Neprilysin, a Novel Target for Ultraviolet B Regulation of Melanogenesis Via Melanocortins

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Compelling evidence suggest a role for melanocortins in the regulation of melanogenesis by ultraviolet radiation. Within the epidermis, melanocytes and keratinocytes produce α-melanocyte-stimulating hormone and adrenocorticotropic hormone. The persistence and the strength of the biologic signal delivered by these peptides depend on their local concentration, which is controlled by the rate of peptide production and by the rate of its degradation. In this study, we investigated the mechanism of melanocortin degradation by melanocytes and the effect of ultraviolet on this process. We have focused our attention on a neutral endopeptidase, neprilysin, which has been implicated in the ending of numerous peptidergic signals. We have shown that this enzyme is expressed at the surface of human melanocytes. Interestingly, its activity and its expression are dramatically downregulated by ultraviolet B treatment. Moreover, in the presence of phosphoramidon, a stable inhibitor of neprilysin, we observed an increased efficiency of α-melanocyte-stimulating hormone and adrenocorticotropic hormone to stimulate both tyrosinase activity and microphthalmia expression. Taken together, these data indicate that neprilysin expressed by melanocytes has a physiologic role in the regulation of melanogenesis by proopiomelanocortin peptide. Further, its downregulation by ultraviolet B irradiation shed light on a new and appealing mechanism of ultraviolet B induced melanogenesis via the control of melanocortins degradation. ***Key words: adrenocorticotropic hormone/human melanocytes/neutral endopeptidase/α-melanocyte-stimulating hormone. J Invest Dermatol 115:381–387, 2000***

In humans, pigmentation results from the synthesis and distribution of melanin in the skin. This process called melanogenesis takes place in melanocytes after differentiation of the nonpigmented precursors, the melanoblasts (Le Douarin *et al*, 1992). Three melanocyte-specific enzymes (tyrosinase, TRP1, and TRP2) and a melanocyte-specific transcription factor (microphthalmia) are involved in the regulation of melanogenesis (Yasumoto *et al*, 1994; Yavuzer *et al*, 1995; Bertolotto *et al*, 1998).

Melanogenesis is regulated by ultraviolet (UV) B radiation that can act directly on melanocytes or indirectly through the release of keratinocyte-derived factors such as cytokines, eicosanoids, growth factors, nitric oxide, or melanotropic hormones derived from proopiomelanocortin (POMC) peptides (Swope *et al*, 1991; Abdel-Malek *et al*, 1995; Wintzen and Gilchrest, 1996; Romero-Graillet *et al*, 1997).

POMC was detected primarily in the pituitary gland, but was more recently found in several other regions of the brain and various organs including the skin. In epidermis, keratinocytes would appear to be the major source of POMC peptides such as α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) (Slominski *et al*, 1993; Bhardwaj and Luger, 1994; Wintzen and Gilchrest, 1996; Chakraborty *et al*, 1999), although they are also produced in melanocytes (Wakamatsu *et al*, 1997) and Langerhans cells (Morhenn, 1991). Melanocortins are pleiotropic hormones and until now five subclasses of melanocortin receptors (MC-R) have been described and cloned. To exert their melanogenic effects, α-MSH and ACTH bind to the MC1-R of the melanocytes positively coupled to the cyclic adenosine monophosphate pathway. (Donatien *et al*, 1992; Mountjoy *et al*, 1992; De Luca *et al*, 1993; Hunt *et al*, 1994a).

Among keratinocyte-derived agents, the melanotropic hormones α-MSH and ACTH appear to be very potent stimulators of human pigmentation. Indeed, *in vivo* experiments showed that these peptides could increase skin darkening in humans (Lerner and McGuire, 1961; Levine *et al*, 1991). ACTH also increases melanogenesis and dendricity of human melanocytes in culture (Hunt *et al*, 1994b). One of the most abundant POMC peptides in human skin is $ACTH_{1-17}$ (Thody and Graham, 1998). This peptide binds to the human MC-1R with an affinity comparable with that of acetylated α-MSH and is even more potent in stimulating melanogenesis than α-MSH and ACTH (Wakamatsu *et al*, 1997). Interestingly, there is strong evidence demonstrating that expression and release of POMC peptides by cultured keratinocytes is upregulated by UV light, probably at a transcription level (Schauer *et al*, 1994; Chakraborty *et al*, 1995; Wintzen and Gilchrest, 1996). Further, on melanoma cells, UV radiation has also been shown to increase the expression of the MC-1R that mediates the melanogenic effect of α-MSH and ACTH (Chakraborty *et al*, 1991, 1995). Taken together these data support the hypothesis that UV involves melanocortins and MC1-R in the regulation of photo-induced skin pigmentation.

