α-Melanocortin and Endothelin-1 Activate Antiapoptotic Pathways and Reduce DNA Damage in Human Melanocytes

Ana Luisa Kadekaro,[1] Renny Kavanagh,[1] Hiromi Kanto,[1] Silva Terzieva,[1] Jennifer Hauser,[2] Nobuhiko Kobayashi,[4] Sandy Schwemberger,[3] James Cornelius,[3] George Babcock,[3] Howard G. Shertzer,[2] Glynis Scott,[5] and Zalfa A. Abdel-Malek[1]

Departments of [1]Dermatology, [2]Environmental Health, and [3]Surgery, University of Cincinnati College of Medicine and Shriners' Burns Institute, Cincinnati, Ohio; [4]Department of Dermatology, Nara Medical University, Nara, Japan; and [5]Department of Dermatology, University of Rochester, Rochester, New York

Abstract

UV radiation is an important etiologic factor for skin cancer, including melanoma. Constitutive pigmentation and the ability to tan are considered the main photoprotective mechanism against sun-induced carcinogenesis. Pigmentation in the skin is conferred by epidermal melanocytes that synthesize and transfer melanin to keratinocytes. Therefore, insuring the survival and genomic stability of epidermal melanocytes is critical for inhibiting photocarcinogenesis, particularly melanoma, the most deadly form of skin cancer. The paracrine factors α-melanocortin and endothelin-1 are critical for the melanogenic response of cultured human melanocytes to UV radiation. We report that α-melanocortin and endothelin-1 rescued human melanocytes from UV radiation–induced apoptosis and reduced DNA photoproducts and oxidative stress. The survival effects of α-melanocortin and endothelin-1 were mediated by activation of the melanocortin 1 and endothelin receptors, respectively. Treatment of melanocytes with α-melanocortin and/or endothelin-1 before exposure to UV radiation activated the inositol triphosphate kinase-Akt pathway and increased the phosphorylation and expression of the microphthalmia-related transcription factor. Treatment with α-melanocortin and/or endothelin-1 enhanced the repair of cyclobutane pyrimidine dimers and reduced the levels of hydrogen peroxide induced by UV radiation. These effects are expected to reduce genomic instability and mutagenesis. (Cancer Res 2005; 65(10): 4292-9)

Introduction

Cutaneous pigmentation is the major photoprotective mechanism against the carcinogenic effect of solar UV radiation (UV) on human skin (1). Pigmentation is the outcome of melanin synthesis by epidermal melanocytes, and the transfer of melanin-containing organelles, termed melanosomes, from melanocytes to surrounding keratinocytes (2). The most drastic effects of UV exposure are photoaging and photocarcinogenesis. Melanin guards against the photodamaging effects of UV by acting as a filter that limits the penetration of UV rays into the epidermal layers (3, 4). Melanin also acts as a scavenger of UV-induced reactive oxygen species that cause lipid peroxidation and damage proteins and DNA (5). Acute exposure of the skin to solar UV results in DNA damage, which if not repaired, would lead to cell death as evidenced by the appearance of sunburn cells (i.e., apoptotic keratinocytes), or to mutations (6). One mechanism by which melanocytes *in situ* resist apoptosis is by expressing constitutively the antiapoptotic Bcl2, recently shown to be regulated by the microphthalmia-related transcription factor, Mitf (7–9). However, *in vitro,* melanocytes respond to UV with dose-dependent growth arrest and reduced survival (10–12). Melanocytes have a low proliferation capacity, and their significance in photoprotection makes it crucial to insure their survival and prevent apoptosis.

Many of the effects of UV on human skin are indirectly mediated by up-regulation of various growth factors and cytokines, some of which function as paracrine or autocrine regulators of melanocytes (13–15). Among those factors are α-melanocortin (α-MSH) synthesized by keratinocytes and melanocytes and endothelin-1 (ET-1), which is produced by keratinocytes. Both α-MSH and ET-1 are mitogenic and melanogenic for human melanocytes and are important participants in the melanogenic response of melanocytes to UV (11, 12, 16). The results hereby presented define a novel role for α-MSH and ET-1 as antiapoptotic factors for melanocytes. We show that α-MSH and ET-1 promote melanocyte survival and inhibit the UV-induced apoptosis by activating the inositol triphosphate (IP3) kinase-Akt pathway, increasing the levels of Mitf and counteracting the reduction in Bcl2.

The IP3 kinase pathway is an important survival pathway in many cell types, such as neurons, fibroblasts, and keratinocytes, and mediates the effects of various survival factors, such as insulin-like growth factor (IGF), nerve growth factor, platelet-derived growth factor, mast cell growth factor, epidermal growth factor (EGF), and hepatocyte growth factor (17–23). Activation of IP3 kinase results in increased levels of phosphatidylinositol-3,4,5-triphosphate and inositol 3,4-biphosphate, which ultimately lead to the activation of the serine-threonine kinase Akt/PKB (24). Activated Akt inhibits apoptosis, particularly the intrinsic apoptotic pathway, by phosphorylating and inactivating the pro-apoptotic Bad and caspase 9, and by transactivating nuclear factor-κB (NFκB) (20, 25, 26). The cyclic AMP (cAMP)–dependent pathway, the main signaling pathway activated by α-MSH, also stimulates Akt/PKB activity (27, 28).

Recently, the transcription factor Mitf, known to be critical for melanocyte survival and melanogenesis, was found to regulate Bcl2 expression (9, 29–31). Bcl2 is an important regulator of melanocyte survival, as *Bcl2* knockout mice are hypopigmented due to melanocyte apoptosis (32). Because UV-induced apoptosis occurs in cells with extensive DNA damage, we have hypothesized that the antiapoptotic effects of α-MSH and ET-1 are due to reducing initial DNA damage and/or enhancing DNA repair, which should diminish UV-induced mutagenesis and carcinogenesis. Our results show that

Requests for reprints: Zalfa A. Abdel-Malek, Department of Dermatology, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267-0592. Phone: 513-558-6242; Fax: 513-558-0198; E-mail: abdelmza@email.uc.edu.

α-MSH and ET-1 reduce UV-induced DNA photoproducts and the levels of released hydrogen peroxide. These effects, which are mediated by the respective receptors of α-MSH and ET-1, are expected to contribute to the maintenance of genomic stability and inhibit the transformation of melanocytes to melanoma. These findings may offer an explanation for the association of loss-of-function mutations in the *melanocortin 1 receptor* (*MC1R*) gene, which codes for the melanocortin receptor expressed on melanocytes, with increased risk for melanoma (33, 34).

Materials and Methods

Melanocyte culture. Primary human melanocyte cultures were established from neonatal foreskins or from adult skin biopsies obtained after informed consent and maintained as described (35). To test the effects of ET-1 and/or α-MSH, melanocytes were maintained in medium devoid of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), which down-regulates the endothelin receptors (36), and bovine pituitary extract, which contains high concentrations of melanocortins (35), for 24 hours before and for the duration of the experiments. Early-passage cultures (<10) were used for all experiments to insure minimal genetic drift *in vitro*.

