DNA Damage and Cellular Stress Responses

Molecular Cancer Research

Alpha-Melanocyte–Stimulating Hormone Suppresses Oxidative Stress through a p53-Mediated Signaling Pathway in Human Melanocytes

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Abstract

Epidermal melanocytes are skin cells specialized in melanin production. Activation of the melanocortin 1 receptor (MC1R) on melanocytes by α-melanocyte–stimulating hormone (α-MSH) induces synthesis of the brown/black pigment eumelanin that confers photoprotection from solar UV radiation (UVR). Contrary to keratinocytes, melanocytes are slow proliferating cells that persist in the skin for decades, in an environment with high levels of UVR-induced reactive oxygen species (ROS). We previously reported that in addition to its role in pigmentation, α-MSH also reduces oxidative stress and enhances the repair of DNA photoproducts in melanocytes, independent of melanin synthesis. Given the significance of ROS in carcinogenesis, here we investigated the mechanisms by which α-MSH exerts antioxidant effects in melanocytes. We show that activation of the MC1R by α-MSH contributes to phosphorylation of p53 on serine 15, a known requirement for stabilization and activation of p53, a major sensor of DNA damage. This effect is mediated by the cAMP/PKA pathway and by the activation of phosphoinositide 3-kinase (PI3K) ATR and DNA protein kinase (DNA-PK). α-MSH increases the levels of 8-oxoguanine DNA glycosylase (OGG1) and apurinic apyrimidinic endonuclease 1 (APE-1/Ref-1), enzymes essential for base excision repair. Nutlin-3, an HDM2 inhibitor, mimicked the effects of α-MSH resulting in reduced phosphorylation of H2AX (γ-H2AX), a marker of DNA damage. Conversely, the p53 inhibitor pifithrin-α or silencing of p53 abolished the effects of α-MSH and augmented oxidative stress. These results show that p53 is an important target of the downstream MC1R signaling that reduces oxidative stress and possibly malignant transformation of melanocytes. *Mol Cancer Res; 10(6); 778–86. ©2012 AACR.*

Introduction

The melanocortin 1 receptor (MC1R) is best known for its role in stimulating eumelanin synthesis in melanocytes (MC). In the skin, presence of eumelanin is considered the major photoprotective factor against UV radiation (UVR)-induced DNA damage and carcinogenesis. Eumelanin creates not only a physical barrier against the deep penetration of UVR into the epidermal and dermal layers (1) but also reduces oxidative stress by scavenging free radicals (2–4). MC1R signaling also plays important roles in reducing the burden of UVR-induced DNA damage. This is affected by enhancing the repair of DNA photoproducts (5, 6) and by inhibiting the generation of reactive oxygen species (ROS), as well as by upregulating the activity and expression of antioxidant enzymes (7, 8).

We have reported that *in vitro* exposure of cultured melanocytes to UVR results in increased ROS generation and oxidative DNA damage (7). Reactive oxygen harms DNA by producing lesions such as damaged bases, apurinic-apyrimidinic sites, and strand breaks (9–11). These lesions, if not repaired, induce mutations and chromosomal instability, events associated with the process of carcinogenesis (12). Several studies relate ROS and oxidative stress to increased susceptibility to melanoma (13–16). Loss of *CDKN2A*/*p16*, a high-penetrance melanoma susceptibility gene, diminishes the capability of melanocytes to counteract ROS (17). The findings that loss of function of MC1R and loss of p16 are associated with increased susceptibility to melanoma suggest that oxidative stress is an underlying mechanism contributing to melanoma formation (18, 19).

This study aimed at elucidating the signaling pathways activated by the MC1R that mediate the antioxidant effect

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of α-melanocyte–stimulating hormone (α-MSH) in melanocytes. We show that the tumor suppressor p53, the universal sensor of cellular stress and regulator of the DNA damage response, is one target of the MC1R signaling pathway and a mediator of the antioxidant effects of α-MSH. Our results unraveled a mechanism involving α-MSH and p53 in the adaptive response of melanocytes to UVR. Others have shown that p53 increases the expression of the melanogenic enzymes tyrosinase and tyrosine-related protein 1 (TYRP-1), suggesting its role in regulating melanogenesis (20, 21). In mouse skin, p53 stimulates the expression of proopiomelanocortin (POMC), the precursor of α-MSH and adrenocorticotropic hormone (ACTH; refs. 22, 23). Collectively, findings by others and the results that we herein report underscore the significance of p53 in the DNA damage response of epidermal cells.

Materials and Methods

Culture of human melanocytes

Primary cultures of melanocytes were established from discarded neonatal foreskins as described previously (24). The Institutional Review Board at the University of Cincinnati (Cincinnati, OH) has deemed the protocol for obtaining the skin samples as exempt. The primary culture of melanocytes derived from a patient with oculocutaneous albinism type 2 (OCA2) that expressed inactivating mutation of the *P* gene was a generous gift from Dr. Raymond Boissy (Department Of Dermatology, University of Cincinnati). These cells express functional MC1R but are deficient in melanin production due to impairment of *P* gene. All the melanocyte cultures were maintained in medium containing growth factors, except for the experiments carried out with α-MSH, in which the cells were kept in medium devoid of bovine pituitary extract (BPE) known to contain significant levels of α-MSH.

Transfection of primary culture of melanocytes expressing loss-of-function MC1R with the wild-type MC1R

Melanocytes naturally expressing loss-of-function *MC1R* gene (genotype R160W/D294H) were transfected with pcDNA3 containing the wild-type *MC1R* gene (pMC1R-WT, a gift from Dr. Garcia-Borron, University of Murcia, Murcia, Spain), as described earlier (8).

