Alpha-melanocyte-stimulating hormone protects from ultraviolet radiation-induced apoptosis and DNA damage

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Key words: alpha-melanocyte-stimulating hormone - melanocytes - apoptosis - ultraviolet radiation - DNA damage

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Summary

Ultraviolet (UV) radiation is a well established epidemiologic risk factor for malignant melanoma. This observation has been linked to the relative resistance of normal melanocytes to UVB-induced apoptosis which consequently leads to accumulation of UVB-induced DNA lesions in melanocytes. Therefore, identification of physiologic factors regulating UVB-induced apoptosis and DNA damage of melanocytes is of utmost biological importance. We show that the neuropeptide α-melanocyte-stimulating hormone (α-MSH) blocks UVB-induced apoptosis of normal human melanocytes in vitro. The anti-apoptotic activity of α-MSH is not mediated by filtering or induction of melanin synthesis in melanocytes. α-MSH neither leads to changes in the cell cycle distribution nor induces alterations in the expression of the apoptosis-related proteins Bcl2, Bclx, Bax, p53, CD95 (Fas/APO-1) and CD95L (FasL). In contrast, α-MSH markedly reduces the formation of UVB-induced DNA damage as demonstrated by reduced amounts of cyclobutane pyrimidine dimers, ultimately leading to reduced apoptosis. The reduction of UV-induced DNA damage by α-MSH appears to be related to induction of nucleotide excision repair (NER) since UV-mediated apoptosis was not blocked by α-MSH in NER-deficient fibroblasts. These data, for the first time, demonstrate regulation of UVB-induced apoptosis of human melanocytes by a neuropeptide which is physiologically expressed within the epidermis. Besides its ability to induce photoprotective melanin synthesis, α-MSH appears to exert the capacity to reduce UV-induced DNA damage and thus may act as a potent protection factor against the harmful effects of UV radiation on genomic stability of epidermal cells.

1Abbreviations: ACTH, adrenocorticotropin; BPE, bovine pituitary extract; CPD, cyclobutane pyrimidine dimers; FCS, fetal calf serum; HDF, human dermal fibroblasts; HGF, hepatocyte growth factor; IL, interleukin; MC-R, melanocortin receptor; MSH, melanocyte-stimulating hormone; NER, nucleotide excision repair; NHK, normal human keratinocytes; NHM, normal human melanocytes; PI3K, phosphatidylinositol 3-kinase; POMC, proopiomelanocortin; UV, ultraviolet light; XP, xeroderma pigmentosum.
Introduction

Apoptosis of epidermal cells by ultraviolet B (UVB, 290-320 nm) radiation is a well-described phenomenon in vitro and in vivo and has been extensively studied in keratinocytes, the major target cell of solar UV radiation. It is considered as a protective mechanism to minimize survival of cells with irreparable DNA damage (1), thereby preventing malignant transformation. The molecular pathways leading to UVB-induced apoptosis include formation of cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts (2,3), activation of death receptors including CD95 (Fas/APO-1) (2,4), release of death ligands, e. g. tumor necrosis factor-α (5,6), and formation of reactive oxygen species (7). These pathways are orchestrated by positive and negative factors which act within the epidermis in an autocrine and/or paracrine fashion. For example, hepatocyte growth factor (HGF)/scatter factor produced by dermal fibroblasts inhibits UVB-induced apoptosis of human keratinocytes via the phosphatidylinositol 3-kinase (PI3K)/AKT pathway (8). Insulin-like growth factor-1 which is expressed by melanocytes and fibroblasts (9) delays UVB-induced apoptosis in human keratinocytes via the same mechanism (10). Recently, we reported that the immuno-modulatory cytokine interleukin (IL)-12 significantly reduced DNA lesions induced by UVB irradiation in vitro and in vivo. IL-12 did not reduce the number of sunburn cells in mice being deficient in nucleotide excision repair (NER), indicating that the DNA damage reducing effect of IL-12 may be linked to NER (11).

In the epidermis one of the most biologically relevant protective mechanisms against UVB-induced DNA damage is provided by melanocytes. These neural crest-derived cells deliver melanin in melanosomes via dendrites to adjacent keratinocytes. Failure to produce melanin, for example due to loss of function of tyrosinase like in ocular cutaneous albinism type 1, leads to accumulation of UV-induced DNA damage that finally results in a dramatically increased risk for squamous and basal cell carcinoma as well as malignant melanoma.
Little is known about the natural paracrine or autocrine factors that regulate melanocyte apoptosis upon UVB exposure. Only a limited number of peptide growth factors induce proliferation and melanogenesis in vitro. These mitogens include basic fibroblast growth factor (bFGF), HGF/scatter factor, mast cell growth factor and endothelin-1 (12,13). Another group of melanocyte peptide growth factors belong to the evolutionary conserved family of melanocortins which include α-, β- and γ-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropin (ACTH) (14). These bioactive peptides were initially characterized as pituitary gland-derived peptides that are structurally derived from a common precursor, proopiomelanocortin (POMC) (15). Both α-MSH and ACTH bind with similar affinity to the human melanocortin receptor-1 receptor (MC-1R) being expressed by human melanocytes (16). MC-1R belongs to the superfamily of G-protein coupled receptors with 7 transmembrane domains and which activate adenylate cyclase (17). In the last years, however, it has become apparent that the skin as well as as the majority of skin cell types in vitro express POMC and are capable of generating POMC peptides including α-MSH and ACTH (18,19). Pro-inflammatory cytokines and UV exposure were identified as physiological inducers of the skin POMC system leading to the concept of a cutaneous analogon of the hypothalamic-pituitary-adrenal axis (19). Moreover, MC-1R has been demonstrated to be expressed by a variety of extra-pigmentary cell types of the skin including dermal microvascular endothelial cells (20), epidermal keratinocytes (21), secretory epithelial cell types (22-24) and dermal fibroblasts (25).