Determination of melanocyte survival and proliferation. Human melanocytes were plated onto 60-mm dishes at a density of 2.5×10^5 cells per dish. On days 3 and 5 after plating, the growth medium lacking TPA and bovine pituitary extract was changed and melanocytes were treated with 0 (control), 1 nmol/L α-MSH, 0.1 nmol/L ET-1, 1 nmol/L α-MSH plus 0.1 nmol/L ET-1, or 1 μmol/L forskolin for a total of 4 days, and immediately after exposure to a single dose of 21 mJ/cm^2 UV emitted by a bank of FS-20 fluorescent UV lamps that have a continuous emission spectrum with a peak at 313 nm (National Biological, Twinsburg, OH), as described (10–12). Percent of cell death was determined on days 2 and 4 after UV irradiation by calculating the number of dead melanocytes that detached and incorporated trypan blue dye, as described (10). The cell number in each dish (triplicate dishes in each group) was counted using a Coulter Counter (model ZM). Differences between the control and UV-irradiated groups and in the responses of the cultures to UV alone, versus UV plus treatment with α-MSH and/or ET-1 were compared using ANOVA, followed by SNK ($P < 0.05$). Adult human melanocytes were plated and treated with α-MSH and/or UV, as described above. The cell numbers were determined 2 days after irradiation, and the data were statistically analyzed using Student's t test.

Determination of melanocyte apoptosis by Annexin V staining. Melanocytes were plated and treated with 0, α-MSH, and/or ET-1 and irradiated with UV as described above. Twenty-four hours after UV irradiation, melanocytes were harvested and stained with APC-Annexin (BD Pharmingen, San Diego, CA) and propidium iodide (Sigma Chemical Co., St. Louis, MO). Samples were analyzed by flow cytometry immediately after staining on BD LSR, and the data were analyzed by CELLQuest software.

To verify the significance of the endothelin receptor in the survival effect of ET-1, melanocytes were treated for 4 days before and immediately after UV exposure with 150 nmol/L PD 145065, a potent inhibitor of the ET-A and ET-B receptors (Sigma Chemical; ref. 37). For determining the significance of the MC1R in mediating the effects of α-MSH, four different melanocyte cultures expressing loss-of-function mutations in the *MC1R* gene were tested (38). The significance of melanin in the survival effects of ET-1 and α-MSH was investigated using tyrosinase-negative albino melanocytes that lack the ability to synthesize melanin due to abnormal expression of tyrosinase protein and absence of tyrosinase activity (a gift from Raymond Boissy, Department of Dermatology, University of Cincinnati; ref. 39). The involvement of the IP3 kinase in the survival effects of α-MSH and ET-1 was first determined by treating normal melanocytes for 45 minutes before and immediately after UV exposure with 15 μmol/L LY 294002, a selective inhibitor of IP3 kinase (Calbiochem, La Jolla, CA; ref. 40). The extent of apoptosis was determined by Annexin V staining as described above.

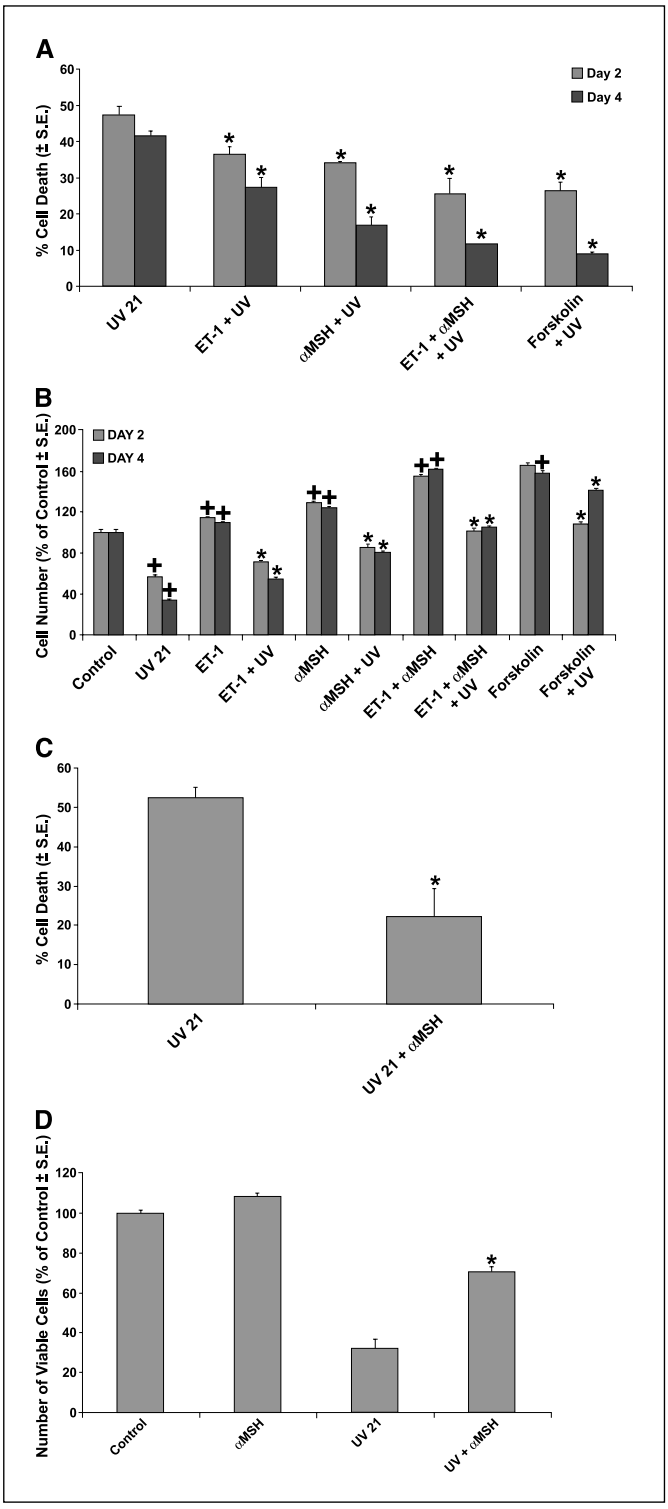
Akt kinase activity assay. Melanocytes were plated at a density of 1.0 to 1.5×10^6 cells/100-mm dish, and 72 hours later were treated as described above. Eighteen hours before UV irradiation, melanocytes were kept in unsupplemented MCDB medium with or without ET-1 and/or α-MSH. Cell extracts were prepared 1 hour after irradiation, and Akt kinase activity was determined using a commercially available assay kit, using purified Bad as a substrate (Upstate Biotechnology, Lake Placid, NY). Briefly, Akt was immunoprecipitated from 300 to 500 μg of total protein by incubation overnight at 4°C with 4 μg of anti-Akt/PKBα PH domain antibody covalently bound to agarose beads. The washed immunoprecipitates were suspended in kinase assay buffer containing 3 μg of purified Bad protein, and the reaction mixture was incubated for 10 minutes at 37°C to allow phosphorylation of the exogenously added substrate, Bad, by Akt. Western blot analysis was done using the phospho-specific Bad (Ser^{136}) antibody, and the bands were visualized by chemiluminescence.

