC signaling**

To analyze the functional consequences of POMC peptides processing by NEP or ACE for the activation of EC MC1 receptors, HMEC-1 cells were stimulated with various concentrations of α-MSH or ACTH1–39. After 15 min, a dose-dependent induction of cAMP was observed (Fig. 6, A and B). Notably, when HMEC-1 cells were transfected with a HA-tagged-MC1 expression vector, the cAMP induction by POMC peptides but not that induced by the adenylyl cyclase-stimulating agent forskolin (FSK) was significantly increased in comparison with unstimulated controls, and cells not transfected with HA-MC1 (Fig. 6, A and B). To determine, whether proteolytic processing of α-MSH or ACTH1–39 potentially modulates MC1-signaling, cells were incubated with synthetic α-MSH2–12 as a model peptide, which was identified by MS/MS in supernatants after incubation of α-MSH or ACTH1–39 with both rhuNEP and NEP-expressing HMEC-1 cells (Tables 1–3 and Fig. 4A). In MC1-transfected HMEC-1 (Fig. 6C) or HEK293 cells (Fig. 6D) α-MSH2–12, but not the C-terminal tripeptide KPV (α-MSH11–13) is still capable of inducing cAMP amounts comparable with native α-MSH or ACTH1–39 with both rhuNEP and NEP-expressing HMEC-1 cells (Tables 1–3 and Fig. 4A). In MC1-transfected HMEC-1 (Fig. 6C) or HEK293 cells (Fig. 6D) α-MSH2–12, but not the C-terminal tripeptide KPV (α-MSH11–13) is still capable of inducing cAMP amounts comparable with native α-MSH or full-length ACTH1–39 (Fig. 6, C and D). Thus, a C- and N-terminal truncation of α-MSH does not impair MC1 signaling. Stimulation of MC1-transfected HMEC-1 with POMC peptides in the presence of PA also significantly increased the intracellular content of cAMP in comparison with cells not treated with this NEP inhibitor (Fig. 6E), indicating that NEP inhibition augments functional MC1 activity. Noteworthy,

**TABLE 2.** Cleavage of α-MSH by rhuNEP after 30 and 120 min

<table>
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<th>Mass [M+H]+</th>
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<td>1535.75</td>
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<tr>
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<td>2–12</td>
<td>YSMHEFWKGKPV</td>
</tr>
<tr>
<td>1187.57</td>
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<td>790.43</td>
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All masses are displayed and calculated as monoisotypic with one proton (mass = 1) added [M+H]+.
TABLE 3. Qualitative intensity values of α-MSH fragmentation after digestions with rhuNEP (1, 0.1 μg) or human microvascular membranes (100, 10 μg)

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<th>Peptide</th>
<th>m/z</th>
<th>Sequence</th>
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<th>NEP 0.1</th>
<th>NEP 1.0 PA/TP</th>
<th>NEP 0.1 PA/TP</th>
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<th>HM 10</th>
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<td>100</td>
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<td>100</td>
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<td>100</td>
</tr>
<tr>
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</table>

Qualitative intensity values of α-MSH fragmentation after digestion with rhuNEP (1, 0.1 μg) or human microvascular endothelial cell line-1 (HM) membranes (100, 10 μg). NEP cleavage occurs preferentially N-terminal of hydrophobic amino acids (bold underlined letters). The five most intense fragments are highlighted in bold.

NEP inhibition also increased activity of MSH<sub>2–12</sub> on the MC<sub>1</sub> suggesting that this peptide may subject to further degradation by PA-inhibited enzymes (Fig. 6F). Moreover, ACTH<sub>1–39</sub>, α-MSH<sub>2–12</sub> and FSK induced luciferase activity in HMEC-1 cells transfected with the luciferase cis-reporter plasmid pCRE-Luc (Fig. 6F). Thus, cAMP accumulation induced by POMC peptides after MC<sub>1</sub> activation results in downstream activation of CRE-regulated gene expression.