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Abbreviations: α-MSH, α-melanocyte stimulating hormone; ACTH, adrenocorticotropic hormone; POMC, proopiomelanocortin; PA, phosphoramidon; NHM, normal human melanocytes

Noteworthy, is that the biologic function of POMC-derived peptides can be regulated at other levels than the transcription one. Indeed, the melanotropins, α-MSH, ACTH, β-lipotropin, and β-MSH are a family of peptides derived from POMC (Eipper and Mains, 1980). Proteolytic enzymes, known as prohormone convertases PC1 and PC2, with distinct cleavage specificities are required for the processing of melanotropins from POMC. PC1 cleaves POMC into ACTH and β-lipotropin, whereas PC2 cleaves POMC into β-endorphins and N-terminally extended ACTH, which is further cleaved into smaller fragments such as $ACTH_{1–17}$ and desacetyl α-MSH (Benjannet *et al*, 1991). Following proteolytic cleavage, some of the POMC peptides become acetylated ensuring better secretion and biologic action (Dores *et al*, 1993).

Finally, the local concentration of α-MSH and ACTH in epidermis and the persistence of the biologic signal are dependent on the degradation of the peptide by resident proteases. Interestingly, α-MSH is degraded by neprilysin leading to fragments that have lost their ability to bind the MC1-R (Deschodt-Lanckman *et al*, 1990). Neprilysin, also termed enkephalinase, CD10 or neutral endopeptidase 24.11 (EC 3.4.24.11) is a 90–110 kDa zinc-dependent metallopeptidase that cleaves peptide bonds on the amino side of hydrophobic amino acids and is identical to the common acute lymphoblastic leukemia antigen (Shipp *et al*, 1989). Neprilysin inactivates a variety of physiologically active peptides, including neurotensin, met-enkephalin, substance P, bombesin and endothelin-1, thereby reducing local concentrations of peptides available for receptor binding and signal transduction (Kenny, 1993; Roques *et al*, 1993). Neprilysin is normally expressed by a wide range of tissues and cells, including human melanoma cells (Carrel *et al*, 1983).

We report here that neprilysin is highly expressed by human melanocytes and that its expression and catalytic activity are downregulated by UVB light. In addition, we show that α-MSH and ACTH are specific substrates for neprilysin and that specific inhibition of neprilysin increases the melanogenic activity of these peptides on human melanocytes. These data indicate that neprilysin inactivation by UVB in melanocytes may contribute to enhance the POMC paracrine loop mediating UV-induced pigmentation.

MATERIALS AND METHODS

Cell culture Normal human melanocytes (NHM) were grown in MCDB 153 supplemented with 2% fetal bovine serum (Hy-Clone, Logan, UT), 1 ng basic fibroblast growth factor per ml (Promega, Cergy Poutoise, France), 10 μM forskolin, 8 nM phorbol-12-myristate-13-acetate (TPA) (Sigma, Saint Quentin Fallavier, France), 10 μg bovine pituitary extract per ml (Gibco, Life Technologies, Charbonnières, France), 5 μg insulin per ml, 0.5 μg hydrocortisone per ml (Sigma), and penicillin/streptomycin (100 i.u./50 μg per ml) in a humidified atmosphere containing 5% CO_2 in air at 37°C. Before experiments, cells were placed in minimal growth medium composed of MCDB 153 containing 2% fetal bovine serum, 1 ng basic fibroblast growth factor per ml, 5 μg per ml insulin and 0.5 μg hydrocortisone per ml. MeWo human melanoma cells were maintained in Dulbecco's modified Eagle's medium containing 7% fetal bovine serum.

Irradiation procedure The source of ultraviolet radiation was a Bio-Link BLX-312 (Vilber Lourmat, Marne la Vallée, France) fitted out with a UVB irradiation source composed of five tubes of 8 W at 312 nm wavelength and a UV energy programming system in joules per cm^2. Cells were irradiated in phosphate-buffered saline (PBS), the culture dish lid being removed.

Immunofluorescence microscopy Cells were seeded on glass coverslips 2 d before the experiments, fixed in 3% paraformaldehyde and then incubated with 50 mM NH_4Cl. NHM and MeWo cells were labeled first with anti-neprilysin monoclonal antibody (mouse anti-human CD10, Immunotech, Marseille, France) diluted $1/50^e$ in PBS containing 0.1% bovine serum albumin at 4°C overnight, followed by biotinylated horse anti-mouse immunoglobulins (Dako, Trappes, France) diluted 1/150 for 1 h, and avidin conjugated Texas red, diluted 1/300 for 30 min. Cryosections of human skin were labeled with anti-TRP1 polyclonal antibody diluted 1/100 in PBS containing 0.1% bovine serum albumin at 4°C overnight, followed by fluorescein isothiocyanate conjugated goat anti-rabbit immunoglobulins diluted 1/100 for 1 h. Same section was labeled with anti-neprilysin monoclonal antibody as described above. Immunofluorescence was observed and photographed with a Zeiss-Axiophot fluorescence microscope (Carl Zeiss, Thorwood, NY) by using Kodak T-Max 400 isofilm.