Immunoblot analysis of total Akt, Mitf, and Bcl2. Human melanocytes were plated onto 100-mm dishes at a density of 1.5×10^6 cells and treated with 0, α-MSH, and/or ET-1 as described above. For the analysis of total Akt or Mitf, cells were kept in unsupplemented MCDB medium to down-regulate Akt or Mitf activity, with or without ET-1 and/or α-MSH for 18 hours before and after UV irradiation. Cell extracts were prepared 1 or 1.5 hours after UV exposure for detection of total Akt or Mitf, respectively, using RIPA buffer containing a cocktail of protease inhibitors. Western blot analysis for total Akt and Mitf was carried out using Akt antibody immunoglobulin G (rabbit polyclonal, Cell Signaling, Beverly, MA) and monoclonal C5 Mitf antibody (a gift from David Fisher, Dana-Farber Institute, Harvard School of Medicine), respectively. Western blot analysis of Bcl2 was carried out on cell extracts that were prepared 48 hours after UV exposure, using monoclonal Ab-1 antibody (Calbiochem). For all three proteins, the membranes were then incubated with the appropriate horseradish peroxidase–conjugated anti-mouse or anti-rabbit immunoglobulin G (Calbiochem). Membranes were also reacted with actin-horseradish peroxidase immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA) to control for loading. The respective bands were visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Densitometric analysis of the bands was carried out using Alpha Innotech Imaging System and the AlphaEase FC StandAlone Software (San Leandro, CA).

Southwestern blot analysis of cyclobutane pyrimidine dimers. Melanocytes were plated onto 100-mm dish and treated for 4 days with 0, α-MSH, or ET-1, as described above. Melanocytes were then irradiated with 0 or 21 mJ/cm^2 followed by the appropriate treatment with growth factors. Cells were harvested immediately after UV exposure (T0) to determine induction of cyclobutane pyrimidine dimers (CPD), or 10 and 24 hours after UV irradiation to determine the rate of CPD repair. Genomic DNA was isolated from each group (at least 2×10^6 melanocytes per group) using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). For Southwestern blot analysis, triplicate lanes (0.5 μg DNA per lane) were loaded for each group, and monoclonal antibody TDM2 directed against CPD (a gift from Toshi Mori, Nara University, Japan) was used, as described by Schwarz et al. (41). Each membrane was incubated with horseradish peroxidase–conjugated anti-mouse antibody (Calbiochem) followed by reacting with 3,3′-diaminobenzidine peroxidase substrate (Vector Laboratories, Burlingame, CA). The resulting bands were quantified by densitometry, as described above. The data were statistically analyzed using Student's t test ($P \leq 0.01$). In some experiments, the results obtained by Southwestern blot analysis were confirmed by determining CPD in the same DNA samples using RIA, as described by Mitchell (42). Briefly, 2 μg of heat-denatured DNA samples were incubated overnight at 4°C with 10 pg of DNA labeled with [^{32}P]dTTP and purified anti-CPD IgG. The immune complex was precipitated with goat immunoglobulin and carrier serum (Calbiochem), and the radioactivity counted by scintillation counter. The frequency of CPD (CPD/10^7 Da) was calculated using a standard curve based on measuring irradiated salmon sperm DNA.

Determination of hydrogen peroxide generation by UV-irradiated melanocytes. Melanocytes were plated onto 100-mm dish and irradiated in PBS with 0 or 21 mJ/cm^2 UV, which is expected to result in the generation of photoproducts as well as reactive oxygen species (43). Immediately after UV exposure, melanocytes were treated with 0, 1 nmol/L α-MSH, and/or 0.1 nmol/L ET-1. The release of hydrogen peroxide was measured

immediately (1-2 minutes), 30, 45, and 60 minutes after UV exposure by determining the luminescence of luminol by the released hydrogen peroxide as follows. At the indicated time, 240 μL aliquots of PBS from each dish (triplicate dishes per group) were transferred to tubes containing 240 μL respiratory buffer consisting of 70 mmol/L sucrose, 220 mmol/L D-mannitol, 2 mmol/L HEPES, 2.5 mmol/L KH_3PO_4, 2.5 mmol/L $MgCl_2$, 0.5 mmol/L Na_4-EDTA, 1 mmol/L D(+)-glucose. Horseradish peroxidase (10 μg/mL) and 5 μmol/L luminol (Sigma Chemical) were added to the tubes and luminescence was measured, immediately after, in a Turner Luminometer, model TD10e. One UV-irradiated group was treated with 300 units/mL catalase, which degrades hydrogen peroxide to water and oxygen, to show the specificity of the reaction. Luminescence readings were plotted against a standard curve generated using known concentrations of hydrogen peroxide, and the data were expressed as pmol hydrogen peroxide/10^5 cells. Data were statistically analyzed using ANOVA, followed by SNK ($P < 0.05$).



Results

UV-induced apoptosis of human melanocytes and its inhibition by ET-1 and α-MSH. Irradiation of cultured human melanocytes with 21 mJ/cm^2 UV resulted in marked cytotoxicity, evidenced by an increase in cell death and reduction in the number of viable melanocytes (Fig. 1). Treatment with 1 nmol/L α-MSH or 0.1 nmol/L ET-1 for 4 days before and immediately after irradiation with 21 mJ/cm^2 UV, significantly reduced the UV-induced cell death and increased the viability of neonatal melanocytes (Fig. 1*A* and *B*). Concomitant treatment with α-MSH and ET-1 reduced the cytotoxic effect of UV to a greater extent than treatment with either agent alone (Fig. 1*A*). Forskolin, a direct activator of adenylate cyclase, also reduced the cytotoxic effect of UV on melanocytes, suggesting the significance of the cAMP pathway in melanocyte survival (Fig. 1*A* and *B*). These responses to UV, α-MSH, ET-1, and forskolin were observed in neonatal melanocyte cultures with different melanin contents. The anti-apoptotic effect of α-MSH was also evident in adult human melanocytes and was not strictly dependent on its mitogenic effect, because 1 nmol/L α-MSH did not significantly increase the proliferation of adult melanocytes, yet reduced cell death and increased the number of viable melanocytes by >50% following UV exposure (Fig. 1*C* and *D*).

The UV-induced melanocyte death occurred by apoptosis, as determined by Annexin V staining, and treatment with ET-1 and/or α-MSH reduced the apoptotic effect of UV (Fig. 2*A*). The anti-apoptotic effects of ET-1 and α-MSH were mediated by activating the ET-1 receptors and the MC1R, respectively. Treatment of melanocytes with 150 nmol/L PD 145065, the universal endothelin receptor antagonist, blocked the anti-apoptotic effect of ET-1 and the contribution of ET-1 to the survival of melanocytes concomitantly treated with ET-1 and α-MSH (Fig. 2*B*). In addition, the anti-apoptotic effect of α-MSH but not that of ET-1 was absent in melanocytes expressing loss-of-function mutations in the *MC1R* gene (Fig. 2*C*). The survival effects of ET-1 or α-MSH were not due to increased melanin synthesis, because they were evident in tyrosinase-negative albino melanocytes that lack the ability to synthesize melanin (Fig. 2*D*).

Activation of the inositol triphosphate kinase-Akt and Mitf pathways in UV-irradiated melanocytes by ET-1 and α-MSH. To investigate the role of the IP3 kinase pathway in the survival effects of α-MSH and ET-1, melanocytes were treated with 15 μmol/L

Figure 1. ET-1 and α-MSH reduce the death and increase the survival of UV-irradiated neonatal and adult human melanocytes. Neonatal melanocytes (*A* and *B*) and adult melanocytes (*C* and *D*) were treated with 0 (*control*), 0.1 nmol/L ET-1 and/or 1 nmol/L α-MSH, or 1 μmol/L forskolin for a total of 4 days and were either sham irradiated or irradiated with 21 mJ/cm^2 UV, with or without further treatment with ET-1 and/or α-MSH. The number of dead cells (*A* and *C*) and viable cells (*B* and *D*) in each dish (triplicate dishes per group) was determined on days 2 and 4 after UV exposure, as described in Materials and Methods. The data in (*A*) and (*B*) were analyzed using ANOVA followed by SNK. *, $P < 0.01$, significantly different from UV group; +, $P < 0.01$, significantly different from control group. The data in (*A*) and (*B*) are representative of at least 10 experiments in which different neonatal melanocyte cultures were used. The results presented in (*C*) and (*D*) were analyzed statistically by Student's *t* test ($P < 0.05$) and are representative of three independent experiments using three different adult melanocyte cultures.

