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MC1R Expression in HaCaT Keratinocytes Inhibits UVA-Induced ROS Production via NADPH Oxidase- and cAMP-Dependent Mechanisms

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Ultraviolet A (UVA) radiations are responsible for deleterious effects, mainly due to reactive oxygen species (ROS) production. Alpha-melanocyte stimulating hormone (α-MSH) binds to melanocortin-1 receptor (MC1R) in melanocytes to stimulate pigmentation and modulate cutaneous inflammatory responses. MC1R may be induced in keratinocytes after UV exposure. To investigate the effect of MC1R signaling on UVA-induced ROS (UVA-ROS) production, we generated HaCaT cells that stably express human MC1R (HaCaT-MC1R) or the Arg151Cys ($R_{151}C$) non-functional variant (HaCaT-$R_{151}C$). We then assessed ROS production immediately after UVA exposure and found that: (1) UVA-ROS production was strongly reduced in HaCaT-MC1R but not in HaCaT-$R_{151}C$ cells compared to parental HaCaT cells; (2) this inhibitory effect was further amplified by incubation of HaCaT-MC1R cells with α-MSH before UVA exposure; (3) protein kinase A (PKA)-dependent NoxA1 phosphorylation was increased in HaCaT-MC1R compared to HaCaT and HaCaT-$R_{151}C$ cells. Inhibition of PKA in HaCaT-MC1R cells resulted in a marked increase of ROS production after UVA irradiation; (4) the ability of HaCaT-MC1R cells to produce UVA-ROS was restored by inhibiting epidermal growth factor receptor (EGFR) or extracellular signal-regulated kinases (ERK) activity before UVA exposure. Our findings suggest that constitutive activity of MC1R in keratinocytes may reduce UVA-induced oxidative stress via EGFR and cAMP-dependent mechanisms.

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UVA radiations (320–400 nm) have cutaneous deleterious effects and are potent inducers of reactive oxygen species (ROS) (Ichihashi et al., 2003). Increased level of ROS in the skin is a major cause of cellular damage that leads to apoptosis, lipid peroxidation, photoaging, and skin cancers (Nishigori, 2006). Alpha-melanocyte stimulating hormone (α-MSH) is derived from pro-opiomelanocortin (POMC) and is expressed both in the central nervous system and peripheral tissues including skin (Slominski et al., 2000). Recent studies have demonstrated that α-MSH could inhibit intracellular peroxidation in keratinocytes and melanoma cells (Haycock et al., 2000), decrease oxidative burst in human macrophages (Sarkar et al., 2003) and inhibit the production of superoxide radicals (Oktar et al., 2004), thus indicating that it may stimulate a quick anti-oxidant defense (Song et al., 2009). The actions of α-MSH are mediated by its binding to all the known melanocortin receptors (i.e., MC1, -3, -4, and -5R) except MC2R. MCRs are G protein-coupled, seven-pass transmembrane receptors (GPCRs) that activate cAMP-dependent pathways (Busca and Ballotti, 2000; Catania et al., 2004; Garcia-Borron et al., 2005). Melanocortin-1 receptor (MC1R) expression is not restricted to melanocytes and has been observed in several other cell types including keratinocytes, fibroblasts, monocytes, dendritic, and endothelial cells suggesting other functions for this receptor (Luger et al., 2000; Curry et al., 2001; Catania et al., 2004). Indeed, several data show that α-MSH is involved in the regulation of apoptosis, inflammation and UV-induced immune suppression (Lipton and Catania, 1997; Luger et al., 2003; Bohm et al., 2005). Furthermore, recent findings demonstrate that, like other GPCRs, MC1R may display agonist-independent activity, which results in increased levels of cAMP (Sanchez-Mas et al., 2004; Garcin et al., 2007, 2009).

Abbreviations: α-MSH, alpha-melanocyte stimulating hormone; DMEM, Dulbecco's modified Eagle's medium; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; IBMX, 3-isobutyl-1-methylxanthine; MC1R, melanocortin-1 receptor; NHK, normal human keratinocyte; Nox, NADPH oxidase; NoxA1, Nox activator 1; PKA, protein kinase A; RHC, red hair color; RIPA buffer, radioimmunoprecipitation buffer; ROS, reactive oxygen species.

The authors have declared that no conflict of interest exists.

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The human *MC1R* gene is highly polymorphic (Garcia-Borron et al., 2005) and several variants, such as the $Arg_{151}Cys$ ($R_{151}C$) mutation, result in loss of function of MC1R (Ringholm et al., 2004) and are associated with the red hair color phenotype (RHC) (Valverde et al., 1995; Box et al., 1997; Healy et al., 2000; Rees, 2004), poor tanning ability, and elevated risk of melanoma and carcinoma (Palmer et al., 2000; Kennedy et al., 2001; Sturm et al., 2003; Soufir et al., 2009). These variants can bind to α-MSH but are unable to activate adenylate cyclase in cultured cells (Healy et al., 2001; Ringholm et al., 2004; Newton et al., 2005, 2007; Sanchez-Laorden et al., 2007; Herraiz et al., 2009). Under the action of α-MSH, MC1R regulates the amount and type of pigment production (Hunt et al., 1994) and is a major determinant of the skin phototype and sensitivity to UV light (Im et al., 1998; Kadekaro et al., 2003a,b, 2005). Moreover, after UV irradiation, both mRNA and protein expression of MC1R are induced in vitro and in vivo at the cell surface of keratinocytes (Chakraborty et al., 1999; Schiller et al., 2004).