UV irradiation and H_2O_2 pulse treatment of melanocytes

Melanocytes were irradiated with a bank of FS-20 lamps, with 75% UVB and 25% UVA emissions. UVC rays were blocked by Kodacel filter (Eastman Kodak). Cells were irradiated with a dose of 105 mJ/cm^2 UVR unless otherwise specified, as described before (8). For the experiments using H_2O_2, cells were washed in PBS and incubated for 30 minutes at 37°C in the presence of 100 μmol/L of H_2O_2, or another concentration of H_2O_2 as indicated, and then replaced by fresh medium.

Assessment of DNA damage by single-cell electrophoresis (Comet assay)

Single-cell electrophoresis was conducted to determine the extent of DNA damage induced by UVR or H_2O_2, as previously described by Song and colleagues (7). Briefly, melanocytes kept in medium deprived of BPE (−BPE) were pretreated with 1 nmol/L α-MSH for 4 days before 105 mJ/cm^2 UVR or pulse treatment with 100 μmol/L H_2O_2 (unless otherwise indicated). Two hours after UVR or H_2O_2, cells embedded in 1% low–melting point agarose gel were subjected to electrophoresis. Under these conditions, the undamaged DNA remains in the nucleus and the DNA containing strand breaks (alkali-labile sites) streams toward the anode. The extension of each tail moment, defined as the product of DNA in the tail and the mean distance of its migration, was analyzed using a computerized image analysis system (TriTek CometScore Freeware). The tail moment values obtained from a minimum of 50 randomly selected cells from each slide were expressed as the mean value ± SEM, and data were analyzed by ANOVA followed by Student–Newman–Keuls (SNK; $P \leq 0.05$).

Measurement of UVR-induced hydrogen peroxide

Melanocytes were plated at a density of 1×10^6 cells, irradiated 4 days later with 105 mJ/cm^2 UVR, and treated with 1 nmol/L α-MSH immediately thereafter. The release of H_2O_2 was measured at different time points after UVR, as described (7). Data were expressed as mean of H_2O_2 in pmol/mL/10^6 cells ± SEM, and data were analyzed by ANOVA followed by SNK ($P \leq 0.05$).

Analysis of protein expression by Western blotting

Melanocytes were treated as indicated for Comet assay, and cell extracts were obtained at the indicated time points after UVR or H_2O_2 exposure. Western blotting was carried out using the following antibodies: p53 (DO-1) anti-mouse (Santa Cruz); phospho-p53 serine 15 anti-rabbit (Cell Signaling); phospho-p38 Thr180/Tyr182 (Cell Signaling); p21 (Waf1/Cip1) anti-mouse (Cell Signaling); GADD45α anti-mouse (Santa Cruz); phospho-ATR Ser428 anti-rabbit (Cell Signaling); DNA-PK anti-mouse (Santa Cruz); phospho-DNA-PK anti-rabbit (Santa Cruz); OGG1anti-rabbit (Novus Biologicals); APE-1/Ref-1 anti-mouse (Novus Biologicals); and actin horseradish-conjugated (Santa Cruz); pan-p63 antibody (4A4) against all p63 isoforms and specific antibody anti-alpha-p63 (H-129). Aliquots of 50 μg from total cell lysates obtained with radioimmunoassay buffer supplemented with protease inhibitors (RIPA buffer) were used for Western blotting, as described (25).

Effects of α-MSH and PKA on p53 phosphorylation on serine 15

To determine the effect of α-MSH on p53 phosphorylation and activation, melanocytes kept in −BPE medium were pretreated with 0 and 2 μmol/L H-89, a PKA inhibitor (Sigma Chemical Co.), before UVR exposure. Western

blotting of p53 phosphorylated on serine 15 (p53-Ser15) was conducted 1 hour after UVR.

Effect of α-MSH on the expression of phosphorylated p38 and the role of p38 in the α-MSH–induced phosphorylation of p53 on serine 15

Melanocytes were kept in medium without any growth factors for 18 hours before exposure to UVR. Expression of phospho-p38 was determined by Western blotting 1 hour after UVR. The role of p38 in the phosphorylation of p53 was determined by pretreating melanocytes with 10 μmol/L SB203580, a p38 inhibitor (Sigma Chemical Co.), for 18 hours before UVR and 1.5 hours after irradiation.

Immunofluorescence of p53

Melanocytes rescued for wild-type MC1R expression were plated at the density of 0.15×10^5 cells per coverslip and treated for 4 days with 1 nmol/L α-MSH before irradiation with 105 mJ/cm^2 UVR. Cells were kept in fresh medium supplemented with 0 or 1 nmol/L α-MSH for 2 hours after UVR and then fixed with acetone: methanol (1:1) for 10 minutes. Coverslips were incubated with 10% bovine serum albumin (BSA) in PBS for 1 hour at room temperature, followed by 1-hour incubation at 37°C with antibody against p53 (1:200) and anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (1:200; Jackson Laboratories). Immunofluorescence was visualized using a fluorescence microscope and images acquired with a computer-assisted program.