LY 294002, a selective IP3 kinase inhibitor, for 45 minutes before and immediately after UV exposure. Treatment of melanocytes with LY 294002 augmented the UV-induced apoptosis and abrogated the anti-apoptotic effect of α-MSH and ET-1 (Fig. 3*A*). Additionally, exposure of UV-irradiated human melanocytes to α-MSH and/or ET-1 markedly increased the activity of the serine-threonine kinase Akt, the substrate for IP3 kinase, above that of control or UV-irradiated melanocytes, as determined by Akt kinase activity assay using exogenous Bad as a substrate (Fig. 3*B*). By Western blot analysis of total Akt, we found that treatment of melanocytes with α-MSH and/or ET-1, or exposure to UV did not change the total levels of Akt (Fig. 3*C*).

Regulation of Mitf and Bcl2 expression by UV, α-MSH, and ET-1. Western blot analysis of Mitf revealed that in human melanocytes that were irradiated with UV, the level of phosphorylated Mitf, the slower mobility form, was increased by 50% above control (Fig. 4*A*). Treatment with α-MSH alone or in combination with ET-1 resulted in further increase (84%) of phosphorylated Mitf in the UV-irradiated groups. As for the unphosphorylated form of Mitf, the faster mobility form, it was increased by α-MSH as well as ET-1, with the highest increase achieved upon concomitant treatment with both agents. Irradiation of melanocytes with UV reduced the levels of the anti-apoptotic Bcl2, known to be regulated by Mitf, by 25% (Fig. 4*B*; ref. 9). Treatment of UV-irradiated melanocytes with ET-1 or α-MSH suppressed the UV-induced reduction in Bcl2 levels and restored them back to control, or above control levels, respectively.

Effects of α-MSH and ET-1 on the induction and repair of DNA photoproducts following UV irradiation. Irradiation of human melanocytes with UV resulted in a dose-dependent induction of DNA photoproducts, mainly in the form of CPD (data not shown). Melanocytes irradiated with 21 mJ/cm^2 UV encountered considerably high levels of CPD, of which <25% were removed 24 hours after irradiation, as determined by densitometric analysis of the bands (triplicate/group) obtained by Southwestern blotting (Fig. 5). Pretreatment with α-MSH alone or in combination with ET-1 enhanced the rate of removal of CPD, an effect that increased significantly by 24 hours when >45% of CPD were repaired (Fig. 5). In some experiments, ET-1 or α-MSH reduced the initial induction of CPD. In the experiment presented in Fig. 5, ET-1 reduced the induction of CPD by about 30%. Enhancement of CPD removal by α-MSH was absent in melanocytes expressing loss-of-function MC1R (data not shown).

Generation of hydrogen peroxide by human melanocytes in response to UV irradiation and inhibition of this response by α-MSH and ET-1. Exposure of cultured human melanocytes to UV, known to induce the generation of reactive oxygen species (43), resulted in a dose-dependent release of hydrogen peroxide (data not shown). Release of hydrogen peroxide occurred instantly after irradiation of melanocytes with 21 mJ/cm^2 UV and increased by >3-fold within 30 minutes after UV exposure (Fig. 6). The levels of released hydrogen peroxide dropped sharply by 45 minutes

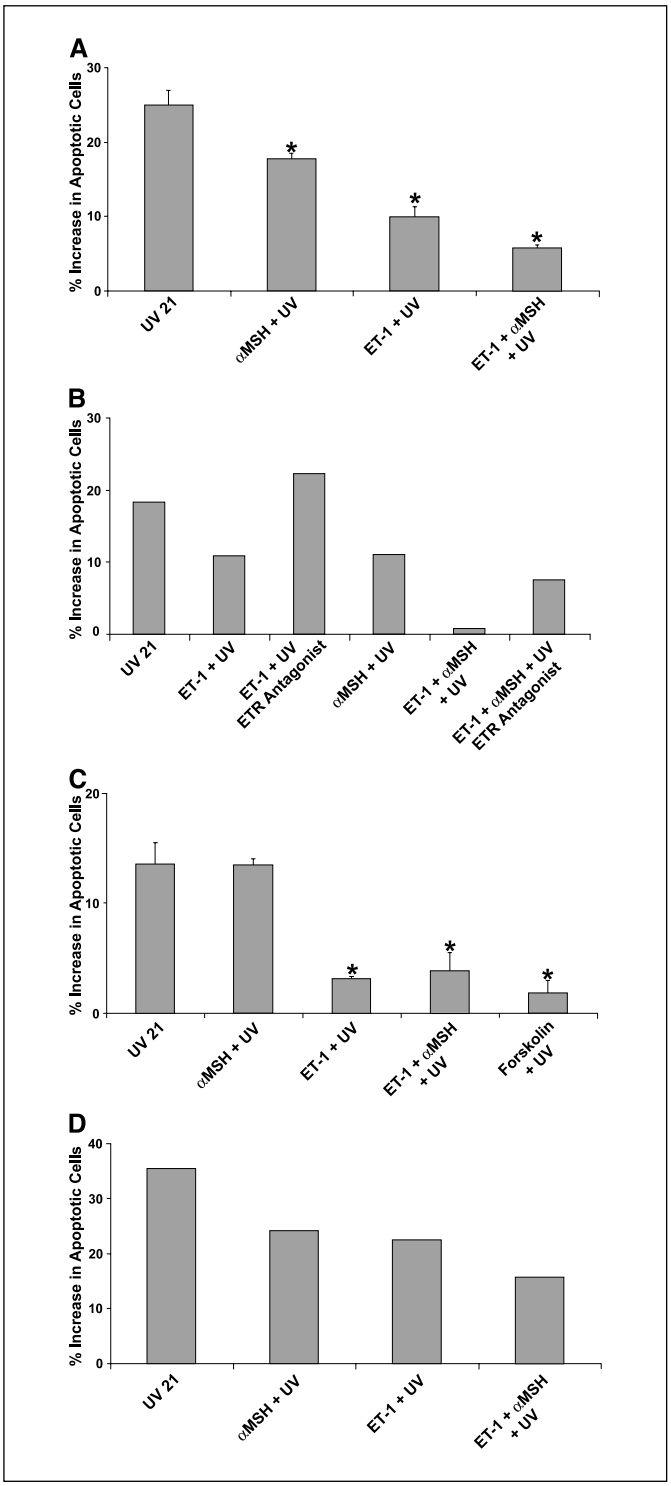


Figure 2. ET-1 and α-MSH inhibit UV-induced apoptosis of human melanocytes by receptor-mediated mechanisms, independently of melanogenesis. Melanocytes were treated with 0, ET-1, and/or α-MSH and irradiated with UV, harvested 24 hours later, stained with APC Annexin V and propidium iodide, and analyzed by flow cytometry, as described in Materials and Methods. *A,* responses of human melanocytes with functional MC1R. *Columns,* means of three separate experiments in which three different melanocyte cultures were tested; *bars,* ±SE. *, $P < 0.05$, significantly different from the UV group as determined by ANOVA followed by SNK. *B,* significance of the endothelin receptors in the antiapoptotic effect of ET-1. Some groups were treated with the ET receptor antagonist PD 145065 4 days before UV exposure and for an additional 24 hours thereafter. This experiment was repeated twice with similar findings. *C,* apoptotic responses of human melanocytes with loss-of-function mutations in the MC1R. *Columns,* mean of three experiments in which three different cultures expressing different loss-of-function mutations in the MC1R were tested; *bars,* ±SE. *, $P < 0.05$, significantly different from the UV group as determined by ANOVA followed by SNK. *D,* apoptotic response of a tyrosinase-negative albino melanocyte culture that lacked the ability to synthesize melanin. This experiment was repeated twice with similar findings.