In this study, we investigated the effect of MC1R signaling on UVA-induced ROS (UVA-ROS) production in keratinocytes that express high level of MC1R at the cell surface. For that purpose, we used the immortalized human keratinocyte cell line HaCaT (Fusenig and Boukamp, 1998), stably transfected with wild-type human *MC1R* (HaCaT-MC1R) or the non-functional variant $R_{151}C$ (HaCaT-$R_{151}C$) (Garcin et al., 2007, 2009). We focused our analysis on UVA radiations since most of the mutagenic and carcinogenic effects of UVA appears to be mediated through ROS production (de Gruijl, 2000).

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), L-glutamine, penicillin–streptomycin, and trypsin–EDTA were from Lonza (Verviers, Belgium). Fetal bovine serum (FBS), carboxy-H_2DCF-DA, and MitoSox were obtained from Invitrogen (Groningen, NL). Forskolin, 3-isobutyl-1-methylxanthine (IBMX), NaOH, 1,10-phenanthroline, and propidium iodide (PI) were from Sigma (St Louis, MO). H-89, apocynin, PD98059, PD153035, and gp91 ds-tat were obtained from Calbiochem (San Diego, CA). Bovine serum albumin (BSA) fraction V was purchased from Euromedex (Souffelweyersheim, France) and sodium dodecylsulfate (SDS) from BioRad (Marnes la Coquette, France). α-MSH was obtained from the Institut des Biomolécules Max Mousseron (IBMM, UMR-CNRS 5247, Montpellier, France) and ^{125}I [Nle^4, D-Phe^7]-α-MSH (^{125}I-NDP-MSH) from PerkinElmer Life Sciences (Boston, MA). The rabbit polyclonal anti-NoxA1 antibody was obtained from Abnova (Taipei, Taiwan). The rabbit polyclonal anti-phospho-(Ser/Thr) protein kinase A (PKA) substrate antibody and the anti-rabbit horseradish peroxidase (HRP)-linked IgG were purchased from Cell Signaling Technology (Beverly, MA). The ECL Plus™ Western blotting detection reagents and Protein A Sepharose ™ 4 Fast Flow were obtained from Amersham Bioscience (Freiburg, Germany).

Cell culture

The HaCaT cell line was obtained from N.E. Fusenig (Institute of Biochemistry, German Cancer Research Center, Heidelberg, Germany). Cells were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 25 U/ml penicillin, and 25 μg/ml streptomycin and maintained at 37°C in 5% CO_2 in a humidified environment. HaCaT-MC1R (clones 35 and 53) and HaCaT-$R_{151}C$ (clone 30) were previously established in our laboratory (Garcin et al., 2007, 2009). HaCaT-MC1R and HaCaT-$R_{151}C$ cells were cultured like parental HaCaT cells.

Binding assays

Parental and transfected HaCaT cells were plated in 24-well plates (1×10^5 cells per well). After 24 h, cells were incubated at 37°C with increased concentrations of α-MSH ranging from 10^{-11} to 10^{-6} M and 100,000 cpm of ^{125}I-NDP-MSH in DMEM, 0.5% BSA, and 0.3 mM 1,10-phenanthroline for 2 h. Radioactivity was measured with a γ counter and results were fitted by nonlinear regression analysis using the GraphPad Prism software (San Diego, CA).

Cyclic AMP assays

Parental and transfected HaCaT cells were plated in 96-well plates (1,000 cells per well) and incubated at room temperature with 1 μM α-MSH, 0.5 μM PD153035, 20 μM PD98059, or 10 μM forskolin for 1 h. All experiments were performed in the presence of IBMX (1 mM). cAMP concentration was measured with the LANCE cAMP kit (PerkinElmer Life Science) according to the manufacturer's instructions using a Victor plate reader (Wallac, PerkinElmer Life Science, Boston, MA).

UVA irradiation

Parental and transfected HaCaT cells were seeded (1×10^6 cells per 100 mm Petri dishes) in PBS containing 1% FBS and exposed to UVA radiation at doses ranging from 1.5 to 9 J/cm^2. Cells were irradiated with a Waldmann 800k light source composed of UVA Philips 40 W lamps (Herbert Waldmann, Werk für Lichtechnik Schwenningen, Bischheim, Germany). The irradiance (4 mW/cm^2) was measured using a UV light meter (Herbert Waldmann, Werk für Lichtechnik Schwenningen).

Detection of intracellular ROS production

Cells exposed to UVA radiation and non-irradiated controls were resuspended in 500 μl PBS and 2.5 μl of 1 mg/ml propidium iodide (PI) was added to each sample immediately before flow cytometry analysis. Parental and transfected HaCaT cells were treated with 1 μM α-MSH, 10 μM apocynin, 5 μM H-89, 0.5 μM PD153035, and 20 μM PD98059 for 1 h or with 5 μM gp91 ds-tat for 2 h and then exposed to UVA radiation at doses ranging from 1.5 to 9 J/cm^2. Immediately after UVA exposure, cells were incubated at 37°C under dark conditions with the carboxy-H_2DCF-DA ROS-sensitive probe (10 μM) for 1 h. In order to perform an optimal assessment of intracellular ROS production, the fluorescence intensity of the specific probe was gated on viable, PI-negative cells. Indeed, PI-positive cells have a permeable plasma membrane that allows the release of reduced carboxy-H_2DCF-DA in the extracellular medium, resulting in a underestimation of the signal. To detect mitochondrial ROS, cells were incubated with 5 μM MitoSox at 37°C under dark conditions for 10 min (Kudin et al., 2004; Mukhopadhyay et al., 2007a,b). Cell fluorescence was quantified by flow cytometry analysis using a FacScan cytometer (Becton-Dickinson, Le Pont-de-Claix, France). Data were collected with the DakoCytomation (Fort Collins, CO) software. Results are shown as variations of fluorescence intensities (arbitrary units) of the probe relative to control cells.