Colocalization of components that modulate MC<sub>1</sub> signaling in membrane rafts

Signaling and endocytosis of G protein-coupled receptor (GPCR) frequently requires association of receptor components in membrane microdomains (rafts) enriched for cholesterol and caveolin (30). In HA-MC<sub>1</sub> transfected HMEC-1, treatment with the cholesterol-binding and -depleting agent filipin impaired the capability of α-MSH and the proteolysis-resistant analog [Nle<sup>8</sup>, p-Ph<P>]-α-MSH to induce cAMP (Fig. 7A), suggesting that intact, cholesterol-rich membrane microdomains are required for MC<sub>1</sub> signaling. Next, HA-MC<sub>1</sub>-transfected HMEC-1 cells were lysed in the cold in the presence of Triton X-100 and subjected to sucrose gradient fractionation. Interestingly, after SDS-PAGE and Western blotting, MC<sub>1</sub> IR could be detected in low-density gradient fractions positive for caveolin-1 (Fig. 7B) and other membrane markers such as Gαq (data not shown). We also detected NEP IR in these low-density but also in high-density fractions, favoring the idea that NEP and MC<sub>1</sub> may be colocalized in distinct membrane rafts.

Effect of selected POMC peptides on ACD in mice

α-MSH is an established, highly potent antiinflammatory and immunosuppressive mediator (31). To examine, whether α-MSH<sub>2–12</sub> in addition to its MC<sub>1</sub>-activating properties in vitro is still antiinflammatory in vivo, we analyzed its capacity to modulate murine ACD responses in comparison with ACTH and α-MSH. Accordingly, when DNFB-sensitized mice were epicutaneously treated with the respective peptides before and after antigen ear challenge, α-MSH<sub>2–12</sub> in addition to α-MSH and/or ACTH<sub>1–39</sub>, displayed a significant anti-inflammatory activity on ACD elicitation as revealed by reduced allergic ear swelling responses in comparison with mice not treated with the peptides (Fig. 8). Reduced ear-swelling responses were accompanied by clearly reduced histological signs of inflammation 48 h after antigen challenge in ears treated with the peptides (data not shown). The effect of peptide application on the ear swelling without antigen challenge as well as DNFB treatment of mice not sensitized with DNFB did not exert any significant change in ear swelling (data not shown). Thus, the truncated NEP-derived fragment α-MSH<sub>2–12</sub> retains its antiinflammatory properties in this model of skin inflammation.

Discussion

In this study we demonstrate that NEP and ACE, two important ectopic peptides expressed by ECs, are highly relevant for the proteolytic processing of POMC peptides such as ACTH<sub>1–39</sub>, ACTH<sub>1–24</sub> and α-MSH. We used membranes from HDMECs and a HDMEC-derived EC line (32) that share EC-phenotypical properties (33) but notably differ in their reciprocal expression and regulation of NEP and ACE (34, 35). A similar inversely correlated regulation of NEP and ACE expression has been observed in spontaneously hypertensive rats (36), and the opposing roles of ACE and NEP due to a competitive cleavage of vasoactive peptides such as angiotensin or bradykinin are highly relevant for cardiovascular homeostasis. Thus, the above cells constitute a suitable EC model to analyze the role of a differential peptidase expression on the processing of external mediators.

Incubation of ACTH<sub>1–39</sub> with EC membranes clearly decreased the ACTH IR in the membrane supernatants over time, causing a temporary NEP-dependent increase in α-MSH IR. A similar processing of ACTH<sub>1–39</sub> or a parasite-
derived POMC-like hormone by NEP-expressing human granulocytes or invertebrate immunocytes, respectively, has been reported (37–39). Although this suggested a NEP-dependent generation of bioactive \( \alpha\)-MSH, the authors did not rule out an endogenous \( \alpha\)-MSH release in their cell system. In contrast, we excluded the possibility that EC-derived POMC peptides (19) may interfere with the analysis of POMC peptide processing by using a cell-free system. Moreover, MALDI-TOF MS and ESI-MS analysis did not reveal the presence of \( \alpha\)-MSH (acetyl-ACTH\(_{1–13}\)-amid) after incubation of ACTH\(_{1–39}\) with EC membranes or rhuNEP, thus excluding a direct ACTH-to-\( \alpha\)-MSH conversion. In addition,