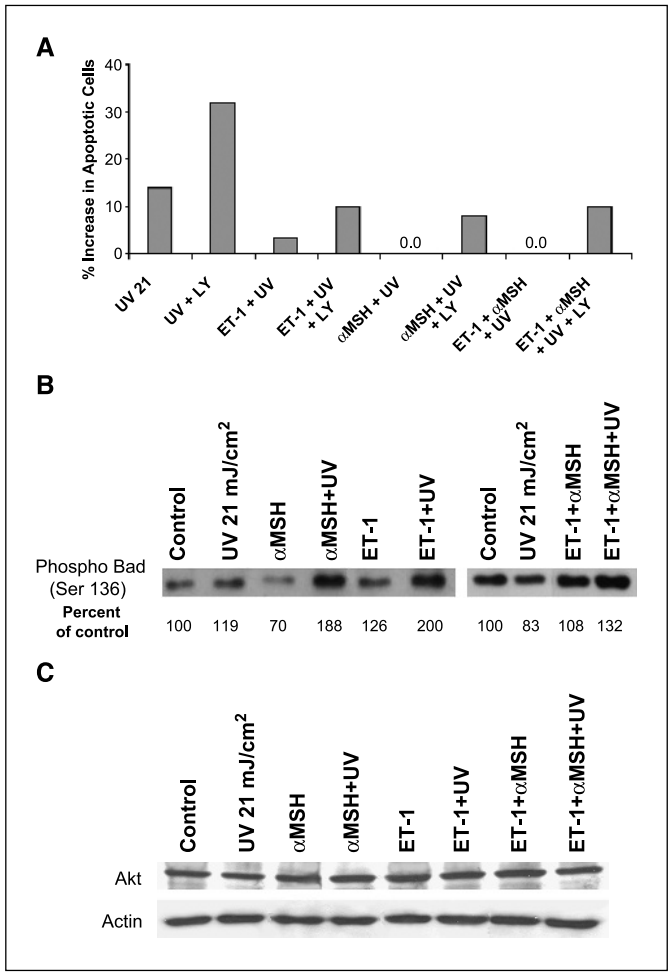


Figure 3. Involvement of the IP3 kinase pathway in the survival effects of α-MSH and/or ET-1. *A,* effect of the IP3 kinase inhibitor LY 294002 on the apoptosis of UV-irradiated melanocytes, in the presence or absence of ET-1 and/or α-MSH was determined using Annexin V staining, as described in Materials and Methods. This experiment was repeated twice with similar results. *B,* kinase activity of Akt, the substrate of IP3 kinase, was determined as described in Materials and Methods, using purified Bad as substrate. The resulting bands were quantified by densitometry, and the change in Akt activity was expressed as % of control. Those experiments were repeated five times with similar results. *C,* Western blot analysis of total Akt and actin (to control for loading), to verify that the observed change in Akt activity is not due to increased levels of Akt by treatment with UV, α-MSH, or ET-1.

after irradiation. Unirradiated melanocytes did not release any hydrogen peroxide in the presence or absence of α-MSH or ET-1. Treatment of human melanocytes with 1 nmol/L α-MSH, 0.1 nmol/L ET-1, or combination of both, immediately after UV irradiation prevented the increase in hydrogen peroxide released within 30 minutes after UV exposure. Treatment with α-MSH alone, or concomitantly with ET-1 continued to reduce significantly the release of hydrogen peroxide 45 minutes after irradiation. In all the UV-irradiated groups, the release of hydrogen peroxide was minimal 60 minutes after UV exposure. Treatment of human melanocytes to 300 units/mL catalase totally abrogated the UV-induced hydrogen peroxide release (data not shown).

Discussion

Epidermal melanocytes confer photoprotection to the skin by the synthesis of melanin and distribution of melanin-containing melanosomes to the neighboring keratinocytes (1, 2). Unlike keratinocytes that have a high self-renewal capacity, epidermal melanocytes are differentiated cells with a low proliferation potential. Given the significance of melanocytes in photoprotection and prevention of photocarcinogenesis, it is crucial to insure their viability and genomic stability in the epidermis. A main goal of this study is to elucidate some of the pathways that promote melanocyte survival and genomic integrity following UV exposure.

Irradiation of cultured human melanocytes with UV results in dose-dependent induction of DNA photoproducts, reduction in proliferation due to G_1 arrest, and apoptosis (refs. 10–12; Figs. 1, 2, and 5). The melanocortins and ET-1 determine the melanogenic response of melanocytes to UV and stimulate their proliferation by overcoming the UV-induced G_1 arrest (11, 12). The mitogenic and melanogenic effects of α-MSH on human melanocytes are mediated by binding and activating the MC1R, a G_s protein–coupled receptor with seven transmembrane domains (28). The effects of ET-1 on human melanocytes are elicited by activating the endothelin receptors, G_q protein–coupled receptors with seven transmembrane domains, particularly the predominant ETBR (12). ET-1 might indirectly affect human melanocytes by up-regulating the mRNA levels of the *MC1R* gene (12, 44). The latter effect is expected to sustain and possibly enhance the ability of melanocytes to respond to melanocortins.

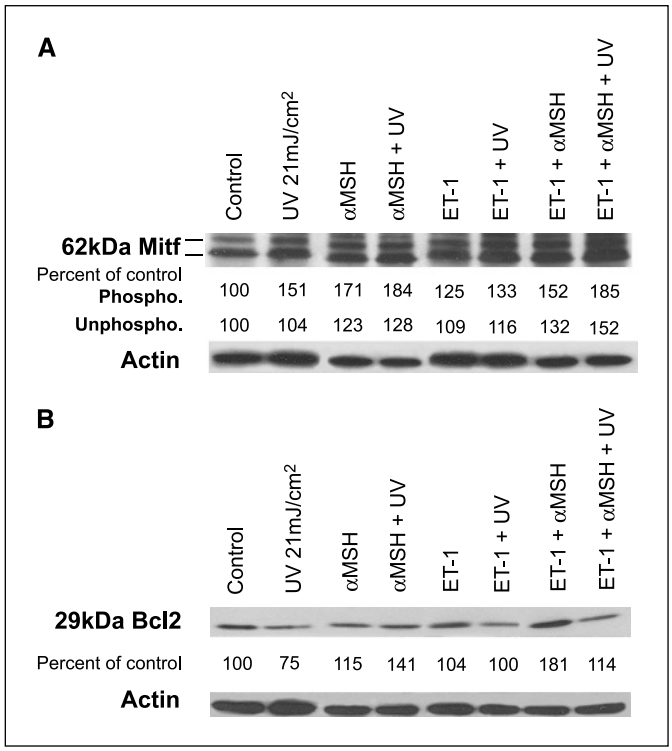


Figure 4. Modulation of Mitf and Bcl2 expression by UV, ET-1, and/or α-MSH. Melanocytes were treated with ET-1 and/or α-MSH and irradiated with UV. *A,* 90 minutes after UV exposure, cell lysates were prepared and Western blot analysis for Mitf was carried out as described in Materials and Methods. *B,* expression of Bcl2 was determined 48 hours after UV exposure, using Western blot analysis as described in Materials and Methods. For Mitf and Bcl2, Western blot analysis for actin was carried out as a loading control. Each of those experiments was repeated four times with similar results. The resulting bands were quantified by densitometry, and change in expression of the respective proteins was expressed as percent of control, after correcting for the difference in loading.