Coimmunoprecipitation and immunoblotting

Parental and transfected HaCaT cells were plated in Petri dishes and incubated with 1 μM α-MSH, 0.5 μM PD153035, or 20 μM PD98059 for 1 h or with 5 μM H-89 overnight. Cells were lysed in RIPA buffer and then left on ice for 15 min. Whole-cell lysates were incubated with anti-NoxA1 antibody (1:500) at 4°C overnight. The resulting complexes were captured with 10% protein A-Sepharose beads at 4°C for 1 h. Immunoprecipitates were resolved on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Biosciences). Blots were blocked with Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris, pH 7.6, and 2 mM $MgCl_2$) containing 0.1% Tween-20, 5% non-fat milk, and 1%

BSA at room temperature for 1 h. Blots were incubated with anti-NoxA1 (1:1,000) and anti-phospho-(Ser/Thr) PKA substrate (1:1,000) primary antibodies at 4°C overnight. Primary antibody binding was detected by incubation with anti-rabbit secondary antibody linked to HRP. Blots were visualized with Amersham ECL Plus™ Western blotting detection reagents according to the manufacturer's instructions. Blots were analyzed with the Bio1D image analysis software (Vilber Lourmat, Marne La Vallée, France) and results were expressed as relative optical densities (ROD).

Data analysis

Data were expressed as mean ± SEM (standard error of the mean) of three independent experiments and differences were analyzed for significance using a paired Student's *t*-test. Error bars represent standard deviations of the mean. $^{*}P$ values <0.05 were considered significant.

Results

Generation of HaCaT cells that stably express functional MC1R

To investigate the effects of MC1R signaling on UVA-ROS production, we used HaCaT cell lines that stably express human wild-type *MC1R* (HaCaT-MC1R cells) or the *MC1R* variant $R_{151}C$ (HaCaT-$R_{151}C$ cells) (Garcin et al., 2007, 2009). First, we assessed the functional properties of MC1R and of the $R_{151}C$ variant in these cells by using the ^{125}I-NDP-MSH synthetic α-MSH analogue in competitive radioligand binding assays. In agreement with Roberts et al. (2006), we did not observe any specific binding of ^{125}I-NDP-MSH in parental HaCaT cells (Fig. 1A). Conversely, the affinity of radioligand was similar in all transfected HaCaT cells indicating that MC1R binding to α-MSH was unaffected by the $R_{151}C$ mutation (Fig. 1A). On the other hand, incubation with 1 μM α-MSH for 1 h strongly increased cAMP production only in HaCaT-MC1R cells similarly to previous studies (Sanchez-Mas et al., 2004; Garcin et al., 2007, 2009), but not in parental HaCaT and HaCaT-$R_{151}C$ cells (Fig. 1B). Incubation with 10 μM forskolin for 1 h, which directly activates adenylyl cyclase, increased cAMP production in all cell lines.

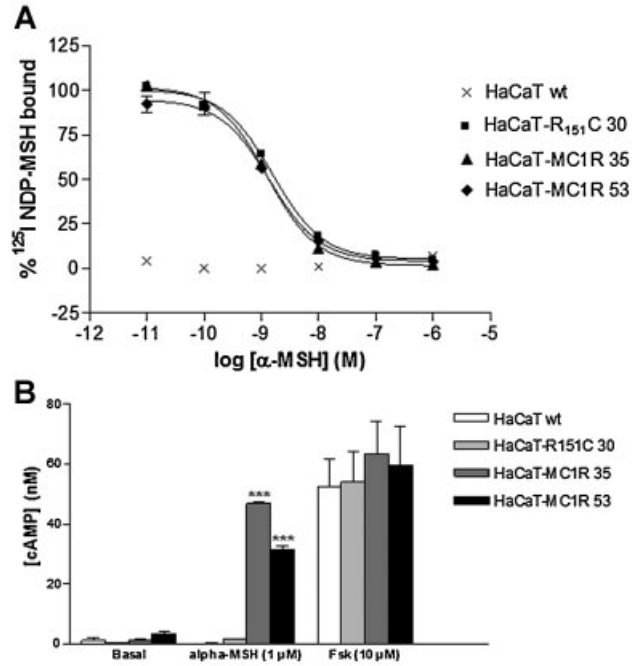

Fig. 1. Parental HaCaT and HaCaT-$R_{151}C$ cells do not express functionally activated MC1R. A: Competition binding data. Parental HaCaT (wt), HaCaT-MC1R (clones 35 and 53) and HaCaT-$R_{151}C$ (clone 30) were incubated at 37°C with 100,000 cpm ^{125}I-NDP-MSH and increasing concentrations (from 10^{-11} to 10^{-6} M) of α-MSH for 2 h. The specifically bound radioactivity was measured on a γ counter. IC_{50} (clone 30) = 1.89 nM; IC_{50} (clone 35) = 1.26 nM and IC_{50} (clone 53) = 1.43 nM. B: Functional coupling of MC1R and $R_{151}C$. HaCaT cells were challenged with 1 μM α-MSH, or 10 μM forskolin (Fsk, positive control) in combination with 1 mM IBMX, a phosphodiesterase inhibitor, for 1 h. cAMP concentration was determined using the LANCE cAMP kit as described in the Materials and Methods Section. Data are expressed as the mean ± SEM of three independent experiments. $^{***}P < 0.001$ (Student's *t*-test) relative to parental HaCaT with the same treatment.