Determination of neprilysin activity Neprilysin activity was determined as previously described (Mari *et al*, 1992). At harvest, cells were washed with PBS and scraped in 0.1 M phosphate buffer pH 6.8, containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) (1 mM), leupeptin (5 μg per ml), and aprotinin (2 μg per ml). This cellular suspension was sonicated on ice, centrifuged at $11000 \times g$ for 5 min and the pellet resuspended in the same phosphate buffer containing 1% Triton X-100. Protein content of this crude membrane preparation was determined by detergent compatible protein assay Bio-Rad (Ivry sur Seine, France). Ten to 50 μg proteins were incubated at 37°C, in 200 μl PBS containing 1 mM Suc-Ala-Ala-Phe-pNA (Bachem, Voisins le Bretonneux, France) as the substrate and 10 μg per ml purified aminopeptidase N (Roche Molecular Biochemicals, Meylan, France). Neprilysin cleaved the substrate at the Ala–Phe bond. Paranitroaniline was then liberated from Phe-pNA by exogenous aminopeptidase N. At different times, endopeptidase activity was determined by measuring the absorbance of paranitroaniline at 410 nm using reaction mixture without membrane proteins as blank. Specific activities were expressed as nanomoles per milligram protein per minute and represent the average of at least three separate measurements. When specified, neprilysin inhibitors were preincubated for 15 min before determination of enzymatic activity.

Determination of tyrosinase activity Tyrosinase activity was estimated by measuring the rate of oxidation of L-Dopa (Takahashi and Parsons, 1992). Two days before the experiment, NHM were placed in minimal growth medium. Then cells were stimulated for 4 d with different concentrations of α-MSH, [Nle^4,D-Phe^7]α-MSH or $ACTH_{1–17}$ in the presence or in the absence of 10 μM phosphoramidon (PA). At the end of treatment period, cells were washed in PBS and lyzed in 0.1 M phosphate buffer pH 6.8 containing 1% Triton X-100, 1 mM AEBSF, leupeptin (5 μg per ml), and aprotinin (2 μg per ml). Protein content was determined by detergent compatible protein assay Bio-Rad and 20 μl (10–30 μg protein per ml) was incubated with 100 μl of L-Dopa solution (2 mg per ml) at 37°C. At different times, Dopa-oxidase activity was determined by the measure of absorbance at 560 nm and a plot against time was represented for each condition. The final activity was expressed in ΔOD per minute per milligram of protein and results are presented as a percentage of basal activity (control condition).

Determination of melanin content Two days before the experiment, NHM were placed in minimal growth medium. Then cells were stimulated for 4 d with 10^{-8} M of α-MSH or $ACTH_{1–17}$ in the presence or in the absence of 10 μM PA. At the end of the treatment period, cells were washed in PBS, trypsinized, and an aliquot was set apart for cell counting using a Coulter counter. The remaining was centrifuged and the pellet dissolved in NaOH 1 M for 2 h at 65°C. Melanin concentration was determined by measurement of OD_{475nm} and comparison with a synthetic melanin standard curve.

Western blots Cells were treated as indicated in figure legends of **Figs 4** and **6**. At the end of each treatment period, cells were washed in PBS and lyzed in 0.1 M phosphate buffer pH 6.8 containing 1% Triton X-100, AEBSF (1 mM), leupeptin (5 μg per ml), and aprotinin (2 μg per ml). Protein content was determined by detergent compatible protein assay Bio-Rad and equal amount of protein extracts was resolved using 7.5 or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Following transblotting on to nitrocellulose membranes and blocking in 5% nonfat milk in saline buffer, the membranes were incubated with monoclonal antibodies directed against neprilysin (K50) at a dilution of 1/4000 or directed against microphthalmia (C5) at a dilution of 1/10. To check equivalent loading and transfer efficiency, anti-ERK2 antibodies were used (1/3000). The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit or mouse IgG (Dako) at a dilution of 1/4000. The immunoreactive bands were detected by chemiluminescence, using the ECL Amersham kit.