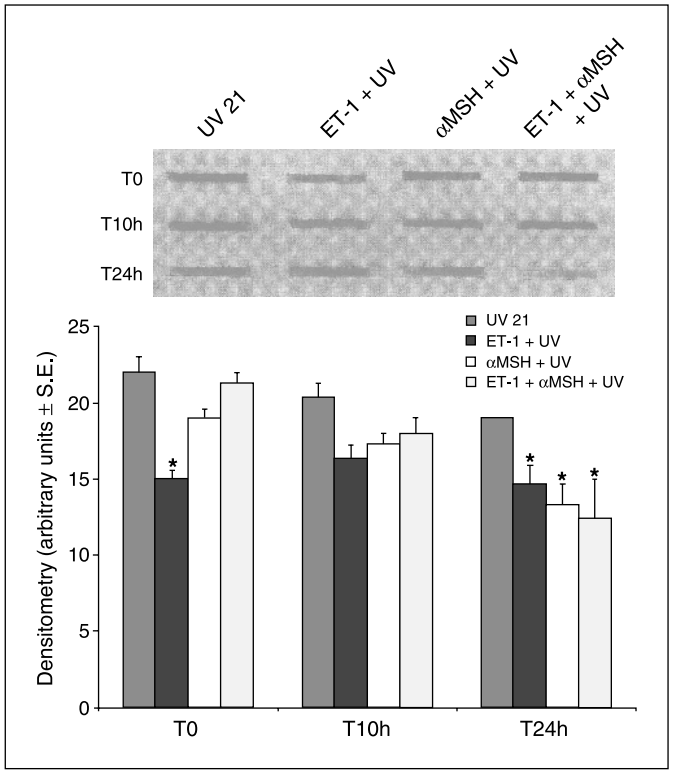


Figure 5. α-MSH and ET-1 enhance the repair of UV-induced CPD in human melanocytes. Southwestern blot analysis was employed to determine induction of CPD immediately (i.e., few minutes) after UV exposure (T0) and their repair 10 and 24 hours after irradiation as described in Materials and Methods. Densitometry of the bands in each group revealed gradual reduction of CPD over time and significant enhancement of their removal in the groups treated with α-MSH or α-MSH plus ET-1 24 h after irradiation. These results were reproduced in at least three experiments using different melanocyte cultures.

The data presented in this study ascribe a new role for ET-1 and α-MSH as survival factors that rescue melanocytes from UV-induced apoptosis (Figs. 1 and 2*A*, *B*, and *D*). The anti-apoptotic effects of ET-1 and α-MSH are independently and strictly mediated by activation of the endothelin receptor and MC1R, respectively. Blocking the endothelin receptors abrogated the survival effect of ET-1, but the response to α-MSH was still evident (Fig. 2*B*). Loss-of-function mutations in the *MC1R* abolished the anti-apoptotic effect of α-MSH but not that of ET-1 (Fig. 2*C*). The pro-survival effects of α-MSH or ET-1 were not dependent on their mitogenic or melanogenic effects on human melanocytes and were evident in adult melanocytes that did not exhibit a significant increase of proliferation in response to treatment with 1 nmol/L α-MSH (Fig. 1*C* and *D*), and in tyrosinase-negative albino melanocytes that lacked the ability to synthesize melanin (Fig. 2*D*). These findings suggest that activation of survival pathways is independent of regulation of melanocyte proliferation and melanogenesis.

The observed increase in survival of UV-irradiated melanocytes in response to ET-1 or α-MSH treatment is due to inhibition of apoptosis and activation of survival pathways. Inhibition of apoptosis was shown by reduction in the percent of melanocytes that stained positively with Annexin V and by reversing the inhibitory effect of UV on Bcl2 levels (Fig. 2*A*, *B*, *D* and Fig. 4*B*). α-MSH and ET-1 activated the IP3 kinase pathway, as well as Mitf, a helix-loop-helix transcription factor that is crucial for melanocyte survival and the regulation of genes that code for important melanogenic enzymes, such as tyrosinase and tyrosinase-related protein 1 (*Tyrp1*; Figs. 3 and 4; refs. 29–31).

It has been proposed that whereas UV induces apoptosis in human skin *in vivo*, and in cultured human keratinocytes *in vitro*, it also activates survival pathways (i.e., IP3 kinases and its substrate Akt/PKB) via activation of the EGF receptor, to limit the extent of cell death (21). Activation of Akt/PKB results in inhibition of the mitochondrial apoptotic pathway, evidenced by phosphorylation of the proapoptotic Bcl2 family member Bad, and thus inhibits its dimerization with Bcl_{xL} (25). This study reveals that activation of the IP3 kinase pathway is critical for limiting apoptosis of melanocytes in response to UV and for the survival response of melanocytes to ET-1 and α-MSH. Blocking IP3 kinase activity by LY 294002 reduced the antiapoptotic effects of α-MSH and ET-1 (Fig. 3*A*). Furthermore, treatment of UV-irradiated melanocytes with α-MSH and ET-1 activated Akt, the substrate for IP3 kinase (Fig. 3*B*). The observed change in Akt activity was not accompanied by change in the levels of total Akt (Fig. 3*C*). Recently, IP3 kinase was reported to mediate the survival effect of stem cell factor on human melanocytes treated with TRAIL, further suggesting the importance of the IP3 kinase pathway in the maintenance of melanocyte survival (45). Our results show the significance of this pathway in mediating the survival

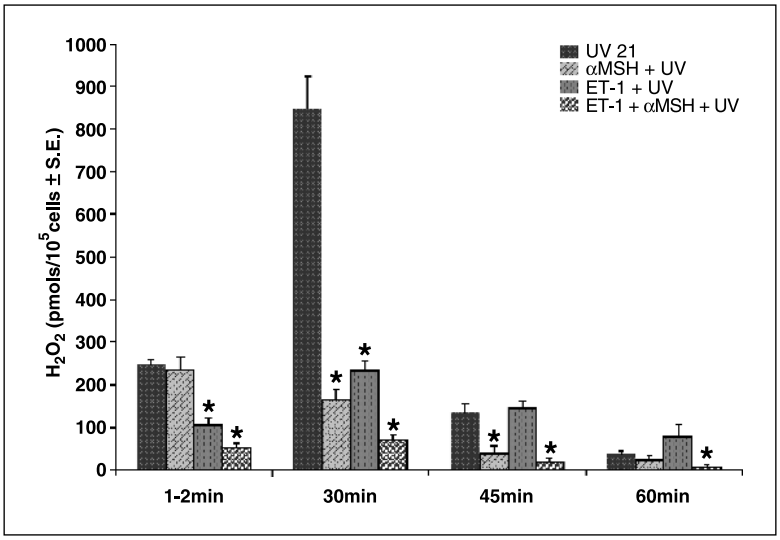


Figure 6. Reduction in the levels of hydrogen peroxide produced by UV irradiated melanocytes by α-MSH and ET-1. Melanocytes were irradiated with 21 mJ/cm^2 UV and immediately treated with 0, 1 nmol/L α-MSH, and/or 0.1 nmol/L ET-1. The generation of hydrogen peroxide was determined by measuring the luminescence of luminol by hydrogen peroxide that is released by melanocytes immediately (1-2 minutes), 30, 45, and 60 minutes after UV exposure as described in Materials and Methods. Similar results were obtained in three separate experiments using three different melanocyte cultures. *, $P < 0.05$, significantly different from UV using ANOVA followed by SNK.

effects of α-MSH and ET-1 but do not rule out the possibility that these two factors might influence other pathways to promote melanocyte viability.