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**Fig. 6.** Modulation of MC\(_1\) signaling in HMEC-1 and HEK293 cells. HMEC-1 were transfected with a HA-tagged expression construct containing the full-length cDNA of human MC\(_1\) (HA-MC\(_1\)). Transfected and untransfected control cells pretreated with IBMX were stimulated for 15 min with various concentrations of \( \alpha\)-MSH (A), ACTH\(_{1–39}\) (B), or the \( \alpha\)-MSH peptides \( \alpha\)-MSH\(_{2–12}\) and \( \alpha\)-MSH\(_{11–13}\) (C), and the intracellular cAMP content was determined by enzyme immunoassay. Treatment with 10 \( \mu \)M FSK served as control in these experiments. HEK293 were HA-MC\(_1\)-transfected (D) and stimulated with 10 \( \mu \)M of the indicated peptides. ACTH\(_{1–39}\), \( \alpha\)-MSH, \( \alpha\)-MSH\(_{2–12}\), but not \( \alpha\)-MSH\(_{11–13}\) elicited a cAMP response in transfected but not untransfected HEK293 cells. HA-MC\(_1\)-transfected HMEC-1 cells were treated with 10 \( \mu \)M ACTH\(_{1–39}\), \( \alpha\)-MSH, or \( \alpha\)-MSH\(_{2–12}\) in the presence of 1 \( \mu \)M of the NEP inhibitor PA (E). HMEC-1 cells transfected with the luciferase reporter plasmid pCRE-Luc were stimulated with ACTH\(_{1–39}\), \( \alpha\)-MSH\(_{2–12}\), or FSK for 15 min, and luciferase activity was determined after 4 h. (F). The cAMP contents (A–D and F) or the luciferase activity (F) are expressed in percent as mean ± SEM with unstimulated controls set to 100% (n = 3 independent experiments). *, \( P < 0.05 \) vs. control; **, \( P < 0.01 \) vs. control; ***, \( P < 0.001 \) vs. control (A–D); #, \( P < 0.05 \) vs. cells not treated with the NEP inhibitor (E).
we identified several larger and shorter ACTH fragments that may have caused false-positive results in the α-MSH RIA used.

The specificity of some but not all of the fragments generated from ACTH1-39 or ACTH1-24 by EC membranes could be assigned to NEP or ACE activity by switching the pH to a value suboptimal for these enzymes, applying specific enzyme inhibitors, or comparing the peptide profile with that generated from ACTH1-39 or ACTH1-24 by EC membranes. Likewise, a peptide at m/z 1662 was routinely detected, which did not correspond to any regular proteolytic peptide but could be derived from ACTH1-14 with an intramolecular loss of water. This dehydration process originates from the sample and not from MS processes and the assignment was supported by ESI-MS/MS. However, there are a number of open questions surrounding that peptide and its purification is in progress for further clarification. Importantly, EC membranes also digested α-MSH to peptides such as MSH/ACTH4-12, MSH4-13, MSH/ACTH2-12, or MSH2-13. A preferred cleavage occurred in the N terminus of the ACTH/α-MSH molecule at positions S1-Y2, H6-F7, and K12-V13 (ACTH), which is in good agreement with the proposed cleavage pattern for NEP N terminal of large hydrophobic amino acid residues (40). In addition, rhuNEP and particularly EC membranes also cleaved α-MSH/ACTH1-39 at somewhat unexpected sites (M4-E5, E5-H6, G9-K10, G14-K15, or R17-I18). Thus, ACE and particularly NEP specificity is not always confined to the above cleavage pattern, and with respect to NEP, variations have been described. In general, NEP has broader substrate specificity and cleaves peptides as large as 17 kDa, although true endopeptidase activity preferentially occurs within smaller linear peptides such as tachykinins, gastrin, or cholecystokinin-8 (41).