Impact of accumulation or inhibition of p53 on oxidative DNA damage

To determine the effect of p53 inhibition on oxidative DNA damage, melanocytes were preincubated with 10 μmol/L pifithrin-α (PFT; Sigma Chemical Co.), an inhibitor of p53, for 18 hours before and immediately after UVR or 30-minute pulse-treated with 50 and 75 μmol/L H_2O_2, and the effect on DNA damage determined 2 hours later by Comet assay. The effect of p53 accumulation on oxidative DNA damage was determined by pretreating melanocytes expressing loss-of-function MC1R with 10 μmol/L nutlin-3 (Sigma Chemical Co.), for 18 hours before exposure to 105 mJ/cm^2 UVR or 100 μmol/L H_2O_2. The tail moment values obtained from a minimum of 50 randomly selected cells from each slide were expressed as the mean value ± SEM, and data were analyzed by ANOVA followed by SNK ($P < 0.05$).

Generation of primary cultures of melanocytes silenced for p53 by shRNAi

Neonatal melanocytes (hMC1, hMC2, and hMC3) were used for silencing of p53 with 1.25×10^5 (Infectious Units) short hairpin RNA (shRNA) lentiviral particles (Santa Cruz Biotechnology), according to manufacturer's instructions. Nontargeted scrambled sequence shRNA was used as transduction control and stable infections were obtained with puromycin selection. Confirmation of p53 silencing was verified by Western blotting using p53 antibody.

Determination of DNA damage by γ-H2AX immunofluorescence

Melanocytes were plated at a density of 1×10^6 cells per 100-mm dish and kept in medium with growth factors. Eighteen hours before and immediately after irradiation with 50, 75, and 105 mJ/cm^2 UVR, cells were treated with 10 μmol/L nutlin-3. Two and 4 hours after irradiation, cells were fixed with ice-cold 1% paraformaldehyde for 15 minutes and 70% ethanol for 1 hour. After permeabilization with 0.2% Triton X-100 in 3% BSA/PBS for 10 minutes, nonspecific binding was blocked with 20% normal goat serum for 30 minutes at room temperature. Cells were incubated with 1 μg/mL anti-γ-H2AX antibody [anti-phospho histone H2AX (Ser 139; Millipore)] overnight at 4°C, followed by goat anti-mouse IgG Alexa Fluor 488 (1:200; Invitrogen) for 1 hour at room temperature. After 2 washes with 0.1% Triton X-100 in 1% BSA/PBS and one with PBS, cells were resuspended in PBS containing 0.11 mg/mL RNase A and 6.85 μg/mL propidium iodide and 30 minutes later analyzed by flow cytometer.

Results

Decrease of UVR-induced oxidative DNA damage in melanocytes by α-MSH is dependent on the expression of functional MC1R, but independent of melanin synthesis

We have shown that α-MSH decreases UVR- or H_2O_2-induced oxidative DNA damage in melanocytes (7). To further show that this inhibitory effect is mediated by activation of the MC1R, we compared the responses of melanocytes naturally expressing nonfunctional receptor with that of the same culture stable transfected with the wild-type *MC1R*. Treatment with α-MSH immediately after UVR had no significant effect on the melanocytes lacking functional MC1R (Fig. 1A and B) whereas forskolin, a direct activator of adenylyl cyclase, markedly reduced the UVR-induced H_2O_2 production by 30% and 55% at 0 and 30 minutes, respectively. Similar effects were observed in cells expressing functional MC1R treated with forskolin, with reductions of 42% and 46% at 0 and 30 minutes, respectively (Fig. 1C and D). Transfection with the wild-type *MC1R* (Fig. 1C) not only reduced 6-fold the levels of H_2O_2 generated after UVR compared with cells expressing nonfunctional MC1R (Fig. 1A) but also rescued the responsiveness to α-MSH, as evidenced by the 35%, 51%, and 45% reductions of the UVR-induced H_2O_2 generation at 15, 30, and 45 minutes, respectively.

To exclude the possibility that the observed protective effect of α-MSH was due to an increase in melanin synthesis, we used melanocytes derived from a patient with OCA2, characterized by a mutation in the *P* gene, but functional MC1R. Mutation in the *P* gene results in lack of synthesis of melanin and albino phenotype (23). In the OCA2-MCs α-MSH reduced the UVR-induced H_2O_2 generation by 30%, 33%, 44%, and 62% at 0, 15, 30, and 45 minutes, respectively (Fig. 1E and F). Using the Comet assay, OCA2-MCs treated with 1 nmol/L α-MSH for 4 days before and 2