UVA-ROS production is strongly reduced in HaCaT-MC1R cells and almost completely abrogated after α-MSH treatment

We then analyzed the effects of a broad range of UVA doses (1.5–9 J/cm^2) on the different HaCaT cell lines and observed, immediately after UVA exposure, a dose-dependent and marked increase of intracellular ROS production in parental HaCaT wt and HaCaT-$R_{151}C$ cells (Fig. 2), whereas UVA-ROS production was significantly reduced in the two HaCaT-MC1R clones (Fig. 2). Furthermore, ROS production after UVA irradiation (9 J/cm^2) in HaCaT cells transfected with an empty pcDNA3 vector was not modified compared to parental HaCaT cells (data not shown). In each experiment, the carboxy-H_2DCF-DA fluorescence was evaluated only in PI-negative, viable cells and no difference in cell viability in all tested cell lines was observed before and immediately after UVA irradiation (data not shown). We then investigated whether α-MSH could modulate UVA-ROS production by pre-incubating cells with 1 μM α-MSH for 1 h before UVA irradiation (1.5–9 J/cm^2). α-MSH significantly blocked UVA-ROS production in HaCaT-MC1R cells (clones 35 and 53), whereas it had no effect on UVA-ROS production in parental HaCaT (data not shown) and only a minor effect in HaCaT-$R_{151}C$ cells (Fig. 2).

UVA-ROS inhibition is dependent on the NADPH oxidase and cAMP/PKA pathways

Since UVA is known to stimulate ROS production in keratinocytes mainly via a Nox1-based NADPH oxidase mechanism (Valencia and Kochevar, 2008), we asked whether the impaired ROS production in HaCaT-MC1R cells could result from inhibition of this enzymatic complex. First, HaCaT cells were pre-incubated with 10 μM apocynin which is supposed to inhibit NADPH oxidase activity by suppressing the translocation of the cytoplasmic subunits $p47^{phox}$ and $gp91^{phox}$ (Babior et al., 2002; Lambeth, 2004), for 1 h. Then, cells were UVA-irradiated (9 J/cm^2). Apocynin inhibited UVA-ROS production by (62 ± 4)% in HaCaT wt cells and by (59 ± 1)% in HaCaT-$R_{151}C$ cells ($P < 0.001$; n = 3), confirming that NADPH oxidase plays a major role in UVA-ROS production (Fig. 3A). Apocynin inhibited UVA-ROS production also in HaCaT-MC1R cells but much less strongly, by only (29 ± 9)% (clone 35) and by (28 ± 5)% (clone 53) ($P < 0.05$; n = 3), suggesting that most of the ROS production in these cells is also dependent on a NADPH oxidase mechanism. Since recent findings showed that apocynin is not a specific NADPH oxidase inhibitor (Heumuller et al., 2008), we then used the peptide-based inhibitor gp91 ds-tat which is a more specific NADPH oxidase inhibitor (Rey et al., 2001; El-Benna et al., 2010) that inhibits the interaction of

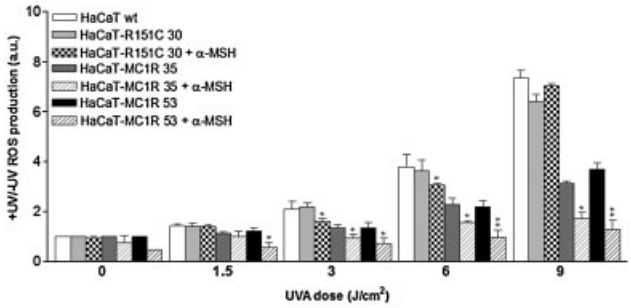

Fig. 2. MC1R expression and α-MSH stimulation inhibit UVA-induced ROS in HaCaT cells. Parental HaCaT (wt), HaCaT-MC1R (clones 35 and 53), and HaCaT-R_{151}C (clone 30) cells were pre-incubated with 1 μM α-MSH for 1 h and exposed to UVA radiation (1.5–9 J/cm²). Immediately after UVA exposure, ROS production was quantified by measuring the fluorescence from the oxidation product of carboxy-H_2DCF-DA, as described in the Materials and Methods Section. For each cell type, non-treated and non-irradiated negative controls were used and results are shown as variations of the fluorescence intensities (arbitrary units, a.u.) of the probe compared to non-irradiated cells. UVA-irradiated and non-irradiated cells were also stained with 5 μg/ml propidium iodide (PI) and only PI-negative cells (viable cells) were used for the analysis. Data are expressed as the mean ± SEM of three independent experiments. *$P < 0.05$ and **$P < 0.01$ (Student's *t*-test) relative to parental HaCaT cells irradiated with the same UVA dose.

gp91^{phox} and p47^{phox} (Rey et al., 2001). HaCaT cells were pre-incubated with 5 μM gp91 ds-tat for 2 h and then UVA-irradiated (9 J/cm²). As expected, gp91 ds-tat inhibited UVA-ROS production by (81 ± 1)% in parental HaCaT cells and by (75 ± 1)% in HaCaT-R_{151}C cells ($P < 0.001$; n = 3). However, it had no effect on HaCaT-MC1R cells, indicating that inhibition of ROS production in these cells is NADPH oxidase-dependent (Fig. 3B).

Since mitochondria may be a potential cellular source of oxidative stress (Batandier et al., 2002; Gauuan et al., 2002; Liu et al., 2002; Kudin et al., 2004), we then investigated the ability of UVA radiation to modulate differentially mitochondrial superoxide anion production in HaCaT cells. Immediately after UVA irradiation, cells were stained with the MitoSox dye which fluoresces when oxidized by superoxide anions in mitochondria of living cells (Kudin et al., 2004; Mukhopadhyay et al., 2007a,b). UVA radiation had no effect on MitoSox fluorescence in the different HaCaT cell lines (data not shown).

The increased cAMP level in HaCaT-MC1R cells could be responsible for the strong reduction of UVA-ROS production in these cells. To verify this hypothesis, HaCaT cells were treated with 5 μM H-89, an inhibitor of PKA (which is activated by cAMP) via competitive binding to the ATP pocket in the kinase catalytic subunit (Engh et al., 1996), for 1 h and then they were UVA-irradiated (9 J/cm²). H-89 had no effect on HaCaT and HaCaT-R_{151}C cells, but markedly increased UVA-ROS production in HaCaT-MC1R cells (Fig. 4). These results indicate that UVA-ROS production inhibition in HaCaT-MC1R cells is linked to their strong cAMP response.