ACE has two structurally related zinc binding sites that both posses endopeptidase and dipetidyl carboxypeptidase activity but differ with respect to pH/chloride dependency and substrate specificity (42, 43). For example, a higher selectivity of the ACE inhibitor captopril described for the N-terminal catalytic site (44) may partly explain why inhibition of ACE by captopril, rather than completely preventing ACE-dependent α-MSH cleavage, resulted in degradation of MSH from the N terminus. Interestingly, NEP inhibition of ACTH degradation by ACEhigh/NEPlow-expressing HDMECs predominately resulted in a loss of N terminus-derived ACTH degradation products, whereas in contrast, ACE inhibition prevented the generation of larger C-terminal fragments (e.g., ACTH18-39 or ACTH15-39). Thus,
NEP and ACE may competitively cleave ACTH, which is a common way of modulating substrate bioactivity. For instance, whereas ACE cleaves Ang I to the hypertensive and proinflammatory peptide Ang II (Ang$_{1-8}$), NEP generates Ang$_{1-7}$, which acts as endogenous inhibitor of Ang II (45). Interestingly, the presence of peptides such as α-MSH$_{1-10}$ or α-MSH$_{2-12}$ after ACE-mediated α-MSH cleavage suggest that the ACE carboxypeptidase activity could be of in vivo relevance for the liberation of the C-terminal tripeptide α-MSH$_{1-13}$ (KPV), which is sufficient to mimic α-MSH-like antiinflammatory and immunosuppressive activities in vivo (46). In line with our findings, melanoma cell lines (47) as well as melanocytes displayed the ability to process α-MSH in a PA-sensitive way indicating a (patho)-physiological role for NEP in malignant melanoma as well as in pigmentation (48). Likewise, ACE pH-dependently metabolizes ACTH$_{1-39}$ in the central nervous system, which may be particularly relevant for pituitary functions (Ref. 16 and references therein). For instance, ACTH$_{1-28}$ was predominantly degraded to ACTH$_{1-16}$ ACTH$_{17-39}$, ACTH$_{22-39}$, and ACTH$_{23-39}$ by rat brain synaptic membranes at pH 8.5 (49), which resembled the ACTH$_{1-39}$ peptide profile obtained from HDMECs or rhuACE in our experiments. The detected ACTH$_{1-15}$ and ACTH$_{1-14}$ could be derived from ACTH$_{1-16}$ trimming by residual ACE carboxypeptidase activity.

MC receptors in mouse and men signal via coupling to heterotrimeric G proteins, resulting in adenylyl cyclase-dependent cAMP synthesis, protein kinase A activation, and CRE-dependent gene expression (reviewed in Refs. 17, 50). In accordance with this notion and similar observations for HDMECs (51), ACTH$_{1-39}$ or α-MSH induced cAMP in NEP/huNEP expressing HMEC-1 as well as luciferase activity in CRE-luciferase reporter-transfected cells. α-MSH and ACTH displayed a biphasic stimulatory effect on cAMP in the micro- as well as the lower nanomolecular range. Although there is no experimental evidence of MC receptors other than MC$_1$ expressed on microvascular ECs (51), the recently observed homo- or heterodimerization of the MC$_1$ (52) with a not-yet-identified partner on EC membranes may account for this effect, resulting in MC receptors with different affinities for one ligand. Importantly, POMC peptide peptidolysis was functionally relevant because the induction of cAMP was significantly augmented by the following conditions: 1) after adding NEP inhibitors, 2) by increasing the number of active binding sites after MC$_1$ transfection, or 3) by using the pro-peptidolysis-resistant α-MSH analog Nle$^3$-d-Phe$^7$-α-MSH (data not shown). The latter is modified at sites particularly susceptible to NEP cleavage, indirectly confirming that NEP is a major player in α-MSH/ACTH degradation. However, we also noted that the NEP-derived ACTH/α-MSH fragment α-MSH$_{2-12}$ retained MC$_1$-activating properties in vitro and antiinflammatory bioactivity in vivo comparable with the parental peptides. In contrast, KPV did not trigger cAMP signaling, even in MC$_1$-transfected HMEC-1 or HEK293 cells. Although KPV-induced Ca$^{2+}$ signaling in HaCaT cells and keratinocytes (53), it does not bind to the MC$_1$ or other MCx and its mode of action is still a matter of debate (50).