RESULTS AND DISCUSSION

Human melanocytes express a functional neprilysin α-MSH and ACTH belong to the family of melanocortins because they are peptides derived from POMC, with melanotropic (or adrenocorticotropic) activity and share an essential tetrapeptide

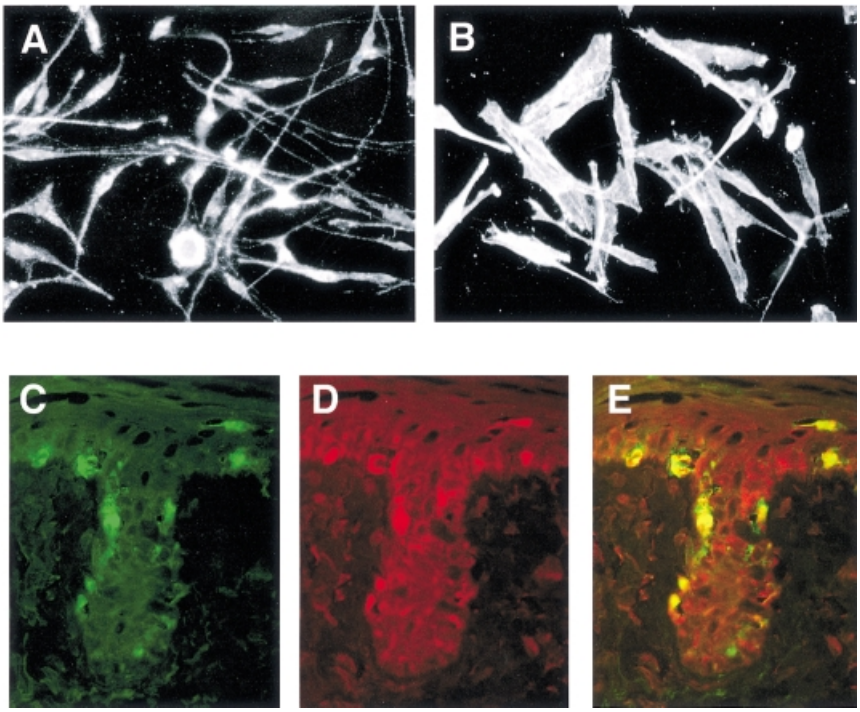

Figure 1. NHM and MeWo cells express Neprilysin. NHM (*A*) and MeWo cells (*B*) were labeled with anti-neprilysin monoclonal antibody as described in *Materials and Methods*. Cryosections of human skin were labeled with anti-TRP1 polyclonal antibody (*C*), same section was labeled with anti-neprilysin monoclonal antibody (*D*). Co-localization of the immunofluorescent stainings is shown in (*E*).

Table I. Neprilysin activity in melanocytes from different donors and in MeWo melanoma cells in the presence or in the absence of 10^{-5} M PA

	Neprilysin activity (nmol per min per mg protein)		
	–PA	+PA	% Inh.
NHM 1	25	1.2	95
NHM 2	50	2	95
NHM 3	7.5	0.3	97
NHM 4	18	0.85	96
MeWo	29	1.5	94

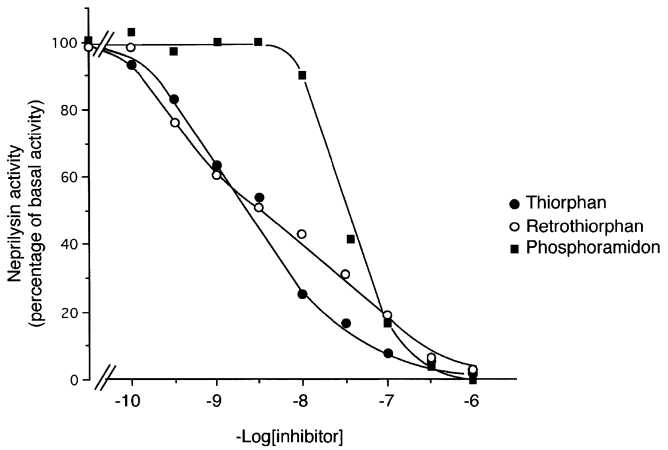

Figure 2. Effect of various protease inhibitors on the hydrolysis of Suc–Ala–Ala–Phe–pNA. NHM were incubated for 15 min at 37°C with different concentrations of thiorphan, retrothiorphan, or PA. Then, crude membrane extracts were prepared and neprilysin activity measured as described under experimental procedure. Thirty micrograms per milliliter proteins were used for each determination. Results are expressed as a percentage of maximal activity (25 nmol per min per mg protein). Data points are means of triplicates.