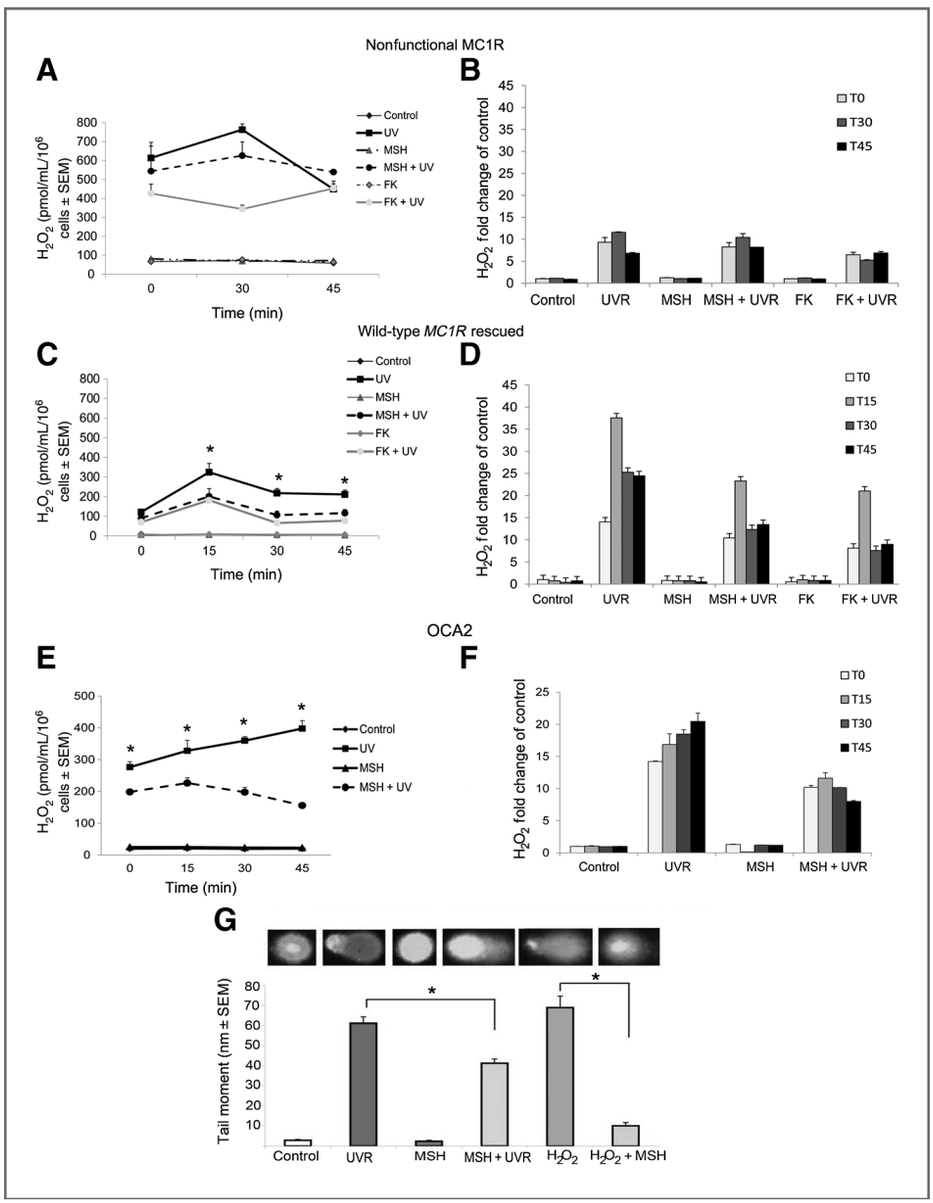

Figure 1. α-MSH decrease of UVR-induced oxidative DNA damage in human melanocytes (MC) is independent of melanin synthesis. MCs expressing nonfunctional MC1R (A and B) were transfected with the wild-type *MC1R* (C and D) and the generation of H_2O_2 in cells treated with 0, 105 mJ/cm^2 UVR ± 1 nmol/L α-MSH, 1 μmol/L forskolin (FK) or vehicle was measured at different time points. B and D, the bar graphs represent the fold increase of H_2O_2 generated in relation to the nonirradiated control group at T0. E and F, generation of H_2O_2 was measured after UVR in the presence and absence of 1 nmol/L α-MSH in OCA2-MCs. G, OCA2-MCs were treated with 1 nmol/L α-MSH for 4 days before and 2 hours following 105 mJ/cm^2 UVR or pulse treated with 100 μmol/L of H_2O_2 for 30 minutes. Cells were immobilized in low–melting point agarose gel and subjected to single-cell electrophoresis. The tail moment, product of the amount of DNA and the extent of its migration within the comet-like figure, was used as a measurement of the extent of DNA damage. *, $P \leq 0.05$; ANOVA followed by SNK.

hours after UVR showed a 33% reduction in the total amount of DNA damage induced by UVR and 85% decrease in the amount of oxidative DNA damage induced 2 hours after acute exposure to H_2O_2 (Fig. 1G).

Induction of accumulation, translocation, and transcriptional activity of p53 by α-MSH

Irradiation of melanocytes with 105 mJ/cm^2 UVR resulted in increased levels of p53 and p53-Ser15, which were further augmented by 1 nmol/L α-MSH in melanocytes expressing functional, but not in melanocytes expressing loss-of-function MC1R (Fig. 2A). The cellular distribution of p53, determined by immunofluorescence, showed that treatment with α-MSH increased the accumulation and translocation of p53 to the nuclei of UVR-irradiated melanocytes (Fig. 2B). The translocation of p53 was consistent with an increase in its transcriptional activity, as evidenced by the greater induction of the p53 targets p21 and GADD45 (Fig. 2C). Because activation of the MC1R increases cyclic AMP (cAMP) levels, we tested the effect of H-89, an inhibitor of the PKA, on p53 phosphorylation. Cells treated with 1 nmol/L α-MSH showed a 69% increase in p53-Ser15 as compared with control. A modest 22% increase was observed in cells exposed to UVR and treated with α-MSH, compared with untreated irradiated cells. Pretreatment of melanocytes with 2 μmol/L H-89 for 18 hours before and 1.5 hours after UVR caused a significant reduction of p53-Ser15 in the UV-irradiated group treated with α-MSH compared with the group treated similarly in the absence of H-89 (Fig. 2D). To rule out the possibility