In light of the broad expression of MC-1R by various cutaneous cell types and the fact that UVB radiation of human skin cells in vitro and in vivo induces POMC expression and α-MSH release (26-28), we hypothesized that α-MSH could serve as a protection factor against UV-induced cell damage. In this study we explored the effect of α-MSH on UVB-induced cell damage of normal human melanocytes (NHM). We have identified a new physiologic pathway which reduces UVB-induced DNA damage in NHM and thus may minimize the risk to develop malignant melanoma.
Materials and Methods

Cell culture

NHM and human dermal fibroblasts (HDF) were established from newborn foreskin and were purchased from CellSystems (St. Katharinen, Germany). NHM cultures were derived from 3 individual donors, and HDF cultures were derived from 2 individual donors, respectively. For experiments on NHK pooled cultures from several donors (CellSystems) were used and maintained in chemically defined Keratinocyte Basal Medium-2 with all supplements according to the manufacturer (CellSystems, St. Katharinen, Germany). NHM were routinely grown in Melanocyte Growth Medium M2 (PromoCell, Heidelberg, Germany). HDF were cultured in RPMI 1640 (PAA, Cölbe, Germany), 1 % L-Gln, 1 % penicillin/streptomycin (both from PAA) and 10 % fetal calf serum (FCS) (PAA). HDF carrying a defective Xpa gene were provided by Dr. Mark Berneburg, University of Tübingen, Germany.

Cell treatment and UV irradiation

For all experiments involving α-MSH we found it essential to deprive the cells from bovine pituitary extract (BPE), the latter commonly used for cell culture of NHM and NHK. Thus, NHM were kept for 3 days in chemically defined Melanocyte Basal Medium (PromoCell) supplemented with 0.5 % FCS, 1 ng/ml bFGF, 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 10 ng/ml phorbol-myristate-acetate, 50 ng/ml amphotericin B and 50 µg/ml gentamicin. NHM were also deprived from BPE for 3 days. HDF were stimulated with α-MSH in absence of FCS. Cells were preincubated with α-MSH (Bachem, Bubendorf, Switzerland) at concentrations as indicated for 6 h prior to UV irradiation. In some experiments the COOH-terminal tripeptide KPV (Bachem) was used. Cells were irradiated through PBS as reported before (2). An irradiation bank consisting of six fluorescent bulbs (TL12, Philips) which emits most of their energy within the UVB range with an emission peak of 313 nm was used. Upon UV irradiation cells were reincubated with α-MSH for indicated time periods as indicated.
Detection of cell death

A cell death detection ELISA Plus from Boehringer (Mannheim, Germany) was used. The assay determines the amount of mono- and oligonucleosomes released into the cytoplasm which are detected by biotinylated anti-histone- and peroxidase-coupled anti-DNA antibodies. NHM, NHK (both 1 x 10^5 per ml) and HDF (0.5 x 10^5 per ml) were seeded into 3.5 cm diameter tissue culture disks. Cells were collected 18 h after UV irradiation. Both adherent and floating cells were combined for sample preparation. Samples were analysed in triplicates and optical density was calculated as mean ± SD. Statistical analysis was performed using the student’s t-test.

Alternatively, apoptosis was detected by Annexin-V staining and flow cytometry as reported previously (29). The rate of apoptosis was determined by comparing the mean fluorescence intensity of Annexin-V stained cells between the different treatment groups.

Spectrophotometry

UV filtering by α-MSH was measured by UV spectrophotometry (Model DU 640, Beckman Instruments, Fullerton, CA). α-MSH was dissolved in PBS at a final concentration of 10^-5 and 10^-6 M. Absorbance was measured within a wide range of the UV spectrum (250-400 nm).

Determination of melanin

Measurement of melanin content was performed on 2 x 10^5 cells after incubation with α-MSH for 6 h. Cells were detached by trypsinization from tissue culture plates, centrifuged, and cell pellets were dissolved in 1 N NaOH. Melanin concentration was determined by measurement of the optical density at 475 nm in relation to a standard curve generated by synthetic melanin (Sigma, Deisenhofen, Germany).