core [His, Phe, Arg, Trp] for binding and activation of their receptor MC1R (Lu *et al*, 1998). Because of its preference for small peptides (no more than 20 amino acids) and its great affinity to cleave a peptidic bond on the amino side of hydrophobic amino acids such as phe, tyr, and trp, we wondered whether neprilysin was expressed by human melanocytes and involved in the degradation of melanocortins. Human melanoma cells express neprilysin but its presence on NHM has not been yet investigated (Deschodt-Lanckman *et al*, 1990). To assess if human melanocytes also expressed a functional neprilysin, we performed immunofluorescence staining of human melanocytes (**Fig 1*A***) and MeWo melanoma cells (**Fig 1*B***) with a specific monoclonal antibody directed against neprilysin. We observed a strong labeling at the cell surface in both cell types whereas an irrelevant monoclonal antibody of the same isotype (IgG1), used as a negative control failed to reveal any fluorescent cells under the same conditions (data not shown). Melanocytes also express neprilysin *in vivo*. Indeed, immunostaining of human epidermis with a melanocyte-specific antibody (anti-TRP1 polyclonal antibody, **Fig 1*C***) and with a specific monoclonal antibody directed against neprilysin (**Fig 1*D***) shows the colocalization of neprilysin with melanocytes (**Fig 1*E***). The ability of human melanocytes and MeWo cells to hydrolyze the neprilysin substrate Suc–Ala–Ala–Phe–pNA was next assessed. **Table I** shows some variability in the levels of neprilysin activity among melanocyte cultures established from different donors, ranging from 7.5 to 50 nmol per min per mg protein; however, the values obtained for human melanocytes and MeWo cells are comparable with the levels of neprilysin activity measured in tissues and cells known to exhibit strong neprilysin expression such as kidney, thymus epithelia, or endothelial cells (Kanazawa and Johnston, 1991; Llorens-Cortes *et al*, 1992; Guerin *et al*, 1997). To confirm unambiguously the hydrolysis activity as neprilysin, several specific inhibitors were used. **Figure 2** shows that PA, thiorphan, and retrothiorphan, three potent neprilysin inhibitors, abrogated enzymatic activity at 10^{-6} M, with IC_{50} at 20, 2, and 3 nM, respectively. These IC_{50} values are in agreement with those previously reported in T lymphocytes and thymus epithelial cells (Mari *et al*, 1992; Guerin *et al*, 1997). Because of its greater stability in culture media as compared with thiorphan or retrothiorphan, we chose PA as the neprilysin inhibitor in the following experiments.

Incubation of membrane extracts from human melanocytes and MeWo cells with PA at 10^{-5} M, resulted in more than 90%

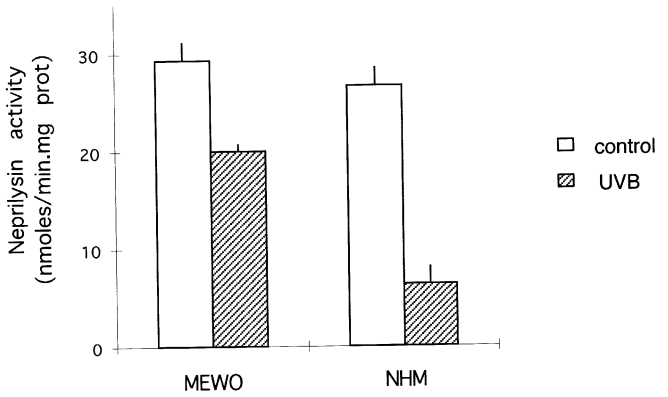

Figure 3. Neprilysin activity is regulated by UVB. Two days before the beginning of the experiment, NHM were placed in growth medium diluted 1:4. Cells were irradiated daily for 2 d with 75 mJ per cm^2 for NHM and 30 mJ per cm^2 for MeWo cells. Cells were irradiated in PBS, then replaced in fresh medium. Twenty-four hours after the last irradiation, neprilysin activity was measured in membrane extracts. Data are mean ± SE of three experiments performed in triplicates.

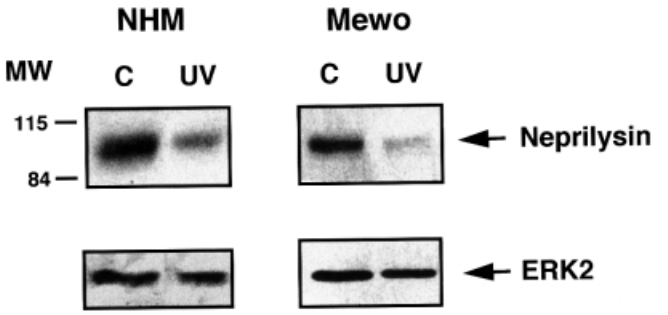

Figure 4. Neprilysin expression is regulated by UVB. Two days before the beginning of the experiment, NHM were placed in growth medium diluted 1 : 4. Cells were irradiated daily for 2 d with 75 mJ per cm^2 for NHM and 30 mJ per cm^2 for MeWo cells. Cells were irradiated in PBS, then replaced in fresh medium. Twenty-four hours after the last irradiation, protein extracts were prepared and 100 μg proteins for NHM and 50 μg proteins for MeWo cells were electrophoresed on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes. Specific detection of neprilysin was then performed. ERK2 detection was used as an internal control for comparable loading and transfer in all lanes.