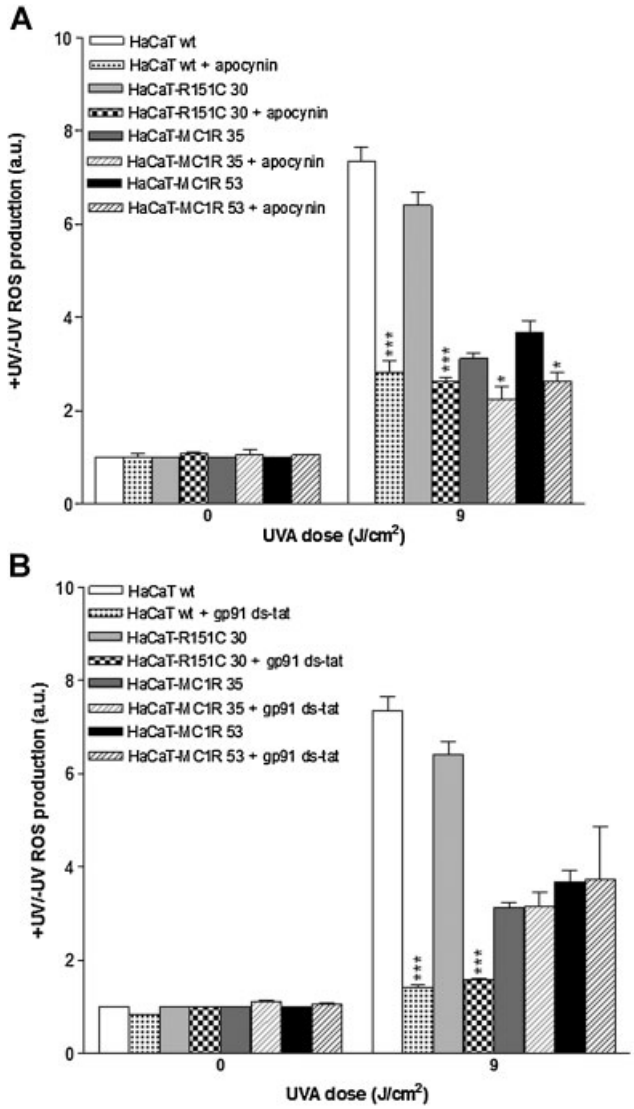

Fig. 3. UVA-induced oxidative stress is dependent on the NADPH oxidase pathway. A: Effect of apocynin. Parental HaCaT (wt), HaCaT-MC1R (clones 35 and 53), and HaCaT-R_{151}C (clone 30) cells were pre-incubated with 10 μM apocynin for 1 h and then exposed to UVA radiation (9 J/cm²). B: Effect of gp91 ds-tat. Parental HaCaT (wt), HaCaT-MC1R (clones 35 and 53), and HaCaT-R_{151}C (clone 30) cells were pre-incubated with 5 μM gp91 ds-tat for 2 h and exposed to UVA radiation (9 J/cm²). Immediately after UVA exposure, ROS production was quantified by measuring the fluorescence from the oxidation product of carboxy-H_2DCF-DA as described in the Materials and Methods Section. For each cell type, untreated and non-irradiated negative controls were used and results are shown as variations of the fluorescence intensities (arbitrary units, a.u.) of the probe relative to non-irradiated cells. UVA-irradiated and non-irradiated cells were also stained with 5 μg/ml propidium iodide (PI) and only PI-negative cells (viable cells) were used for the analysis. Data are expressed as the mean ± SEM of three independent experiments. *$P < 0.05$ and ***$P < 0.001$ (Student's *t*-test) compared with untreated HaCaT cells irradiated with the same UVA dose.

PKA-dependent phosphorylation of NoxA1 is increased in HaCaT-MC1R cells

Nox1 activation depends on interaction with regulatory subunits including the Nox Organisator 1 (NoxO1) and Nox Activator 1 (NoxA1) (Bedard and Krause, 2007). Recent findings suggest that PKA-dependent phosphorylation of the regulatory NoxA1 subunit is a new pathway for inhibition of ROS production (Kim et al., 2007). To verify this hypothesis, NoxA1 was immunoprecipitated in the different HaCaT cell lines and its phosphorylation status was determined by immunoblotting with an anti-phospho-(Ser/Thr) PKA substrate antibody. NoxA1 phosphorylation was higher in HaCaT-MC1R cells than in parental HaCaT or HaCaT-R_{151}C cells (Fig. 5A).