MC-1R genotyping and sequencing

Genomic DNA extraction from NHM, HDF and XPA fibroblasts was performed using a
protocol from Gentra Systems Minneapolis, MN). The MC-IR gene was amplified by PCR using established protocols (30) with forward (5´-ATGGCTG-TGCAGGGATCC -3´) and reverse (5´-TCACCAGGAGCATGTCAGCACC-3´) primers, which were specific for MC1R. The reactions (primers, 2mM MgCl2, 200µM dNTPs, 1 x Optibuffer (Bioline, London, United Kingdom), and Biotaq red (Bioline) were carried out in a Perkin Elmer Cetus 9700 thermal cycler and included an initial denaturation phase at 94°C x 5 min, followed by 35 cycles of 94°C x 1min, 62°C x 1 min, 72°C x 2 min, with a final extension phase of 7 minutes. The products (954bp) were purified using a Qiagen PCR preps column (Qiagen), and the MC-IR coding region sequenced on a model ABI 377 automated DNA sequencer (Applied Biosystems) using a dye terminator cycle sequencing kit (Amersham PharmaciaBiotech, UK), and the following primers; 5´-GCGGTGCTGCAGCAGCTGG-3´, 5´-TGCTGCAGCACCGCAGCC-3´, 5´-ACCACGAGGCACACGGCAGC-3´, 5´-GGCGCTGTCACCCTCACC-3´.

Colony-formation assays

NHM were seeded into 6 cm diameter tissue culture plates at a density of 2 x 10³ cells per ml. Cells were treated with α-MSH at 10⁻⁶ M as outlined above. Following irradiation with UVB doses ranging from 150 to 400 J/m², cells were re-stimulated with α-MSH in chemically defined medium for additional 18 h. Culture medium was subsequently switched to routine Melanocyte Growth Medium M2. Cells were kept in culture for 2-3 weeks with renewal of medium twice weekly. Cultures were monitored daily. When colonies were easily visible, cells were stained with 0.1 % crystal violet. The number and size in mm² (actually the area being covered) of the colonies were analysed using the Image Analysis software Image J (http://rsbweb.nih/jj/docs/index.html). Three adjacent fields (each 3 cm x 3 cm) per 10 cm diameter tissue culture dish per experiment were analysed. Data were analysed statistically by the student’s t-test.
DNA content analysis

NHM were seeded at a density of 1 x 10^5 cells per ml into 6 cm diameter tissue culture plates in chemically defined medium without BPE. Cells were preincubated with α-MSH and irradiated with 600 J/m^2 as described above. After 18 h cells were harvested by trypsinization and DNA content analysis was determined by flow cytometry (31). Cell cycle analysis was performed using the ModFit LT software (Verity, Topsham, ME).

Western immunoblotting

NHM were collected by trypsinization and centrifugation. Pellets were lysed with lysis buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 10 % glycerol, 1 % Triton X-100, 1.5 mM MgCl_2, 1 mM ethylenglycol-bis(beta-aminoethylether)-N,N,N’N’-tetraacetic acid and 0.01 % NaN_2 plus freshly added proteinase inhibitors (25 µg/ml aprotinin, 25 µg/ml leupeptin and 1 mM phenylmethyl-sulfonyl fluoride). After sonication and centrifugation, supernatants were analyzed for protein content using the modified Bradford assay (Bio-Rad, Richmond, CA). An equal volume of 2x SDS loading buffer was then added and the samples were boiled at 95°C for 5 min. Equal protein amounts (10 or 20 µg/lane) were separated by denaturing SDS-PAGE using 4-12 % gradient gels from NuPAGE (Invitrogen, Carlsbad, CA). The polyvinylidene difluoride membranes were blocked over night with 10 % bovine serum albumin followed by immunoprobing with an anti-Bcl_2 antibody (Ancell, Bayport, MN), an anti-Bel antibody (BD PharMingen, San Diego, CA), an anti-Bax antibody (BD PharMingen), an anti-p53 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), an anti-CD95 (Fas/APO-1) antibody (Immunotech, Marseille, France), an anti-CD95L (FasL) antibody (BD PharMingen) or an anti-α-tubulin antibody (Oncogene Research Products, San Diego, CA). Membranes were reacted with a horseradish-peroxidase-conjugated secondary antibody (Amersham Life Science, Freiburg, Germany) followed by enhanced chemiluminescencence kit (Amersham). Membrane stripping was performed as described before (13).
**South Western dot-blot**

Genomic DNA was prepared from 1 x 10⁶ NHM immediately or 8 h after UV irradiation (150 J/m²). 2 µg of genomic DNA was transferred to a nylon membrane by vacuum dot-blotting and fixed by baking the membrane for 15 min at 80°C. CPD were detected by probing the membrane with a monoclonal antibody directed against thymine dimers (1:500; Kamiya Biomedical Comp., Seattle, WA). Bound antibodies were visualized using a horseradish peroxidase-conjugated anti-mouse antibody (Amersham, Piscataway, NJ).