inhibition of enzyme specific activity (**Table I**). The results obtained with the inhibitors of neprilysin confirm that the enzyme activity detected was neprilysin and not a related endopeptidase present in the melanocyte. Indeed, it is noteworthy that human melanocytes are proteolitically active cells with a plasminogen activation system (Bizik *et al*, 1996), matrix metalloproteinases such as gelatinases or collagenases (Durko *et al*, 1997), and other members of the ectopeptidase family such as the protease dipeptidyl peptidase IV (Morrison *et al*, 1993).

neprilysin is regulated by UVB irradiation A previous study made in mouse melanoma cells S91 and in transformed keratinocytes Pam 212 suggested a system of positive feedback regulatory steps as a mechanism for UVB-induced pigmentation (Chakraborty *et al*, 1995). These authors showed that UVB treatment upregulated cellular responsiveness to MSH/MSH receptor system, through increased MSH receptor synthesis as well as production of the signal peptides, MSH and ACTH that can further activate receptors. Because degradation of MSH and ACTH leads to the end of the melanogenic signal, we thought that inhibition of this process could be part of the positive regulatory

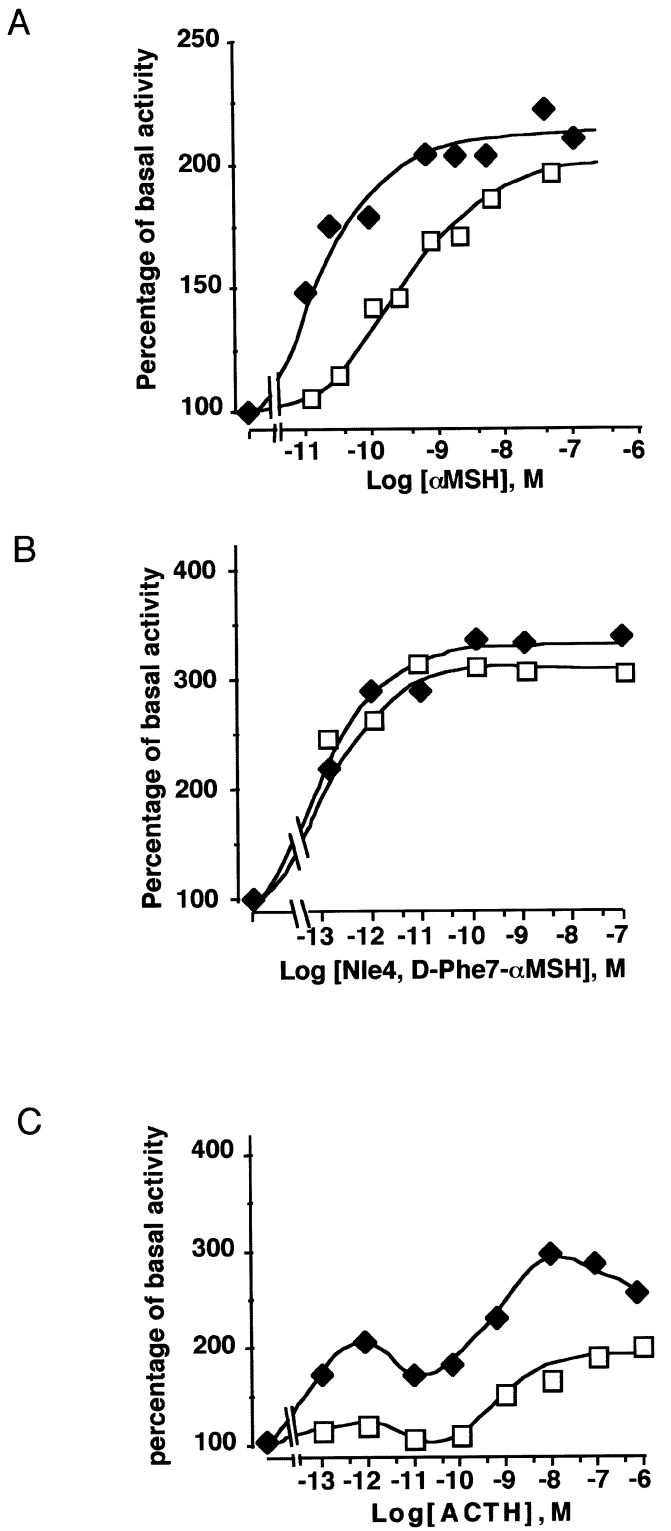

Figure 5. Inhibition of neprilysin by PA enhances tyrosinase activity (Dopa-oxidase activity) stimulated by α-MSH and ACTH in melanocytes. Two days before the beginning of the experiment NHM were grown in minimal growth medium. Cells were incubated for 4 d with increasing doses of α-MSH (*A*), [Nle^4,D-Phe^7]α-MSH (*B*) and $ACTH_{1–17}$ (*C*), in the presence or in the absence of 10^{-5} M PA. Then Dopa-oxidase activity of the tyrosinase was measured. □, Without PA; ◆, with PA, 10^{-5} M.