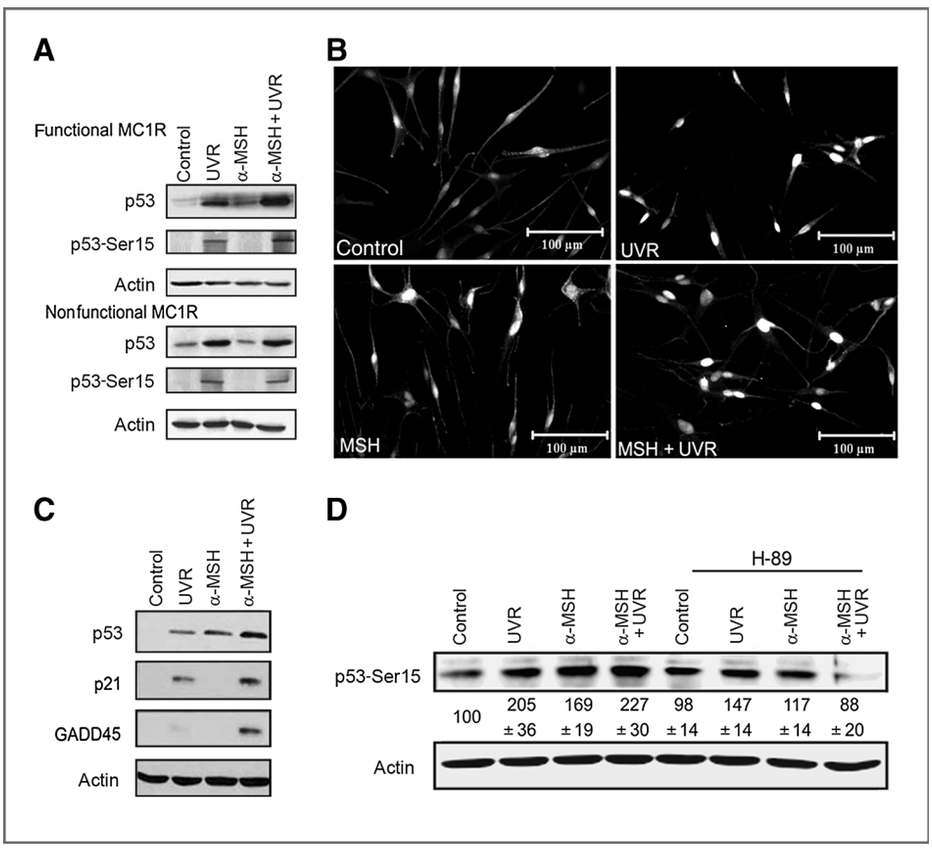

Figure 2. α-MSH induces further accumulation and activation of p53 in UVR-irradiated melanocytes (MC). A, MCs expressing functional or nonfunctional MC1R were pretreated with 1 nmol/L α-MSH for 4 days before and 48 hours after exposure to 105 mJ/cm^2 UVR, and expression of p53 was analyzed by Western blotting. B, MCs were kept in culture with or without α-MSH, as described above, and the cellular distribution of p53 was determined by immunofluorescence 2 hours after UVR. C, melanocytes were pretreated with 1 nmol/L α-MSH for 4 days and 24 hours after exposure to 105 mJ/cm^2 UVR and p21 and GADD45 protein expression determined by Western blotting. D, melanocytes were pretreated with the PKA inhibitor H-89 or vehicle for 18 hours before and immediately after UVR irradiation and expression of p53-Ser15 determined in the presence or absence of 1 nmol/L α-MSH 1 hour after UVR. The numbers represent the mean ± SEM densitometric values normalized by actin as percentage of the experimental control of 3 independent experiments.

that the effects of α-MSH occurred through activation of other p53 family members, the expression of p63 isoforms was investigated by Western blotting. The results indicate that melanocytes do not express any of the p63 proteins (Supplementary Fig. S1) or p73 (data not shown) because no detection was observed even at high protein concentrations (≥70 μg).

α-MSH signaling and the DNA damage response to UVR contribute to p53 phosphorylation on serine 15

The mitogen-activated protein kinase (MAPK) p38 induced by stress phosphorylates p53 on serine 15. Our results showed that p38 was phosphorylated by UVR as shown by the 2-fold increase above control. This phosphorylation was further enhanced to 3-fold above control in the presence of 1 nmol/L α-MSH (Fig. 3A). Pretreatment with SB203580, a p38 inhibitor, abrogated the increase in p53 phosphorylation that was induced by α-MSH and UVR (Fig. 3B). We found that ATR, another upstream activator of p53, was phosphorylated by pulse treatment with H_2O_2 in a time-dependent manner, and a transient enhancement of phosphorylation (30 minutes) was observed in the presence of α-MSH (Fig. 3C). Check 2 (Chk2), the kinase downstream of ATR, was phosphorylated rapidly and transiently (30 minutes) after treatment with H_2O_2. However, in the presence of α-MSH, the phosphorylation of Chk2 was maintained for at least for 1 hour after H_2O_2 treatment (Fig. 3C). Similarly, α-MSH enhanced the phosphorylation of DNA-PK by more than 3-fold above control in melanocytes exposed to UVR (Fig. 3D).

Effect of p53 on oxidative DNA damage in melanocytes exposed to UVR or H_2O_2

Treatment of melanocytes with increasing doses of nutlin-3, a small molecule that blocks the HDM2-binding site to p53, induced a dose-dependent accumulation of p53 (Supplementary Fig. S2). Treatment of melanocytes expressing loss-of-function MC1R with nutlin-3 resulted in similar effects as of α-MSH on melanocytes expressing functional MC1R (Fig. 1G), namely, significant reduction of UV- and H_2O_2-induced DNA damage, as determined by Comet assay (Fig. 4A). Moreover, 10 μmol/L nutlin-3 markedly reduced γ-H2AX formation in melanocytes irradiated with increasing doses of UVR (Supplementary Fig. S3). To further show the significance of p53 in reducing oxidative stress in melanocytes, we investigated the effect of PFT, an inhibitor of p53, on the generation of H_2O_2 following UVR and DNA damage induced by UVR and H_2O_2. Suppression of p53 by 10 μmol/L PFT increased H_2O_2 generation by UVR-exposed melanocytes (Supplementary Fig. S4) and produced more DNA damage in these melanocytes (1.5-fold) and in melanocytes exposed to 50 and 75 μmol/L H_2O_2 (4-fold), as shown by the Comet assay (Fig. 4B). In addition, p53 was silenced using shRNA in 3 different