**Immunofluorescence studies**

Visualization of CPD in NHM by immunofluorescence was performed using a modified protocol from Roza et al. (32). 8 h after UV irradiation (150 J/m²), cells were trypsinized and centrifuged. Cell pellets were resuspended in PBS and processed for cytospin preparation (60-70,000 cells/slide). Cells were rinsed in PBS, fixed with 45 % ethanol for 5 min followed by 70 % ethanol at -20°C for 10 min. Cells were subsequently permeabilized with 0.3 % Triton X for 30 min. DNA denaturation was performed by treating the cells with 0.5 N HCl and 0.05 % pepsine (Sigma) at 37°C for 30 min. Slides were incubated with the antibody against CPD (1:2000) in 5 % FCS and 0.05 % Tween 20 for 1 hr at 37°C. Bound antibodies were visualized with a rabbit anti-mouse antibody coupled to Texas Red (Dianova, Hamburg, Germany). Nuclei were stained with 4,6-diamido-2-phenylindole dihydrochloride (DAPI) at 100 ng/ml in Tris buffer (pH 7.4) for 1 hr at room temperature. Slides were mounted in Mowiol (Hoechst, Frankfurt, Germany). Cells were imaged with a fluorescence microscope (Leica DMRD/RXA, Wetzlar, Germany). Red fluorescence was excited using the TX2 filter (BP 560/40 nm). Emission was measured at 645±75 nm for Texas Red. For DAPI staining an excitation wavelength of 360/40 nm was used. The number of CPD immunoreactive cells per 100 DAPI positive cells was determined in 3 individual high-power viewing fields per experiment. Data were subsequently analysed by the student’s t-test.
Results

\(\alpha\)-MSH protects from UVB-induced apoptosis

To determine the effect of \(\alpha\)-MSH on UVB-induced apoptosis of NHM, we treated cells with \(\alpha\)-MSH (10\(^{-6}\) M) 6 h prior to exposure with UVB (600 J/m\(^2\)). After irradiation cells were incubated for additional 16 h in the presence or absence of \(\alpha\)-MSH followed by determination of nucleosomal DNA fragmentation. In previous experiments and in accordance with others (33,34) it had been noticed that NHM are less vulnerable than NHK to UVB radiation and require higher dosages of UVB (at least 400 J/m\(^2\) \textit{in vitro}) for induction of robust apoptosis.

\(\alpha\)-MSH significantly blocked UVB-induced apoptosis (Figure 1A). In contrast, \(\alpha\)-MSH \textit{per se} did not elicit any effect on DNA fragmentation (Figure 1A). Interestingly, the COOH-terminal tripeptide KPV which retains many immunomodulatory activities of \(\alpha\)-MSH (35,36) did not have a significant effect on UVB-induced apoptosis (Figure 1B). Despite the fact that none of the utilized NHM cultures established from the three individual donors were homozygous for a signaling deficient MC-1R variant (two NHM cultures with both wild type \textit{MC-1R} alleles, one NHM culture with a heterozygous Arg151Cys variant), the anti-apoptotic effect of \(\alpha\)-MSH was not due to an increment in melanin content during the incubation period with \(\alpha\)-MSH prior to UVB exposure (data not shown). Moreover, spectrophotometric analysis within the range of 250-400 nm did not demonstrate any filtering effect of \(\alpha\)-MSH (data not shown). In accordance with an anti-apoptotic effect independent of melanogenesis, \(\alpha\)-MSH blocked apoptosis of NHK exposed to UVB (250 J/m\(^2\)) as shown by death detection ELISA (Figure 1C). To confirm the protective effect of \(\alpha\)-MSH on UVB-induced apoptosis in NHM and NHK by another read-out system, we performed Annexin-V surface staining and flow cytometry. Again, \(\alpha\)-MSH suppressed UVB-induced apoptosis in NHM (mean fluorescence intensity of non-irradiated cells: 10.6 vs. UVB-treated cells: 26.2 vs. cells treated with \(\alpha\)-MSH plus UVB: 17.4) (Figure 1D) as well as in NHK (mean fluorescence intensity of non-irradiated cells: 7.4 vs. UV treated cells: 18.3 vs. cells treated with \(\alpha\)-MSH plus UVB: 9.7) (Figure 1E).
**α-MSH promotes long-term survival after UVB irradiation**

To exclude that α-MSH only delays apoptosis, but indeed enhances survival of NHM, colony forming assays were performed. To this end, cells were preincubated with α-MSH, exposed to UVB and maintained in routine culture medium for several weeks. To allow the survival of a sufficient number of cells suitable of colony formation a lower UVB dose (150 J/m²) was applied than in the previous experiments. A single exposure to this dose drastically reduced the number of colonies detectable after 3 weeks (Figure 2A). α-MSH (10⁻⁶ M) significantly increased the number and size of the visible colonies as compared to UVB-exposed NHM (Figure 2B,C). In contrast, α-MSH alone did not have any robust effect on the number of colonies in sham-irradiated cells. These data demonstrate that α-MSH indeed promotes long-term survival of NHM exposed to a single dose of UVB.