steps triggered by UVB. To test this, MeWo melanoma cells and NHM were UVB irradiated, with 30 and 75 mJ per cm^2, respectively. Forty-eight hours later, neprilysin activity was

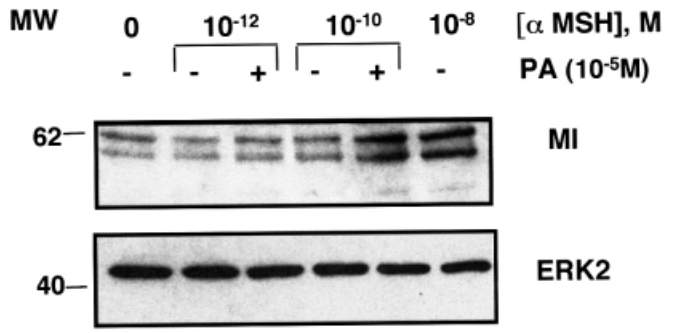

Figure 6. Inhibition of neprilysin by PA enhances the effect of α-MSH on microphthalmia expression. Two days before the beginning of the experiment, NHM were grown in minimal medium. Cells were then incubated for 5 h with different doses of α-MSH in the presence or in the absence of 10^{-5} M PA. Western blot with anti-microphthalmia antibody was performed. ERK2 detection was used as an internal control for comparable loading and transfer in all lanes.

measured in membrane extracts. In both cell types, the enzymatic activity was significantly reduced following UVB treatment as compared with basal conditions (**Fig 3**). The level of inhibition was 35% for MeWo melanoma cells and 70% for human melanocytes.

To elucidate whether UVB negative regulation of neprilysin activity could be attributed only to an inhibition of its enzymatic activity, we examined the expression of neprilysin following UVB irradiation. Western blot analysis using a specific monoclonal antibody directed against neprilysin revealed, in both cell types, decreased levels of neprilysin expression following UVB treatment as compared with basal levels (**Fig 4**). We then studied whether UVB radiation could downregulate the degradation of α-MSH by melanocytes. The addition of α-MSH to melanocyte culture and analysis of the medium by reverse phase high-performance liquid chromatography, gave three degradation products. Unfortunately, after UVB treatment, analysis of melanocytes control medium (without addition of exogenous α-MSH), gave numerous and strong picks in high-performance liquid chromatography that prevented the evaluation of exogenous α-MSH degradation by UV. These picks may represent peptides produced by melanocytes or melanin intermediates whose production or release are stimulated by UVB (data not shown).

UVB has already been described to regulate protein expression in melanocytes, especially enzymes directly involved in melanogenesis, such as tyrosinase, TRP1, and TRP2 (Friedmann and Gilchrest, 1987; Ramirez-Bosca *et al*, 1992; Aberdam *et al*, 1993), but also lysosome-associated membrane proteins (Ota *et al*, 1998) and anti-oxidant enzymes such as catalase (Bessou-Touya *et al*, 1998); however, this is to our knowledge one of the first demonstrations of an inhibitory effect of UVB on a melanocyte protease. Noteworthy, downregulation by UVB does not seem to be a general feature of melanocyte surface peptidases as the activity of dipeptidyl peptidase IV was not found to be modified by UVB treatment (data not shown). Further, expression of matrix metalloproteinase that is increased during malignant transformation of melanocytes and invasive processes, appears to be upregulated in human skin after UV light exposure (Fisher *et al*, 1999).