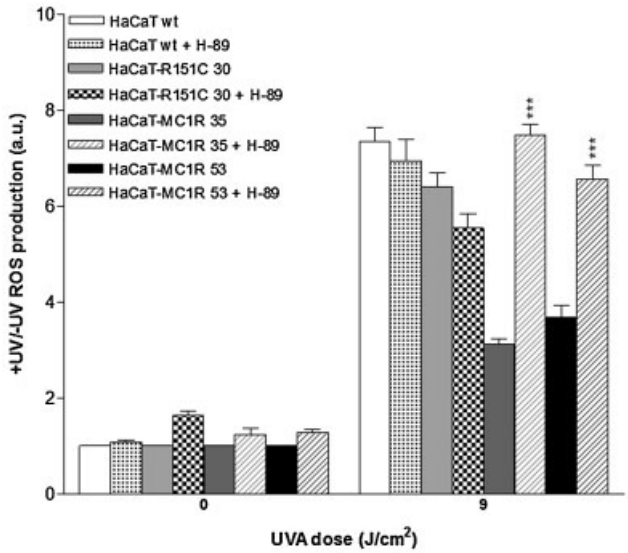

Fig. 4. UVA-induced oxidative stress is inhibited in HaCaT-MC1R cells by a cAMP/PKA-dependent mechanism. Parental HaCaT (wt), HaCaT-MC1R (clones 35 and 53), and HaCaT-R_{151}C (clone 30) were pre-incubated with 5 μM H-89 for 1 h and then exposed to UVA radiation (9 J/cm^2). Immediately after UVA exposure, ROS production was quantified by measuring the fluorescence from the oxidation product of carboxy-H_2DCF-DA as described in the Materials and Methods Section. For each cellular type, untreated and non-irradiated negative controls were added and results are shown as variation of the fluorescence intensities (arbitrary units, a.u.) of the probe relative to non-irradiated and untreated cells. UVA-irradiated and non-irradiated cells were also stained with 5 μg/ml propidium iodide (PI) and only PI-negative cells (viable cells) were used for the analysis. Data are expressed as the mean ± SEM of three independent experiments. ***$P < 0.001$ (Student's *t*-test) compared with untreated HaCaT cells irradiated with the same UVA dose.

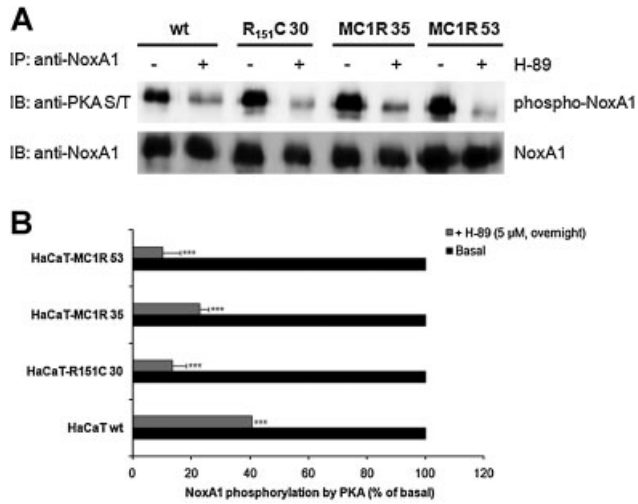

Fig. 5. NoxA1 phosphorylation by PKA is up-regulated in HaCaT-MC1R cells. A: Parental HaCaT (wt), HaCaT-MC1R (clones 35 and 53), and HaCaT-R_{151}C (clone 30) cells were treated or not with 5 μM H-89 overnight and immunoprecipitated with anti-NoxA1 antibody. Cellular extracts (0.5 μg) were resolved by SDS–PAGE electrophoresis, transferred to nitrocellulose membranes, and probed with specific anti-NoxA1 or anti-phospho-(Ser/Thr) PKA substrate antibodies as described in the Materials and Methods Section. B: Expression of phosphorylated NoxA1 was quantified with the Bio1D image analysis software (Vilber Lourmat, Marne La Vallée, France). Data are expressed as the percentage of the relative optical densities (ROD) relative to the expression in untreated HaCaT cells normalized to 100%.

These results indicate that over-expression of functional MC1Rs in HaCaT cells is associated with increased phosphorylation of NoxA1. In order to clearly confirm the PKA-dependent mechanism of phosphorylation of the regulatory NoxA1 subunits, cells were incubated with the PKA inhibitor H-89 (5 μM, overnight). H-89 treatment resulted in a drastic inhibition of NoxA1 phosphorylation in MC1R-transfected cells (Fig. 5B), suggesting that NoxA1 phosphorylation is indeed closely dependent on PKA activation.

Inhibition of EGFR and ERK increases ROS production after UVA exposure in HaCaT-MC1R cells

Since a link may exist between Nox1 activity and epidermal growth factor receptor (EGFR) activation (Chen et al., 2008) and since GPCRs may transactivate EGFR (Bhola and Grandis, 2008), we then investigated the effect of the EGFR inhibitor PD153035 (0.5 μM, 1 h) on UVA-ROS production in the different HaCaT cell lines. EGFR inhibition restored UVA-ROS production in HaCaT-MC1R cells, but had no effects on the parental HaCaT and HaCaT-R_{151}C cell lines (Fig. 6A). Then, as extracellular signal-regulated kinase (ERK) is the major mitogenic pathway initiated by EGFR activation (Blenis, 1993; Marshall, 1995) and is implicated in the regulation of EGF-induced ROS generation (Oh et al., 2010), we treated cells with the specific ERK inhibitor PD98059 (20 μM, 1 h) before UVA irradiation (9 J/cm^2). ERK inhibition also increased UVA-ROS production in HaCaT-MC1R cells, but not in the parental HaCaT and HaCaT-R_{151}C cell lines (Fig. 6B). These results suggest that EGFR, ERK, and MC1R may act in concert to regulate ROS production after UVA exposure.

EGFR and ERK inhibition decrease α-MSH-induced cAMP production in HaCaT-MC1R cells

To test this hypothesis, we then measured cAMP levels in HaCaT cells after incubation with 1 μM α-MSH, 0.5 μM PD153035, or 20 μM PD98059 alone or in combination for 1 h. As expected, α-MSH increased intracellular cAMP levels in HaCaT-MC1R cells, but not in parental HaCaT and HaCaT-R_{151}C cells. Conversely, pre-incubation with PD153035 and PD98059 strongly reduced the α-MSH-induced cAMP production in HaCaT-MC1R cells (Fig. 7), whereas it had no effect on forskolin-induced (10 μM, 1 h) cAMP production (data not shown).