**α-MSH mediated protection of UVB-induced apoptosis is not due cell cycle changes**

The protective effect of α-MSH on UVB-induced apoptosis of NHM could be caused by changes in the cell cycle, giving the cells more time to repair UVB-induced DNA damage. It has been reported that in asynchronously growing NHK and HaCaT cells sublethal doses of UVB irradiation induce G₂ cell cycle arrest (31). On the other hand, NHM were shown to arrest in G₁ after UVB irradiation *in vitro* (37). However, the experimental conditions as presented in this work suggested *a priori* a G₁ arrest in untreated NHM and NHK as these cells had been deprived from BPE prior to treatment with α-MSH and/or UVB (see Material and Methods). DNA content analysis of untreated NHM revealed 88.6±1.8 % of cells being arrested in G₁ and 10.7±1.3 % being arrested in G₂/M (Table I). Albeit α-MSH, UVB and α-MSH plus UVB lead to a small but statistically significant increase in the percentage of cells in S phase as compared to non-treated cells, α-MSH failed to increase the percentage of cells in G₁ or G₂/M compared to UVB alone (Table I). Therefore, it can be concluded that the protective effect of α-MSH from UVB-induced apoptosis is not mediated via accumulation of NHM in the G₁ or G₂ phase.
α-MSH does not alter protein expression of Bcl2, Bclx, Bax, p53, CD95 or CD95L

To further elucidate the mechanisms by which α-MSH protects NHM from UVB-induced apoptosis we analysed the protein expression of a number of molecules crucially involved in regulation of apoptosis. We first examined the expression of Bcl2, a well known anti-apoptotic protein. As determined by Western immunoblotting of total cells lysates obtained 18 h after UVB irradiation (600 J/m²), UV exposure resulted in a clear-cut reduction in protein expression of Bcl2 as compared to sham-irradiated NHM. α-MSH (10⁻⁶ M) only marginally affected this UVB-mediated reduction in Bcl2 expression (Figure 3). Neither UVB irradiation nor α-MSH treatment altered the amounts of Bclx protein, suggesting that this molecule is not involved in protection of UVB-induced apoptosis by α-MSH (Figure 3). In another set of experiments we determined the protein expression of CD95 (Fas/APO-1) and its ligand CD95L (FasL) in NHM treated with UVB and/or α-MSH. CD95 was constitutively expressed in NHM and not induced either by UVB or α-MSH (Figure 3). On the other hand, CD95L protein was undetectable in NHM (Figure 3) and treatment with UVB or α-MSH did not induce its expression (data not shown). UVB irradiation slightly increased the amount of p53 protein as compared to non-irradiated NHM. However, co-treatment with α-MSH did not lead to any significant change in p53 protein expression (Figure 3).

In addition, although α-MSH alone appeared to increase Bax protein expression, it did not alter the relative expression levels of this proapoptotic regulator in UVB-treated NHM as compared to cells irradiated with UVB alone (Figure 3).

α-MSH reduces the amount of UVB-induced CPD

Since UVB-induced DNA damage has been identified as the major trigger for UV-induced apoptosis (2,3), the effect of α-MSH on CPD formation was determined. 8 h after irradiation genomic DNA was extracted and subjected to South Western dot blot analysis using an antibody against CPD. CPD were undetectable in non-irradiated NHM or in cells stimulated with α-MSH alone, respectively (Figure 4A). UVB irradiation strongly induced CPD in NHM. This effect was
markedly suppressed by α-MSH (Figure 4A). Induction of CPD by UV radiation is an immediate event and thus detectable shortly after UV exposure. When NHM were analysed for CPD immediately after UVB exposure no differences were observed in cells treated with α-MSH (Figure 4B). This implies that α-MSH does not prevent induction of CPD, thereby excluding a filtering effect. To further confirm the effect of α-MSH on the CPD induced by UVB we performed immunocytochemical analysis of NHM after *in situ* DNA denaturation and CPD epitope retrieval. The extent of CPD visible as immunoreactive nuclei was related to the total number of cells as demonstrated by double staining with the nuclear marker DAPI (Figure 5A and B). As expected, CPD were absent in non-irradiated NHM. Irradiation with UVB (150 J/m²) resulted in dramatic induction of CPD immunoreactive cells 8 h after irradiation with virtually every nucleus immunoreactive for CPD. α-MSH treatment significantly reduced the number of nuclei immunoreactive for CPD compared with NHM exposed to UVB alone (Figure 5A and B).