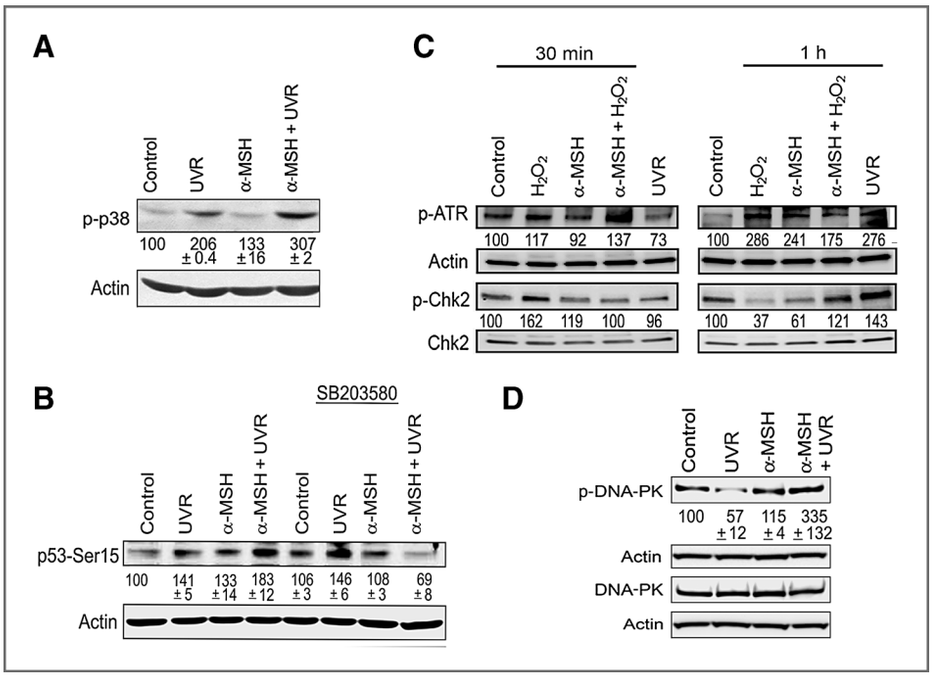

Figure 3. Phosphorylation of p53 on serine 15 is induced by α-MSH signaling and the DNA damage response to UVR. A, melanocytes were exposed to 105 mJ/cm^2 UVR followed by immediate treatment with 1 nmol/L α-MSH or vehicle and phosphorylation of p38 was determined by Western blotting 1 hour after irradiation. B, melanocytes were pretreated with 10 μmol/L SB203580, a p38 inhibitor, for 1 hour before UVR, and the effects of UVR and/or α-MSH on the levels of p53-Ser15 were analyzed by Western blotting 1.5 hours after irradiation. C, the effect of α-MSH on the expression and activation of ATR was analyzed in MCs exposed to a pulse treatment with 100 μmol/L H_2O_2 or 105 mJ/cm^2 UVR. D, effect of α-MSH on the activation of DNA-PK was analyzed 1 hour after UVR. The numbers represent the densitometric values normalized by respective loading controls as percentage of the experimental control. In A, B, and D, the mean values ± SEM of 3 to 4 independent experiments are shown.

melanocyte primary cultures, downregulation of p53 was verified at the functional level by reduced induction of p21 (Supplementary Fig. S5). In melanocytes silenced for p53, α-MSH failed to reduce the levels of UVR-induced H_2O_2 (Fig. 4E and F). Moreover, the initial levels of H_2O_2 generated immediately after UVR exposure (Fig. 4E) were higher than in the shRNA control cells (Fig. 4C). As expected, melanocytes transduced with the nontargeted sequence responded to α-MSH with a significant decrease in UVR-induced H_2O_2 (Fig. 4C and D). In p53-silenced melanocytes, as well as in melanocytes treated with PFT, the H_2O_2- or UVR-induced oxidative DNA damage was not reduced by α-MSH (Fig. 4B and G).

Impact of p53 on melanocyte expression of base excision repair enzymes OGG1 and APE-1

To determine the mechanism by which p53 regulates the repair of oxidative DNA damage, we investigated the expression of OGG1 and APE-1/Ref-1, enzymes essential for base excision repair (BER). The levels of both enzymes increased upon treatment with 100 μmol/L H_2O_2 as well as with 1 n mol/L α-MSH (Fig. 5A and B). In contrast to melanocytes expressing p53, in melanocytes silenced for p53, α-MSH failed to increase the levels of OGG1 or its translocation to the nucleus, as well as prevented the nuclear increase in APE-1, as indicated by the nuclear:cytoplasmic ratios of both proteins (Fig. 5C).

Discussion

The results of our studies unraveled a novel mechanism by which α-MSH, through activation of the MC1R, protects melanocytes from oxidative DNA damage. We show for the first time that activation of the MC1R by α-MSH resulted in increased phosphorylation of p53 on serine 15 in UVR-irradiated melanocytes that leads to p53 accumulation and activation. This increase in p53 was significant in reducing ROS generation, and hence oxidative DNA damage, as well as in regulating the expression of enzymes that have a critical role in BER, the main repair pathway for oxidative DNA damage.