Regulation of neprilysin in other cellular systems has been widely investigated. Among the main activators of neprilysin, are tumor necrosis factor-α, interleukin-1 (van der Velden *et al*, 1998), and transforming growth factor-β (Tharaux *et al*, 1997). Interestingly, these factors are also potent inhibitors of melanogenesis (Swope *et al*, 1991; Martinez-Esparza *et al*, 1997; Englaro *et al*, 1999), suggesting that the mechanisms implied in melanogenesis inhibition could involve degradation of melanogenic peptides via the activation of neprilysin. On the other hand, inhibitors of neprilysin expression and activity such as interferon-gamma (van der Velden *et al*, 1998), TPA (Howell *et al*, 1993), or capsaicin (Kuo and Lu, 1995) are activators of melanocyte differentiation (Fisher *et al*, 1985; Abdel Malek *et al*, 1992; Morre *et al*, 1996). Interestingly, lost or decreased neprilysin expression has been observed in a variety of malignancies including prostate cancer, leukemia, endometrial cancer, invasive bladder cancer, renal cancer, small cell lung cancer, and breast cancer (Nanus *et al*, 1997; Papandreou *et al*, 1998). It has been proposed that peptidergic signals of growth factors would not be terminated in the absence of neprilysin thereby favoring cell growth and malignancy. The dual effect of UVB radiation on human skin is now well established. On the one hand, they increase melanin production which, following transfer to keratinocytes, ensures protection of the genetic material through melanosomes capping above the nuclei. On the other hand, UV radiation promotes photodamage and photocarcinogenesis of epithelial cells. Recently, neprilysin has been reported to be expressed by keratinocytes (Olerud *et al*, 1999). In these cells, the decrease in neprilysin activity and expression induced by UV could lead to the persistence of mitogenic signals and participate in the deregulation of cell growth.

Neprilysin modulates melanogenic effects of melanocortins Because we hypothesized that activation of neprilysin could terminate the melanogenic signal triggered by α-MSH or ACTH, we investigated whether the reduction of the degradation of these two peptides by inhibiting neprilysin would enhance their melanogenic potency. Therefore, we evaluated the tyrosinase activity of NHM in the presence or in the absence of PA. The dopa-oxidase activity of tyrosinase was measured first in melanocytes stimulated by increasing doses of α-MSH. As shown in **Fig 5**(***A***), dopa-oxidase activity was higher when the cells were incubated in the presence of 10^{-5} M PA, suggesting that in melanocytes, constitutent active neprilysin is able to hydrolyze α-MSH. The same experiment was then performed using the superpotent α-MSH analog, [Nle^4,D-Phe^7]αMSH (**Fig 5*B***). In this case, no difference was observed in cells treated or not treated with PA, suggesting that [Nle^4,D-Phe^7]α-MSH is not a substrate for neprilysin. **Figure 5**(***C***) shows dopa-oxidase activity of melanocytes stimulated by $ACTH_{1-17}$, in the presence or in the absence of PA. The characteristic biphasic dose–response curve obtained with ACTH peptides is left-shifted in the presence of PA pointing out an enhancement of the dopa-oxidase activity when neprilysin is inhibited. This also suggests that like α-MSH, $ACTH_{1-17}$ could be a substrate for neprilysin. The final step of melanogenesis is the synthesis of melanin; therefore, we also determined melanin content in melanocytes treated for 4 d with 10^{-8} M α-MSH or ACTH in the presence or in the absence of 10 μM PA. Concentration of melanin increased 260 ± 30% as compared with basal levels in the presence of α-MSH and 740 ± 50% in the presence of α-MSH plus PA, whereas an increase of 210 ± 35% of the content of melanin was observed in the presence of $ACTH_{1-17}$ alone and an increase of 350 ± 20%, when added together with PA. Similar to the previous results, we observed an enhancement of melanin content in the cells when PA inhibited neprilysin.

We finally examined the expression of microphthalmia, a transcription factor involved in the regulation of melanogenesis via the cyclic adenosine monophosphate-dependent pathway, whose expression is regulated by α-MSH (Price *et al*, 1998). For this purpose, normal melanocytes were incubated for 5 h in the presence of different concentrations of α-MSH with or without PA. The western blot experiment was then performed using a monoclonal antibody directed against microphthalmia. **Figure 6** shows that 10^{-12} M α-MSH is not sufficient to elicit an increase in microphthalmia expression even in the presence of PA. Interestingly, 10^{-10} M of α-MSH in combination with PA, increased expression of microphthalmia to the levels obtained with 10^{-8} M α-MSH, whereas α-MSH alone was ineffective at this dose. Taken together, these data show that the inhibition of neprilysin strengthens the effect of POMC peptides on microphthalmia expression and on tyrosinase activity, thereby leading to the potentialization of melanogenesis induced by these peptides.

POMC peptides are probably not the only melanogenic peptides that may be affected by UVB downregulation of neprilysin. Indeed, UVB irradiation increases production by keratinocytes of endothelin-1, which is a mitogenic and melanogenic agent for human melanocytes (Imokawa *et al*, 1992). As in endothelial and mesangial cell systems, neprilysin degrades endothelin-1 thus terminating its regulatory action (Turner and Tanzawa, 1997); therefore, it is worthwhile to propose that neprilysin downregulation would increase endothelin-1 effects on melanocytes.

Taken together, our observations seem to add an element to the regulatory loop that amplifies the melanogenic action of UV signal. Indeed, in addition to an increase in the production of these signal peptides and their related receptors in response to UVB, we can now consider a decrease in their degradation rate through the inhibition of neprilysin.

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