The fact that the amounts of CPD were the same in the α-MSH-treated NHM and untreated samples immediately after UV exposure (Figure 4B) but were remarkably reduced at 8 h after UV exposure (Figure 4A) suggested that α-MSH might accelerate the removal of UVB-induced DNA lesions. Since NER is the major mechanism by which CPD are removed in mammalian cells the effect of α-MSH on UVB-induced apoptosis of NER-deficient cells was determined. The *XPA* gene is an essential component of the NER, thus cells with a mutated *XPA* completely lack a functional NER. Therefore, dermal fibroblasts established from a patient with xeroderma pigmentosum (XP) complementation group A and normal HDF were examined for the effect of α-MSH on UVB-induced apoptosis. It had been previously established that HDF express functional receptors for α-MSH which have been identified by reverse transcription PCR, immunofluorescence analysis and radioligand binding studies as MC-1R (25). α-MSH again significantly blocked UVB-induced apoptosis by 45 % in HDF (Figure 6A). In contrast, XPA fibroblasts were not protected by α-MSH from UVB-induced apoptosis (Figure 6B), indicating that the reduction of CPD by α-MSH may depend on a functional NER. To exclude that the failure of α-MSH to reduce apoptosis in XPA
fibroblasts is due to a non-functional α-MSH receptor, genotyping of the \( MC-1R \) gene was performed. The utilized XPA fibroblasts contained a single variant allele (Val60Leu). Based on the presence of one wild type allele it is highly likely that these cells are able to signal via the wild type receptor upon binding of α-MSH (38).

**Discussion**

UVB-induced apoptosis has been recognized as a protective mechanism since it contributes to the elimination of cells carrying DNA damage, thereby preventing malignant transformation (39). In this context, UVB-induced apoptosis has been most intensively studied in NHK, the major cellular target for solar/UVB radiation. In contrast to NHK which are quite prone to apoptosis, appearing \textit{in situ} as sunburn cells, NHM have been reported to be quite resistant to UV-induced apoptosis (33). This is based on the facts that apoptotic NHM are hardly observed \textit{in situ} and secondly that higher doses are required to induce apoptosis \textit{in vitro} in NHM than in NHK. The resistance to UVB-induced apoptosis of NHM may be due to the high expression of the anti-apoptotic protein Bcl-2 (34,40-43). The higher vulnerability of NHK to UV radiation than that of NHM may also be due to the fact that the former cell type is more likely to undergo active cycling during UV exposure than the normal non-mitotic NHM because cells are prone to undergo apoptosis when synthesizing DNA (44). All these observations gave rise to the speculation that nature may accept a certain degree of risk of mutation induced by UV radiation in NHM in order to maintain their melanin-generating photoprotective role in the skin (33). However, this also implies that severely UV-damaged NHK which are at risk for incomplete repair of DNA and subsequent mutation may be eliminated by apoptosis, whereas similarly damaged NHM are retained and thus at some risk for subsequent mutations. Since the apoptotic protection pathway obviously may not be as efficient in NHM, the identification of any pathway or strategy to reduce the load of DNA damage in NHM may be of relevance for the understanding of the pathogenesis of malignant
melanoma and for the development of preventive/therapeutic measurements. Here, we demonstrate that this may apply for the neuropeptide α-MSH.

The present data demonstrate that α-MSH reduces UV-induced apoptosis of NHM in vitro in a dose dependent manner. Elucidating the underlying mechanism we observed that α-MSH neither exerts its effect on UVB-induced apoptosis via altering the cell cycle distribution nor via changing the expression of apoptosis-related proteins including Bcl2, Bclx, p53, CD95 and CD95L. In contrast, as demonstrated by Southwestern dot blot analysis α-MSH significantly reduced the amounts of CPD, the major DNA lesion induced by UV. The reduction of CPD by α-MSH was detected when DNA was extracted 8 hours after UV exposure. However, when DNA was extracted immediately after UV irradiation no differences were observed between α-MSH-exposed and untreated UV-irradiated NHM. This indicates that the reducing effect of α-MSH is not due to a filtering effect of α-MSH. This is also in accordance with spectrophotometric analyses which did not demonstrate any absorbing capacity of α-MSH within the UVB range.

The fact that the amounts of CPD were the same in the α-MSH-treated and untreated NHM immediately after UV exposure but were remarkably reduced at 8 h after UV exposure can only be explained by the fact that α-MSH might accelerate the removal of UVB-induced DNA lesions. NER is the major DNA repair system in mammalian cells to remove UV-induced DNA damage. To determine whether α-MSH may exert its reducing effect of DNA damage via affecting NER, NER-deficient cells were used. Cells derived from patients suffering from XP either lack or have reduced DNA repair capacity due to genetic mutations in several components of the NER. The XPA complementation type represents the most severe phenotype since the Xpa gene is the most crucial component in the repair process and thus cells lacking the Xpa gene are completely deficient in NER (45,46). Since XPA melanocytes are not available we used XPA fibroblasts for our purposes. Previous studies have demonstrated that HDF also express receptors for α-MSH and are susceptible to the effects of this neuropeptide (25). In fact, the reducing effect of α-MSH on UV-induced DNA damage was observed in wild type but not in XPA fibroblasts. This implies that the effect of MSH...
on DNA damage may be mediated via NER or at least linked to a properly functioning NER system.