Several ACTH/MSH fragments identified after EC membrane or NEP/ACE processing contained the POMC peptide pharmacophore sequence HFRW (ACTH$_{6-9}$), the minimum sequence required for unspecific binding to MC$_{x}$. Outside this sequence, M4 and P12 are of higher importance for MC$_1$ binding, whereas the first three N-terminal amino acids appear to be dispensable. ACTH$_{1-10}$ is a weak agonist for MC$_1$ with EC$_{50}$ values in the lower micromolar range (50). In MC$_1$-transfected HEK293 cells or human melanocytes, α-MSH and ACTH$_{1-17}$ showed similar binding affinity for human MC$_1$, whereas binding affinities for ACTH$_{1-39}$, desacyetyl-α-MSH, and ACTH$_{1-10}$ were lower. ACTH$_{1-12}$ is more potent than acetylated α-MSH in stimulating melanogenesis, indicating that this peptide is a powerful ligand of MC$_1$. In agreement with this observation, site-directed mutagenesis of the human MC$_1$ recently revealed ACTH$_{1-17}$ as the thermodynamically favorable ligand for this receptor (22, 54, 55). The considerable bioactivity of α-MSH$_{2-12}$ in our studies confirms that a slight N- and C-terminal truncation of α-MSH does not interfere with its MC$_1$ activating properties. Notably, we also detected ACTH$_{2-17}$ as an ACTH$_{1-19}$ product of EC membranes and rhuNEP.

Cleavage of ACTH$_{1-39}$ at position R17-R18 and the subsequent trimming of ACTH$_{1-17}$ by carboxypeptidase E activity followed by amidation and acetylation is an important step in the intracellular generation and maturation of α-MSH (3, 56). Importantly, because the digestion after R17 has been exclusively put down to activity of PC2 or related serine proteases, this is the first demonstration of an extracellular peptidase mediating this cleavage. This may partly explain the previously detected α-MSH release by HDMECs in the absence of PC2 and could be highly relevant for EC homeostasis and vascular inflammation (19, 57). Thus, rather than completely inactivating and removing ACTH and α-MSH from the extracellular space, NEP and ACE peptidolysis may generate peptides with novel MC binding and activating properties distinct from the parental peptide. In the absence of the genuine ligands, these ACTH/α-MSH fragments could either function as MC$_1$ agonist or may be local antagonists in the presence of the parental high-affinity peptides. They may also, as in case of KPV, even trigger cellular responses independent from known MC$_{x}$.

A similar biased behavior has been proposed for the NEP substrate SP (58). Accordingly, the SP hydrolysis product SP$_{1-7}$ is mimicking some but opposing other effects of the parental peptide, i.e. the central nociception (59), tumor cell migration, or cancer growth (58, 60–62). Thus, modulation of the bioactivity of neuropeptides by proteolytic products derived from the parental peptide represents a common phenomenon with not yet fully explored physiological consequences (63).

There is increasing evidence that MC$_x$ are capable of forming homo- and heterodimers (52, 64). Such GFCR dimerization and assembly with other signal transduction components frequently occurs in cholesterol-rich membrane rafts. After activation, GPCRs are internalized either in a β-arrestin-dependent manner after traveling to clathrin-coated pits or via specialized lipid raft/caveolae microdomains of the plasma membrane (65). Importantly, cholesterol depletion impaired agonist-induced MC$_1$ signaling in MC$_1$-transfected ECs. Likewise, a colocalized MC$_1$ and NEP IR was detected in low-density membrane fractions positive for membrane and lipid raft markers such as caveolin-1 and G$\alpha_q$. As dem-
onstrated for ACE, NEP, and tachykinin or BK receptors, respectively, the cellular coexpression (66) or a direct sterically close association of receptor and peptidase is important for receptor function and resensitization (67–69). Thus, recruitment of protease and receptor to the same membrane microdomain may also be of functional relevance for MC₁ signaling, although details of a potential association between NEP and MC₁ require further clarification.

In summary, our results demonstrate that the POMC peptides ACTH and α-MSH are substrates for the Zn metalloproteases NEP and ACE, which have an important role in controlling cutaneous inflammation (29, 70) and are expressed in variable levels by dermal microvascular ECs. A local processing of ACTH₁₋₃₉ or longer ACTH precursors derived from various intra- and/or extracutaneous sources, from excessive ACTH precursors present in serum (18) or derived from various intra- and/or extracutaneous sources, is highly relevant for the peripheral physiological and pathophysiological regulation of pigmentation, immunomodulatory, and other responses mediated by this important class of mediators.

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