Inhibition of the EGFR/ERK pathway does not affect PKA-dependent NoxA1 phosphorylation

Since recent findings demonstrate that Ser_{282} of NoxA1 is phosphorylated by ERK in response to EGF (Kroviarski et al., 2010; Oh et al., 2010), we asked whether EGFR/ERK inhibition could affect PKA-dependent NoxA1 phosphorylation in MC1R-transfected HaCaT cells. To this aim, HaCaT MC1R cells (clone 53) were treated with 0.5 μM PD153035 (EGFR inhibitor) for 1 h or with 20 μM PD98059 (ERK inhibitor) for 1 h. Then, NoxA1 was immunoprecipitated and its phosphorylation status was determined by immunoblotting with an anti-phospho-(Ser/Thr) PKA substrate antibody. EGFR/ERK inhibition in either untreated or α-MSH-treated HaCaT-MC1R cells did not modify PKA-dependent NoxA1 phosphorylation (data not shown).

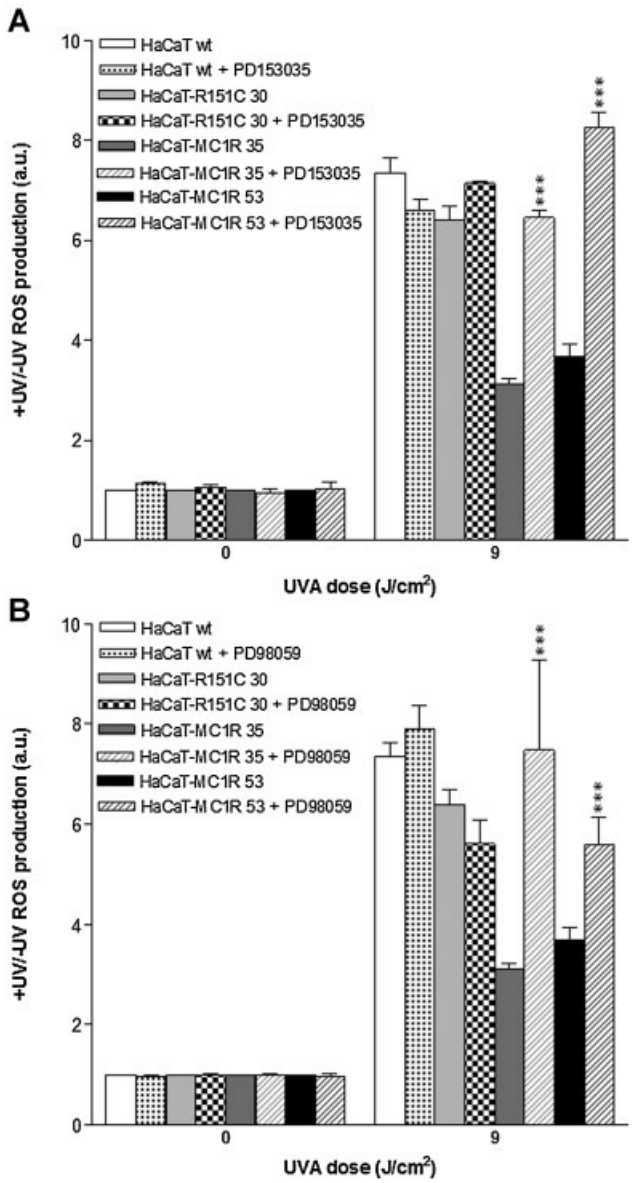

Fig. 6. Effects of EGFR and ERK inhibitors on UVA-induced oxidative stress in HaCaT cells. A: Parental HaCaT (wt), HaCaT-MC1R (clones 35 and 53) and HaCaT-R_{151}C (clone 30) cells were pre-incubated with 0.5 μM PD153035 (EGFR inhibitor) for 1 h and exposed to a 9 J/cm² UVA dose. B: Parental HaCaT (wt), HaCaT-MC1R (clones 35 and 53) and HaCaT-R_{151}C (clone 30) cells were pre-incubated with 20 μM PD98059 (ERK inhibitor) for 1 h and exposed to a 9 J/cm² UVA dose. Immediately after UV exposure, ROS production was quantified by measuring the fluorescence from the oxidation product of carboxy-H_2DCF-DA (10 μM, 1 h) by flow cytometry. For each cell type, untreated and non-irradiated negative controls were added and results are shown as variation of the fluorescence intensities (arbitrary units, a.u.) of the probe compared to non-irradiated and untreated cells. Data are expressed as the mean ± SEM of three independent experiments. ***$P < 0.001$ (Student's t-test) relative to untreated HaCaT cells irradiated with the same UVA dose.

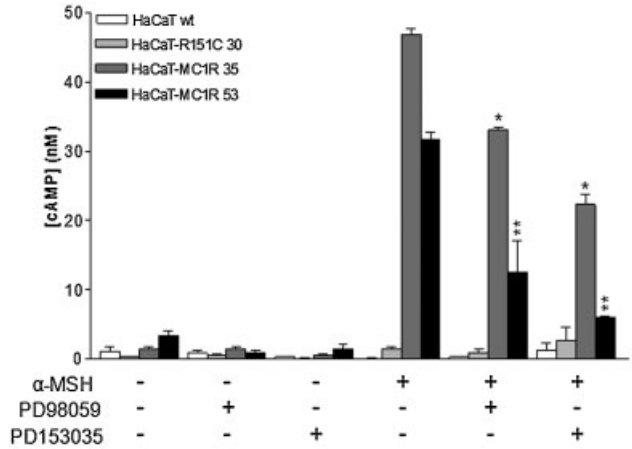

Fig. 7. EGFR and ERK inhibition decrease α-MSH-induced cAMP production in HaCaT-MC1R cells. Parental HaCaT (wt), HaCaT-MC1R (clones 35 and 53), and HaCaT-R_{151}C (clone 30) were stimulated with 1 μM α-MSH, 0.5 μM PD153035 (EGFR inhibitor), or 20 μM PD98059 (ERK inhibitor) for 1 h before determining the cAMP concentration using the cAMP LANCE kit, as described in the Materials and Methods Section. Data are expressed as the mean ± SEM of three independent experiments. *$P < 0.05$ and **$P < 0.01$ (Student's t-test) compared with HaCaT cells stimulated by α-MSH alone.