As a crucial protection mechanism NER was regarded for a long time to be constitutively expressed and not to be regulated by external stimuli. However, it was recently described that the immunomodulatory cytokine IL-12 exhibits the capacity to reduce UV-induced DNA damage most likely via modulation of the NER (11). A similar phenomenon was observed by utilizing small DNA fragment thymidine dinucleotides which enhance the repair of UV-induced DNA damage in a p53 dependent manner (47). In contrast, the effect of IL-12 appeared to be independent of p53, since it was also observed in HaCaT cells which contain two p53 mutations (48). Likewise, we did not observe an alteration of the p53 protein levels by α-MSH, suggesting that the effect may be independent of p53. Our findings are in accordance with data from others who also could not detect a modulating effect of α-MSH on p53 expression in UVB-irradiated NHM (49). However, we can not completely rule out an involvement of p53 in the cytoprotective mechanism of α-MSH. Interestingly, α-MSH is not protective against UVB-induced apoptosis of HaCaT keratinocytes (our own unpublished findings) which carry p53 mutations (50). It is thus possible that the anti-apoptotic pathways targeted by α-MSH exhibit cell-type specific differences. On the other hand, it has been reported that HaCaT keratinocytes exhibit deviations in the signaling events modulated by α-MSH, i.e. the NF-κB pathway (51) being also involved in regulation of apoptosis. Therefore, the lack of an anti-apoptotic effect of α-MSH in HaCaT cells may be related to deviations in the NF-κB signaling pathway. Further studies are needed to fully clarify the mechanistic role of p53 in regulation of UVB-induced apoptosis by α-MSH.

Regarding the protective effect of IL-12 on UVB-induced apoptosis it was shown that IL-12 affects several components of the NER (11). We have not yet checked whether α-MSH exerts a similar effect. The fact that the reduction of DNA damage by α-MSH may be mediated via NER is also supported by the observation that induction of apoptosis of NHM by bleomycin, a radiomimetic DNA damaging anticancer drug inducing both single- and double-strand DNA breaks
which are not repaired by NER (52), is not prevented by α-MSH. In contrast, α-MSH significantly suppressed apoptosis of NHM exposed by cisplatin (our own unpublished findings). Cisplatin-induced DNA lesions are at least partially repaired by NER, thus supporting our assumption that α-MSH may modulate NER.

Nevertheless, at this stage we cannot exclude with absolute certainty that α-MSH may mediate its anti-apoptotic activity via additional mechanisms other than NER. It will be interesting to check whether α-MSH affects other apoptosis regulators such as Bax, Mcl-1, c-IAP1/2, XIAP, Livin and Apaf-1, all of which are expressed in NHM (43). UVB has been recently recognized to be able to induce reactive oxygen species and that this may contribute to UV-induced apoptosis (7). Since α-MSH can modulate the intracellular amount of reactive oxygen species in HaCaT keratinocytes exposed to hydrogen peroxide (53), it also will be worth to investigate if α-MSH reduces UVB-mediated apoptosis by acting on the formation of reactive oxygen species.

Taken together, these data demonstrate that UVB-induced apoptosis of NHM can be modulated in vitro by the neuropeptide α-MSH and that this effect is primarily due to a reduction of UV-induced DNA damage, presumably via activation of the NER. The fact that POMC peptides are expressed in the skin and are induced by UVB radiation in vitro and in vivo (18,19,28) suggests that α-MSH might function as a paracrine or autocrine “protection factor” against the harmful effects of UVB on epidermal homeostasis and genomic stability. This protective effect may be due to its melanin generating capacity primarily affecting NHK but as demonstrated here also due to its capacity to reduce DNA damage. The latter capacity may be of relevance for the homeostatic genomic stability of NHM and may represent a physiological mechanism reducing the risk of malignant transformation of NHM. Whether this indeed of in vivo relevance needs to be demonstrated e.g. in photocarcinogenesis studies using MC-1R deficient mice. Finally, our findings may point towards a novel therapeutic potential of α-MSH or its superpotent analogues such as NDP-MSH, [Nle4-D-Phe7] (54) in UV protection since any strategy which reduces the cumulative
load of UV-damaged DNA in particular in melanocytes may contribute to lower the risk to develop UV-induced skin cancer.

Aknowledgements

This project was supported by a grant to M. B. from the University of Münster, Germany, Innovative Medizinische Forschung (IMF, BÖ 110219) and the Federal Ministry of Environmental Protection (StSch_4373).

References


(1995) Cancer Res. 55, 4041-4046


Table I. α-MSH does not increase the percentage of cells arrested in G1 or G2/M as compared to UVB irradiation alone*

<table>
<thead>
<tr>
<th>Cell cycle phase</th>
<th>Treatment</th>
<th>N/A</th>
<th>M</th>
<th>UVB</th>
<th>M + UVB</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>88.6±1.8</td>
<td>85.1±0.2</td>
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<tr>
<td>G2/M</td>
<td>10.7±1.3</td>
<td>10.5±0.7</td>
<td>8.5±4.5</td>
<td>9.2±1.7</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>0.8±0.5</td>
<td>4.3±1.0²</td>
<td>4.6±1.2¹</td>
<td>3.4±0.4¹</td>
<td></td>
</tr>
</tbody>
</table>