Our results clearly show that reduction of UV-induced H_2O_2 generation and increase in p53 levels by α-MSH treatment required functional MC1R, as these responses were absent in melanocytes expressing loss-of-function receptor. These findings strongly suggest that loss-of-function MC1R, which was associated with diminished accumulation of p53 after UVR exposure renders melanocytes more vulnerable to oxidative stress, increasing the risk for malignant transformation to melanoma. Interestingly, the reduction in oxidative DNA damage by α-MSH was independent of pigmentation because it was present in OCA-MCs that lack the ability to synthesize melanin.

Accumulation of p53 was accompanied by increased phosphorylation of p53 on serine 15 (Fig. 2A), a requirement for its nuclear translocation and transcriptional activity (ref. 26; Fig. 2B and C). Activation of the cAMP signaling

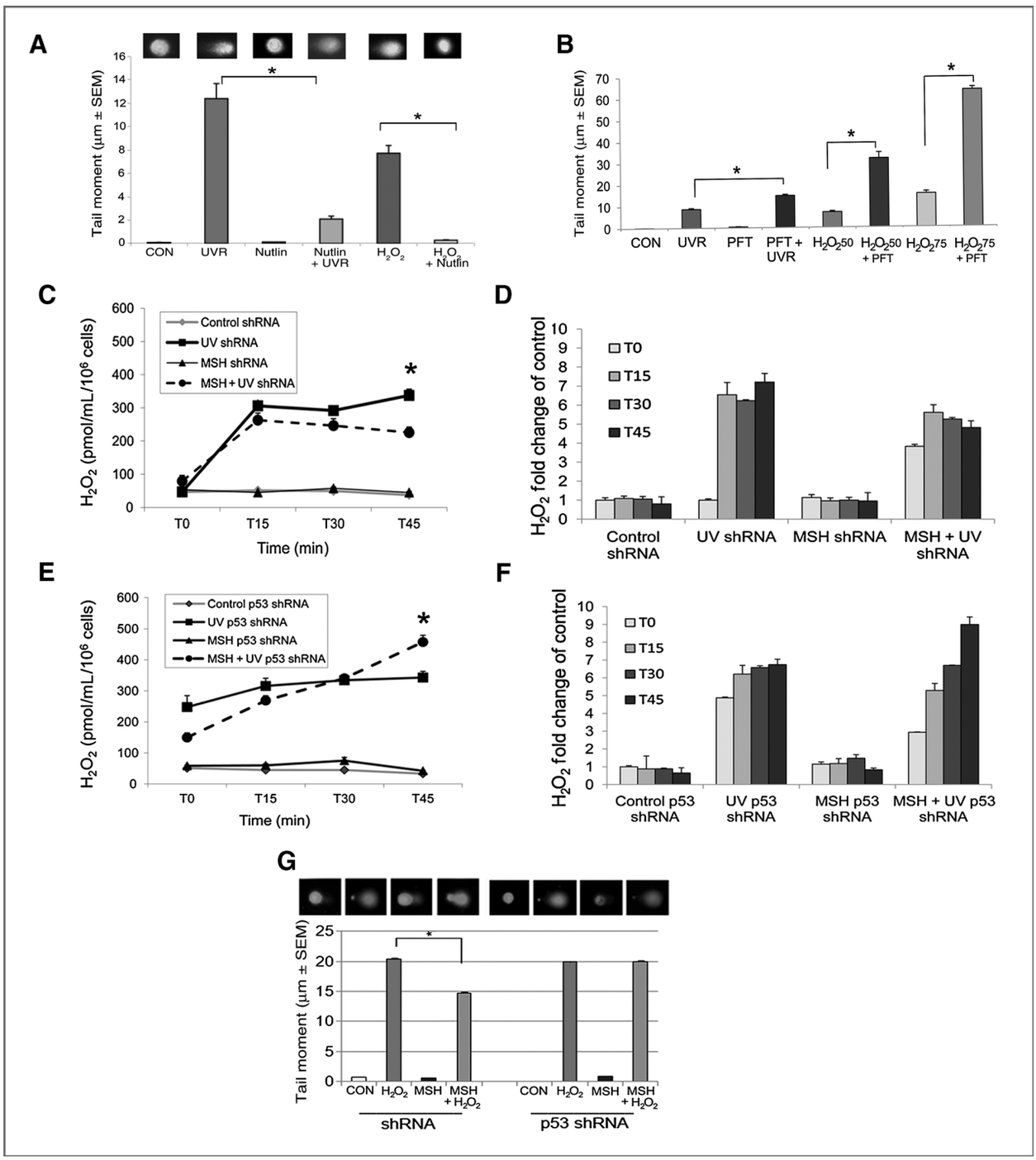

Figure 4. Impact of accumulation or inhibition of p53 on oxidative DNA damage in MCs exposed to UVR or H_2O_2. A, melanocytes expressing loss-of-function MC1R were treated with 10 μmol/L nutlin-3 for 18 hours before exposure to 105 mJ/cm^2 UVR or pulse treatment with 100 μmol/L H_2O_2, and the extent of DNA damage was determined by Comet assay. B, melanocytes expressing functional MC1R were pretreated with 10 μmol/L PFT, an inhibitor of p53, before and immediately after UVR or pulse treatment with H_2O_2, and the effect on DNA damage was determined 2 hours later. C and E, melanocytes silenced for p53 with target shRNA and nontargeted RNA were UV-irradiated, and the levels of H_2O_2 compared in the presence or absence of 1 nmol/L α-MSH. D and F, the bar graphs represent the fold increase of H_2O_2 generated above the nonirradiated control group at T0. G, MCs silenced for p53 with target shRNA and nontargeted RNA were pulse-treated with 100 μmol/L H_2O_2, and the extent of oxidative DNA damage was determined in the presence or absence of 1 nmol/L α-MSH. *, $P \leq 0.05$; ANOVA followed by SNK. CON, control.