Activation of the mitogen-activated protein (MAP) kinases ERK1/2, and subsequently the transcription factor cAMP response element binding protein (CREB), results in the phosphorylation and activation of Mitf in melanocytes (46). Treatment of human melanocytes with ET-1 activates the same MAP kinase pathway and phosphorylates CREB on Ser^{133}; these effects are enhanced in the concomitant presence of α-MSH (47, 48). In human melanocytes, CREB phosphorylation is also induced by UV, in part via activation of the MAP kinase p38 (48). Additionally, CREB is phosphorylated by Akt (49). The data hereby presented show that treatment of human melanocytes with UV, α-MSH, and/or ET-1 resulted in increased levels, particularly of the phosphorylated form of Mitf, downstream of CREB (Fig. 4*A*). Treatment of UV-irradiated melanocytes with α-MSH or ET-1 increased the levels of phosphorylated, as well as unphosphorylated Mitf, and concomitant treatment with both had a greater effect than either factor, or UV alone. Mitf in turn regulates the expression of Bcl2, an important determinant of melanocyte survival, and Akt up-regulates Bcl2 expression through the phosphorylation of CREB, upstream from Mitf (9, 32, 49). Irradiation of melanocytes with UV reduced the levels of Bcl2, and treatment with α-MSH alone, or with α-MSH and ET-1 markedly abrogated this effect (Fig. 4*B*). Collectively, these results suggest that inhibition of the UV-induced reduction in Bcl2 by ET-1 and α-MSH is mediated by activation of Mitf and Akt.

Unlike keratinocytes that are highly proliferative, melanocytes in the epidermis are differentiated cells with a limited proliferation capacity. Melanocyte survival is crucial for cutaneous photoprotection, yet survival is beneficial only if genomic stability is maintained. Generally, apoptosis is a mechanism to eliminate cells with extensive DNA damage that surpasses their DNA repair capacity thus reducing the chance for mutations to occur. Otherwise, the survival of damaged cells would increase the chance for mutagenesis and malignant transformation. Our results clearly show that α-MSH and ET-1 reduce UV-induced DNA photoproducts, suggesting enhancement of nucleotide excision repair (Fig. 5). Therefore, the anti-apoptotic effect of α-MSH and ET-1, which are accompanied by reduced DNA damage, are expected to restore genomic stability and inhibit mutagenesis. The notion that survival factors may enhance DNA repair is supported by two recent studies that showed that interleukin-12, which is synthesized by keratinocytes, particularly after UV exposure, and IGF-I inhibit UV-induced apoptosis and also enhance the rate of removal of DNA photoproducts in keratinocytes (18, 41).

Exposure to UV results in the generation of reactive oxygen species that can cause oxidative DNA damage, as well as lipid peroxidation and protein damage (43). We have found that irradiation of human melanocytes with UV resulted in the generation of hydrogen peroxide (Fig. 6). The levels of released hydrogen peroxide were markedly reduced by α-MSH and ET-1, suggesting that these two factors reduce the UV-induced oxidative stress in melanocytes. Based on the results hereby presented, we propose that increased synthesis of melanocortins and ET-1 in the skin following UV exposure prevents skin cancer via several mechanisms, including reduction in hydrogen peroxide levels, enhancement of DNA repair, preservation of melanocyte survival and stimulation of melanogenesis to insure optimal photoprotection. Our novel findings with α-MSH and ET-1 explain why loss-of-function mutations in the *MC1R* increase the risk for melanoma (33, 34).

Acknowledgments

Received 12/20/2004; revised 2/18/2005; accepted 3/14/2005.

Grant support: R01 ES-09110, (Z. Abdel-Malek), Johnson and Johnson Skin Research Center Training grant ES06096 (Z. Abdel-Malek and A.L. Kadekaro), FAPESP (A.L. Kadekaro), and ES10957 (T32) (J. Hauser).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Toshio Mori, Ph.D. (Radioisotope Research Center, Nara Medical University) for donating the antibody TDM2 against cyclobutane pyrimidine dimers; David Fisher, M.D., Ph.D. (Division of Pediatric Oncology, Dana-Farber Cancer Institute) for donating the C5 Mitf antibody; Raymond Boissy, Ph.D. (Department of Dermatology, University of Cincinnati) for providing the tyrosinase-negative albino human melanocyte culture; and David Mitchell, Ph.D. (Department of Carcinogenesis, University of Texas M.D. Anderson Cancer Center Science Park/Research Division), for doing the RIA for CPD.

References

1. Dillman AM. Photobiology of skin pigmentation. In: Levine N, editor. Pigmentation and pigmentary disorders. Boca Raton: CRC Press, Inc.; 1993. p. 61–94.
2. Pathak MA, Jimbow K, Fitzpatrick T. Photobiology of pigment cell. In: Seiji M, editor. Phenotypic expression in pigment cells. Tokyo (Japan): University of Tokyo Press; 1980. p. 655–70.
3. Kaidbey KH, Poh Agin P, Sayre RM, Kligman AM. Photoprotection by melanin: a comparison of Black and Caucasian skin. J Am Acad Dermatol 1979;1:249–60.
4. Kobayashi N, Nakagawa A, Muramatsu T, et al. Supranuclear melanin caps reduce ultraviolet induced DNA photoproducts in human epidermis. J Invest Dermatol 1998;110:806–10.
5. Bustamante J, Bredeston L, Malanga G, Mordoh J. Role of melanin as a scavenger of active oxygen species. Pigment Cell Res 1993;6:348–53.
6. Murphy G, Young AR, Wulf HC, Kulms D, Schwarz T. The molecular determinants of sunburn cell formation. Exp Dermatol 2001;10:155–60.
7. Plettenberg A, Ballaun C, Pammer J, et al. Human melanocytes and melanoma cells constitutively express the bcl-2 proto-oncogene *in situ* and in cell culture. Am J Pathol 1995;146:651–9.
8. Klein-Parker HA, Warshawski L, Tron VA. Melanocytes in human skin express bcl-2 protein. J Cutan Pathol 1994;21:297–301.
9. McGill GG, Horstmann M, Widlund HR, et al. Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. Cell 2002;109:707–18.
10. Barker D, Dixon K, Medrano EE, et al. Comparison of the responses of human melanocytes with different melanin contents to ultraviolet B irradiation. Cancer Res 1995;55:4041–6.
11. Im S, Moro O, Peng F, et al. Activation of the cyclic AMP pathway by α-melanotropin mediates the response of human melanocytes to ultraviolet B radiation. Cancer Res 1998;58:47–54.
12. Tada A, Suzuki I, Im S, et al. Endothelin-1 is a paracrine growth factor that modulates melanogenesis of human melanocytes and participates in their responses to ultraviolet radiation. Cell Growth Differ 1998;9:575–84.
13. Halaban R, Langdon R, Birchall N, et al. Basic fibroblast growth factor from human keratinocytes is a natural mitogen for melanocytes. J Cell Biol 1988;107:1611–9.
14. Imokawa G, Yada Y, Miyagishi M. Endothelins secreted from human keratinocytes are intrinsic mitogens for human melanocytes. J Biol Chem 1992;267:24675–80.
15. Wakamatsu K, Graham A, Cook D, Thody AJ. Characterization of ACTH peptides in human skin and their activation of the melanocortin-1 receptor. Pigment Cell Res 1997;10:288–97.
16. Imokawa G, Miyagishi M, Yada Y. Endothelin-1 as a new melanogen: Coordinated expression of its gene and the tyrosinase gene in UVB-exposed human epidermis. J Invest Dermatol 1995;105:32–7.
17. Yao R, Cooper GM. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 1995;267:2003–6.
18. Decraene D, Agostinis P, Bouillon R, Degreef H, Garmyn M. Insulin-like growth factor-1-mediated AKT activation postpones the onset of ultraviolet B-induced apoptosis, providing more time for cyclobutane thymine dimer removal in primary human keratinocytes. J Biol Chem 2002;277:32587–95.
19. Romashkova JA, Makarov SS. NF-κB is a target of AKT in anti-apoptotic PDGF signalling. Nature 1999;401:86–90.