*NHM were preincubated with 10⁻⁶ M α-MSH (M) for 6 h or left non-treated (N/A). Cells were then exposed to 400 J/m² or were sham-irradiated. After additional 18 h cell cycle was performed by DNA content analysis as outlined in Material and Methods. Data are means ± SD from triplicates. Data were reproduced twice with qualitatively similar results. §P<0.05 versus N/A.
**Figure 1.** α-MSH protects from UV-induced apoptosis. NHM were deprived from BPE, preincubated with α-MSH (10^{-6} M) for 6 h and irradiated with 600 J/m^2 UVB. Cells were subsequently maintained in presence or absence of α-MSH for additional 18 h followed by determination of mono- and oligonucleosomes using a death detection ELISA (A). In another set of experiments NHM were treated as described above but using the COOH-terminal tripeptide of α-MSH, KPV, at 10^{-6} M (B). NHK were likewise deprived from BPE, preincubated with α-MSH (10^{-6} M) for 6 h and irradiated with 250 J/m^2 UVB. After 18 h in presence or absence of α-MSH apoptosis was measured using the death detection ELISA (C). Suppression of UVB-induced apoptosis of NHM (D) and NHK (E) by α-MSH as shown by Annexin-V staining. Cells were treated exactly as indicated above followed by FACS analysis using an antibody against Annexin-V. Data are representative sets from 3 independent experiments with similar results. *p<0.001 from triplicate analysis. N/A=non-treated cells.

**Figure 2.** α-MSH confers long-term survival of NHM exposed to a single dose of UVB irradiation. NHM were deprived from BPE, preincubated with 10^{-6} M α-MSH for 6 h followed by irradiation with 150 J/m^2 UVB. Cells were kept in presence or absence of α-MSH for additional 18 h. Experimental culture medium was then switched to routine medium and cells were maintained for 14-21 days with medium changes twice weekly. Colonies were visualized by crystal violet staining (A). Number (B) and covered area in mm^2 (representing the size) (C) of the visible colonies were determined and analysed by computerized imaging as outlined in Material and Methods. N/A=non-treated cells. Depicted are means ± SD from triplicate analysis. *p<0.005, **p<0.05.

**Figure 3.** α-MSH does not alter the protein expression of Bcl_2, Bcl_α, Bax, p53, CD95 and CD95L in NHM irradiated with UVB. NHM were deprived from BPE, preincubated with α-MSH (10^{-6} M), and irradiated with UVB (600 J/m^2). After 18 h in presence or absence of α-MSH total cell lysates were prepared. Identical amounts of proteins (10 µg/lane, upper panel; 20 µg/lane, middle panel)
were subjected to Western immunoblotting. Membranes were sequentially probed with antibodies against Bcl2, CD95 and α-tubulin (upper panel), and Bclx, p53 and α-tubulin (middle panel). As a positive control for the expression of CD95L, HL60 leukemia cells (lower panel) were used. N/A=non-treated cells. Shown are representative panels of at least 3 independent experiments with identical results.

**Figure 4.** α-MSH reduces the amounts of UVB-induced CPD in NHM. Cells were deprived from BPE, preincubated with α-MSH (10⁻⁶ M) for 6 h followed by irradiation with 150 J/m² UVB. Cells were kept in presence or absence of α-MSH for additional 8 h (A), or were immediately harvested after irradiation (B). 2 µg of genomic DNA per sample was subjected to South Western dot-blot using a monoclonal antibody against CPD. The figure set is one out of 3 independent experiments with identical results.

**Figure 5.** In situ detection of CDP in NHM exposed to UVB and treated with α-MSH. NHM were deprived from BPE, pretreated with α-MSH (10⁻⁶ M), and irradiated with UVB (150 J/m²). After 8 h in presence or absence of α-MSH cells were trypsinized, and cytospins were prepared. Cells were fixed and in situ DNA denaturation was performed with pepsin followed by incubation with a CPD antibody and the nuclear marker DAPI (blue fluorescence). Bound antibodies against anti-thymine dimers were visualized by a Texas-Red conjugated secondary antibody (red fluorescence). Assessment of the reduction of UVB-induced CPD by α-MSH was performed by merging the blue and red fluorescence images. Depicted is one out of three independent experiments with similar results (A). The number of CPD immunoreactive cells per 100 DAPI positive nuclei was determined and analysed as described in Material and Methods. Depicted are means ± SD from triplicate analysis. *p<0.05 (B).
Figure 6. Protection from UVB-induced apoptosis by α-MSH in HDF (A) but not in NER deficient XPA fibroblasts (B). Fibroblasts were preincubated in serum-free medium with α-MSH (10^{-6} M) for 6 h in followed by irradiation with 400 J/m² UVB. 18 h after irradiation apoptosis was determined by a death detection ELISA. Figures are representative sets of 3 independent experiments with similar results. *p<0.001 from triplicate analysis. N/A=non-treated cells.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Alpha-melanocyte-stimulating hormone protects from ultraviolet radiation-induced apoptosis and DNA damage
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J. Biol. Chem. published online November 29, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406334200

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