Discussion

In this study we assessed the effects of UV light on keratinocytes that express high levels of MC1R at the cell surface as it may occur in human skin after solar exposure by using HaCaT cell lines that stably express MC1R or the non-functional $Arg_{151}Cys$ ($R_{151}C$) variant. Although MC1R expression in HaCaT cells is controversial, our results are in agreement with those by Roberts et al. (2006) and confirm that HaCaT keratinocytes do not express MC1R. Our findings indicate that in HaCaT-MC1R cells (but not in HaCaT-R_{151}C cells) early ROS production after UVA irradiation is reduced and almost abrogated after α-MSH treatment.

MC1R is a GPCR and its activation leads to the interaction of activated G-proteins with adenylate cyclase causing an accumulation of cAMP. Constitutive activity may be a feature of GPCRs and high agonist-independent MC1R activity, which results in increased levels of cAMP, has been described in human cells (Sanchez-Mas et al., 2004; Garcin et al., 2007, 2009). Our data confirm these findings and demonstrate that stable expression of MC1R in HaCaT cells is sufficient to strongly inhibit ROS production after UVA exposure. These effects are probably mediated by increased levels of intracellular cAMP because ROS production was not inhibited in HaCaT-R_{151}C cells, which express a non-functional variant of MC1R, and the H-89 inhibitor of PKA (which is activated by cAMP) had no effect on HaCaT-R_{151}C cells. Although cAMP production and PKA activation have been implicated in the negative regulation of ROS production by phagocytes (Lin et al., 2005; Crawford et al., 2006), little is known about their role in Nox1 activity. It has recently been reported that PKA phosphorylation of NoxA1, one of the regulatory subunits of Nox1 (Bedard and Krause, 2007), results in inhibition of Nox1 activity (Kim et al., 2007). Similarly, the finding that NoxA1 phosphorylation was higher in HaCaT-MC1R cells than in parental HaCaT or HaCaT-R_{151}C cells suggests that inhibition of ROS production in HaCaT-MC1R cells is due to down-regulation of Nox1 activity induced by an increase of cAMP and subsequent PKA activation. It is not known why NoxA1 is phosphorylated in HaCaT-R_{151}C cells (Fig. 5). It may be due to the low basal level of cAMP in these cells (Fig. 1B) and to the tightly regulated cAMP-PKA signaling that involves scaffolding PKA and local phosphodiesterases (Dessauer, 2009; Skroblin et al., 2010). Alternatively, MAP kinases may play a role since

the $R_{151}C$ variant of MC1R can efficiently stimulate ERK activity (Herraiz et al., 2009, 2011). Thus, basal PKA phosphorylation and reduced functional coupling with the cAMP pathway in HaCaT-$R_{151}C$ cells may account for their response to α-MSH with significant reduction in ROS following irradiation with 3 or 6 J/cm^2 UVA (Fig. 2).

We then show that pre-treatment with the EGFR inhibitor PD153035 or with the ERK inhibitor PD98059 restored the ability of HaCaT-MC1R cells to produce ROS after UVA exposure, indicating that the regulation of UVA-ROS production in HaCaT-MC1R cells might depend on cross-talk between MC1R and the EGFR/ERK signaling pathways (Stork and Schmitt, 2002). Indeed, many examples of cross-talk between GPCRs and EGFRs have been described and mechanisms regulating these interactions depend on both the GPCR and the cell type (Rozengurt, 2007). For instance, GPCRs can transactivate EGFRs through intracellular signaling pathways that might include cAMP and PKA activation (Barbier et al., 1999; Drube et al., 2006; Gerits et al., 2008). EGFR activation may regulate GPCR internalization through phosphorylation of GPCR kinases (GRKs) (Chen et al., 2008) and over-expression of GRK2 enhances ERK activation induced by EGF stimulation (Gao et al., 2005). Thus, although most of the published reports concern EGF activation through GPCR stimulation, one cannot exclude that MC1R might be activated via EGFR signaling.

In conclusion, our data demonstrate that MC1R expression is sufficient to strongly inhibit UVA-ROS production and that α-MSH stimulation enforces this inhibition. The mechanisms involved are certainly multiple but they seem to depend mainly on cAMP production and subsequent NoxA1 phosphorylation, which results in down-regulation of Nox1 activity. The present findings are based on over-expression studies and the physiological relevance of our results has to be demonstrated. Moreover, UV light effects on HaCaT cells should be interpreted with caution since this cell line presents several abnormalities including p53 double mutation, p16 promoter hypermethylation, and alterations in some of the molecular events necessary for NF-κB activation (Chaturvedi et al., 1999; Qin et al., 1999; Lewis et al., 2006). Nevertheless, our results suggest that the MC1R genotype may modulate ROS production in the epidermis after UV exposure and that MC1R signaling in keratinocytes may play an important role in the regulation of UV-induced inflammation and carcinogenesis through its agonist-independent activity. Recent findings indicate that MC1R protects from UV light in vivo by a combination of pigmentary and non-pigmentary effects (Robinson et al., 2010). Furthermore, MC1R gene variants, particularly loss-of-function variants, are associated with strongly increased risk of severe photoaging (Elfakir et al., 2009). The skin phototype (related to the *MC1R* allele) may be correlated with UV-induced inflammation through expression of MC1R by keratinocytes and, consequently, the diminished level of UV-ROS production in subjects with functional MC1R could thus protect them against inflammation and malignant transformation.

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