20. Blume-Jensen P, Janknecht R, Hunter T. The Kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser^{136}. Curr Biol 1998;8:779–82.

21. Wan YS, Wang ZQ, Shao Y, Voorhees JJ, Fisher GJ. Ultraviolet irradiation activates PI 3-kinase/AKT survival pathway via EGF receptors in human skin *in vivo*. Int J Oncol 2001;18:461–6.

22. Mildner M, Eckhart L, Lengauer B, Tschachler E. Hepatocyte growth factor/scatter factor inhibits UVB-induced apoptosis of human keratinocytes but not of keratinocyte-derived cell lines via the phosphatidylinositol 3-kinase/AKT pathway. J Biol Chem 2002;277: 14146–52.

23. Dudek H, Datta SR, Franke TF, et al. Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 1997;275:661–4.

24. Kandel ES, Hay N. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Exp Cell Res 1999;253:210–29.

25. Datta SR, Dudek H, Tao X, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997;91:231–41.

26. Cardone MH, Roy N, Stennicke HR, et al. Regulation of cell death protease caspase-9 by phosphorylation. Science 1998;282:1318–21.

27. Filippa N, Sable CL, Filloux C, Hemmings B, Van Obberghen E. Mechanism of protein kinase B activation by cyclic AMP-dependent protein kinase. Mol Cell Biol 1999;19:4989–5000.

28. Suzuki I, Cone R, Im S, Nordlund J, Abdel-Malek Z. Binding of melanotropic hormones to the melanocortin receptor MC1R on human melanocytes stimulates proliferation and melanogenesis. Endocrinology 1996; 137:1627–33.

29. Yasumoto K-I, Yokoyama K, Shibata K, Tomita Y, Shibahara S. Microphthalmia-associated transcription factor as a regulator for melanocyte-specific transcription of the human tyrosinase gene. Mol Cell Biol 1994;14:8058–70.

30. Bentley NJ, Eisen T, Goding CR. Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. Mol Cell Biol 1994;14: 7996–8006.

31. Fang D, Tsuji Y, Setaluri V. Selective down-regulation of tyrosinase family gene TYRP1 by inhibition of the activity of melanocyte transcription factor, MITF. Nucleic Acids Res 2002;30:3096–106.

32. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 1993;75:229–40.

33. Palmer JS, Duffy DL, Box NF, et al. Melanocortin-1 receptor polymorphisms and risk of melanoma: is the association explained solely by pigmentation phenotype? Am J Hum Genet 2000;66:176–86.

34. Duffy DL, Box NF, Chen W, et al. Interactive effects of MC1R and OCA2 on melanoma risk phenotypes. Hum Mol Genet 2004;13:447–61.

35. Abdel-Malek Z, Swope VB, Suzuki I, et al. Mitogenic and melanogenic stimulation of normal human melanocytes by melanotropic peptides. Proc Natl Acad Sci U S A 1995;92:1789–93.

36. Resink TJ, Scott-Burden T, Weber E, Buhler FR. Phorbol ester promotes a sustained down-regulation of endothelin receptors and cellular responses to endothelin in human vascular smooth muscle cells. Biochem Biophys Res Commun 1990;166:1213–9.

37. Doherty AM, Cody WL, He JX, et al. *In vitro* and *in vivo* studies with a series of hexapeptide endothelin antagonists. J Cardiovasc Pharmacol 1993;22 Suppl 8: S98–102.

38. Scott MC, Wakamatsu K, Ito S, et al. Human *melanocortin 1 receptor* variants, receptor function and melanocyte response to UV radiation. J Cell Sci 2002;115: 2349–55.

39. Zhao H, Boissy RE. Distinguishing between the catalytic potential and apparent expression of tyrosinase activities. Am J Med Sci 1994;308:322–30.

40. Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002). J Biol Chem 1994;269:5241–8.

41. Schwarz A, Stander S, Berneburg M, et al. Interleukin-12 suppresses ultraviolet radiation-induced apoptosis by inducing DNA repair. Nat Cell Biol 2002;4:26–31.

42. Mitchell DL. Quantification of DNA photoproducts in mammalian cell DNA using radioimmunoassay. In: Henderson DS, editor. Methods in molecular biology, DNA repair protocols. Totowa: The Humana Press; 1999. p. 165–75.

43. Sander CS, Chang H, Hamm F, Elsner P, Thiele JJ. Role of oxidative stress and the antioxidant network in cutaneous carcinogenesis. Int J Dermatol 2004;43: 326–35.

44. Scott MC, Suzuki I, Abdel-Malek ZA. Regulation of the human melanocortin 1 receptor expression in epidermal melanocytes by paracrine and endocrine factors, and by UV radiation. Pigment Cell Res 2002;15: 433–9.

45. Larribere L, Khaled M, Tartare-Deckert S, et al. PI3K mediates protection against TRAIL-induced apoptosis in primary human melanocytes. Cell Death Differ 2004;11:1084.

46. Wu M, Hemesath T, Takemoto CM, et al. c-Kit triggers dual phosphorylations, which couple activation and degradation of the essential melanocyte factor Mi. Genes Dev 2000;14:301–12.

47. Bohm M, Moellmann G, Cheng E, et al. Identification of $p90^{RSK}$ as the probable CREB-Ser^{133} kinase in human melanocytes. Cell Growth Differ 1995;6:291–302.

48. Tada A, Pereira E, Beitner Johnson D, Kavanagh R, Abdel-Malek ZA. Mitogen and ultraviolet-B-induced signaling pathways in normal human melanocytes. J Invest Dermatol 2002;118:316–22.

49. Du K, Montminy M. CREB is a regulatory target for the protein kinase Akt/PKB. J Biol Chem 1998;273: 32377–9.

α-Melanocortin and Endothelin-1 Activate Antiapoptotic Pathways and Reduce DNA Damage in Human Melanocytes

Ana Luisa Kadekaro, Renny Kavanagh, Hiromi Kanto, et al.

Cancer Res 2005;65:4292-4299.