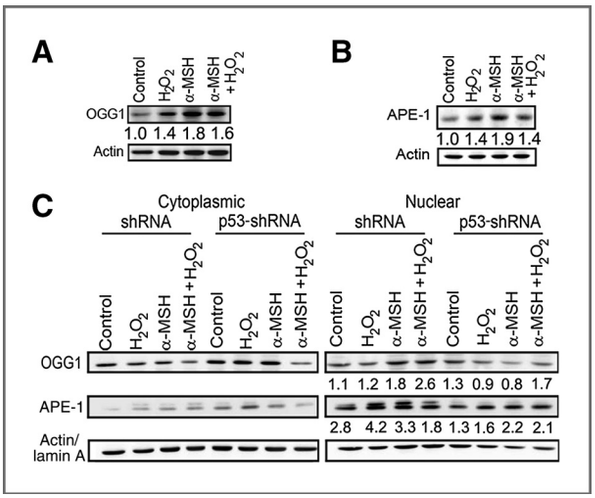

Figure 5. Impact of p53 on expression of BER enzymes OGG1 and APE-1. Melanocytes were pretreated with 1 nmol/L α-MSH for 48 hours before and immediately after exposure to 100 μmol/L hydrogen peroxide and the expression of OGG1 (A) and APE-1 (B) determined after 2 hours by Western blotting, the numbers represent the densitometric values normalized by actin loading control as fold change of control. C, melanocytes were treated as described above and the cytoplasmic versus nuclear distribution of OGG1 and APE-1 was determined by Western blotting 2 hours after H_2O_2 treatment. The numbers represent the nuclear:cytoplasmic ratios of the densitometric values normalized by the loading controls.

pathway was necessary for further phosphorylation of p53 on Ser15, as inhibition of the cAMP-dependent PKA abrogated this effect (Fig. 2D). Similar to findings in melanoma cells and melanocytes in which α-MSH activates the MAPK p38 (27–29), we found that α-MSH increased the UV-induced phosphorylation of the p38 (Fig. 3A), and inhibition of p38 phosphorylation abrogated the increased accumulation of p53 (Fig. 3B). Treatment of melanocytes with α-MSH also increased the phosphorylation of the phosphoinositide 3-kinase (PI3K) ATR and DNA protein kinase (DNA-PK; Fig. 3C and D), the early sensors of DNA damage and activators of p53 (26, 30, 31).

To verify whether p53 is the only player in the antioxidant responses, we examined whether melanocytes express other isoforms of the p53 family members, that is, p63 proteins. However, unlike keratinocytes, melanocytes do not express any of the p63 isoforms (Supplementary Fig. S1).

The significance of p53 in reducing oxidative DNA damage was further confirmed by using 2 strategies to manipulate p53 accumulation: (i) inhibiting its degradation with nutlin-3, an inhibitor of the ubiquitin ligase HDM2 and (ii) inhibiting p53 pharmacologically using PFT, or downregulating p53 expression by shRNA. Treatment of melanocytes expressing loss-of-function MC1R with nutlin-3 had similar effects as treatment of melanocytes expressing functional MC1R with α-MSH, namely, significant reduction in UVR- and H_2O_2-induced oxidative DNA damage (Fig. 4A). The nutlin-3–reduced DNA damage was consistent with the decreased expression of γ-H2AX, an established marker of DNA damage (ref. 32; Supplementary Fig. S3). On the other hand, treatment with PFT (Fig. 4B) or downregulation of p53 by shRNA (Fig. 4E–G) led to a sustained UVR-induced increase in H_2O_2 levels and DNA damage and completely prevented the effect of α-MSH on DNA repair. In addition, silencing of p53 abolished the effects of α-MSH on the increase in OGG1 and APE-1 (Fig. 5C), the major enzymes involved in BER (33, 34). Our results are in agreement with other reports that accumulation of p53 in melanocytes or melanoma cells induces genes that regulate cell-cycle arrest rather than apoptosis and increases the expression of OGG1 in human fibroblasts (35, 36).

It is possible that the regulation of p53 activity and function in melanocytes differ from other cells, in as much as p53 also has important roles that are specific to melanocytes. It has been shown that p53 regulates the expression of genes that play important roles in melanogenesis, such as tyrosinase and TRP-1 (20, 37). Recently, it has been reported that in UV-irradiated mouse skin, p53 increases the expression of the proopiomelanocortin gene that encodes for the precursor of α-MSH and ACTH, the agonists for the human MC1R (22, 23, 38). We hereby present evidence for a new role of p53 in counteracting oxidative damage and in maintaining homeostasis of melanocytes. These findings provide more understanding of the role of the *MC1R* as a melanoma susceptibility gene and how loss of function of the MC1R increases the risk for melanoma. We propose that activation of the p53 pathway could potentially be used as a melanoma preventative strategy. The fact that melanoma tumors express wild-type p53 (39) offers the unique opportunity of using alternative strategies based on targeting p53 for the treatment of advanced stages of the disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

Authors' Contributions

Conception and design: A.L. Kadekaro, Z. Abdel-Malek
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