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2,4,6-Octatrienoic acid is a novel promoter of melanogenesis and antioxidant defence in normal human melanocytes via PPAR-γ activation

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Summary

Given the importance of the tanning response in protecting human skin from the harmful effects of UV radiation, one important research priority is to identify novel molecules that are capable of promoting pigmentation and/or antioxidant defence. Parrodienes share some structural features with carotenoids and retinoids, stimulate cell antioxidant defence and counteract senescence-like phenotype in fibroblasts. We selected the parrodiene-derivative 2,4,6-octatrienoic acid (Octa) to study its impact on key parameters of melanogenesis and antioxidant defence in organ-cultured human skin and in normal human melanocytes. Octa promoted melanogenesis by up-regulating tyrosinase and microphthalmia-associated transcription factor expression. This correlated with an increase of melanin content in both human epidermis in situ and cultured human epidermal melanocytes. Moreover, Octa increased the biological antioxidant potential content and the expression and activity of catalase. Activation of peroxisome proliferator-activated receptor (PPAR)-γ was necessary to evoke these effects. These data strongly encourage the systematic study of Octa as a novel candidate promoter of human skin photoprotection.

Introduction

UV radiation is the main aetiological agent of most types of skin cancer and a key factor responsible for photoaging. Endogenous systems for photoprotection include pigmentation, epidermal thickening, and complex antioxidant and DNA repair systems (Abdel-Malek et al., 2010; González et al., 2008; Wang et al., 2010). Therefore, public health campaigns rightly emphasize the importance of photoprotection (Wang et al., 2010), while dermatologists and pigment biologists have long attempted to promote endogenous protective responses to UV, e.g. by applying exogenous compounds that stimulate pigmentation, antioxidant enzymes, DNA repair enzymes and non-enzymatic antioxidants (Kokot et al., 2009; Song et al., 2009; Svobodová and Vostálová,

Significance

UV exposure is a known key culprit for the development of skin cancer and photoaging. Endogenous photoprotection includes pigmentation, epidermal thickening and antioxidant and DNA repair systems. More research is needed to find new approaches to the prevention of skin damage. There is considerable interest in the use of naturally occurring products able to promote pigmentation and/or antioxidant defence. We provide the first conclusive evidence that the parrodiene, Octa, operates as a novel promoter of melanogenesis and antioxidant defence through PPAR-γ activation. These data suggest future perspectives for Octa employment in sunscreen formulations with the aim to enhance skin photoprotection.

2010). Given the importance of the tanning responses in protecting human skin from the harmful effects of UV radiation, one priority is to identify novel molecules that are capable of promoting pigmentation or antioxidant defence, offering future perspectives for skin photoprotection.

Here, we have explored the working hypothesis that psittacofulvins could be of particular interest in this respect. Psittacofulvins represent a class of pigments found in bird feathers, namely in the red plumage of *Ara macao*, serving physiological functions such as thermoregulation and defence against solar UV rays (Grande et al., 2004). These pigments are not derived from food carotenoids but are endogenously synthesized within the mature feather follicle (McGraw and Nogare, 2004, 2005). As parrodienes, i.e. polyunsaturated synthetic congeners of psittacofulvins, share some structural features with carotenoids and retinoids, it was hypothesized that they might also have antioxidant properties and influence cell proliferation. Parrodienes do exert antioxidant activity as demonstrated by electronic magnetic resonance assessment of inhibition of hydroxyl radical formation in a Fenton-like reaction (Morelli et al., 2003; Stradi et al., 2001). These molecules also inhibit lipoperoxidation of cell membranes and possess anti-inflammatory activities (Pini et al., 2004). Moreover, in a prior study, we observed that selected parrodiene derivatives, such as 2,4,6-octatrienol, increase cell antioxidant defence and counteract senescence-like phenotype in primary cultures of human dermal fibroblasts (Briganti et al., 2010). A recent clinical trial on human subjects with severe facial photoaging also provided suggestive evidence in support of the concept that another parrodiene derivative closely related to 2,4,6-octarienol, i.e. octatrienoic acid (Octa), can significantly modify the photoaged skin synthoms (Giuliani et al., 2010). Furthermore, our preliminary data showed that Octa is able to activate melanogenesis (Flori et al., 2010), suggesting its possible role also in tanning response.

This study aimed to test the working hypothesis that Octa stimulates human epidermal melanogenesis in situ, i.e. in native, organ-cultured human skin. As this turned out to be the case, we then explored how Octa impacts on key parameters of melanogenesis and antioxidant defence in isolated normal human melanocytes (NHMs).

Numerous nuclear receptors (NRs), such as retinoic acid receptor (RAR), retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor-gamma (PPAR-γ), are important in the control of both melanogenesis (Grabacka et al., 2008; Ho et al., 1992; Kang et al., 2004; Lee et al., 2007; Welsh et al., 1999) and antioxidant defence (Ben-Dor et al., 2005; Okuno et al., 2010; Wertz et al., 2005). As Octa shares some structural features with retinoids and carotenoids, we were particularly interested in exploring any effects of Octa on selected nuclear receptors, in particular RAR or RXR-PPAR-γ signalling in vitro.

Results

NHM viability and growth in response to Octa

Starting with proliferation assays, NHMs were first exposed for different time points (48 and 72 h) to increasing concentrations of Octa, ranging from 1 to 100 μM, according to the doses previously employed in normal human fibroblasts (Briganti et al., 2010). No modification in the metabolic activity of NHMs (as assessed by MTT assay) was observed at any dose tested up to 100 μM after 48 h, and 80 and 100 μM after 72 h (Supporting Information Figure S1A). Next, we performed immunofluorescence analysis using an anti-Ki67 polyclonal antibody (Mastrofrancesco et al., 2010), treating NHMs for 48 and 72 h with the highest doses of Octa, which did not affect cell growth (20, 40 and 60 μM). Quantitative Ki-67 immunofluorescence analysis did not show any significant differences in the number of proliferating cells after Octa treatment in comparison with control cells at either 48 or at 72 h (Figure S1B, C). As 40 and 60 μM Octa did not alter NHM proliferation, these doses were selected to perform the subsequent experiments.

Octa induces tyrosinase activity and melanin synthesis

Next, we asked whether Octa stimulates melanogenesis in NHMs. Tyrosinase activity was measured by quantifying 3,4-dihydroxyphenylalanine (DOPA) oxidation, as tyrosinase is the rate-limiting enzyme of melanogenesis (Brenner and Hearing, 2008). We evaluated the DOPA oxidase activity in NHMs treated with Octa (40 and 60 μM) for 72 h. The enzymatic activity of tyrosinase was significantly up-regulated by Octa exposure in a dose-dependent manner (Figure 1A). Forskolin (FSK, 10 μM), a known adenylate cyclase activator, was used as positive control (Bertolotto et al., 1996). The induction of tyrosinase activity was accompanied by a parallel dose-dependent increase of the intracellular melanin content in cells treated for 5 days (Figure 1B).

Octa does not directly modulate tyrosinase activity

To analyse whether Octa was capable of directly influencing tyrosinase activity, we carried out a cell-free, in vitro experiment by adding the test compound to untreated cell lysates, followed by measurement of enzymatic activity. As shown in Figure 1C, Octa was not able to regulate tyrosinase activity directly in a cell-free system.

Octa induces microphthalmia-associated transcription factor (MITF) and tyrosinase gene and protein expression

As Octa did not regulate tyrosinase activity by direct interaction with this key enzyme of melanogenesis, but potently stimulated melanogenesis in isolated NHMs in vitro (Figure 1A–C), we hypothesized that increased

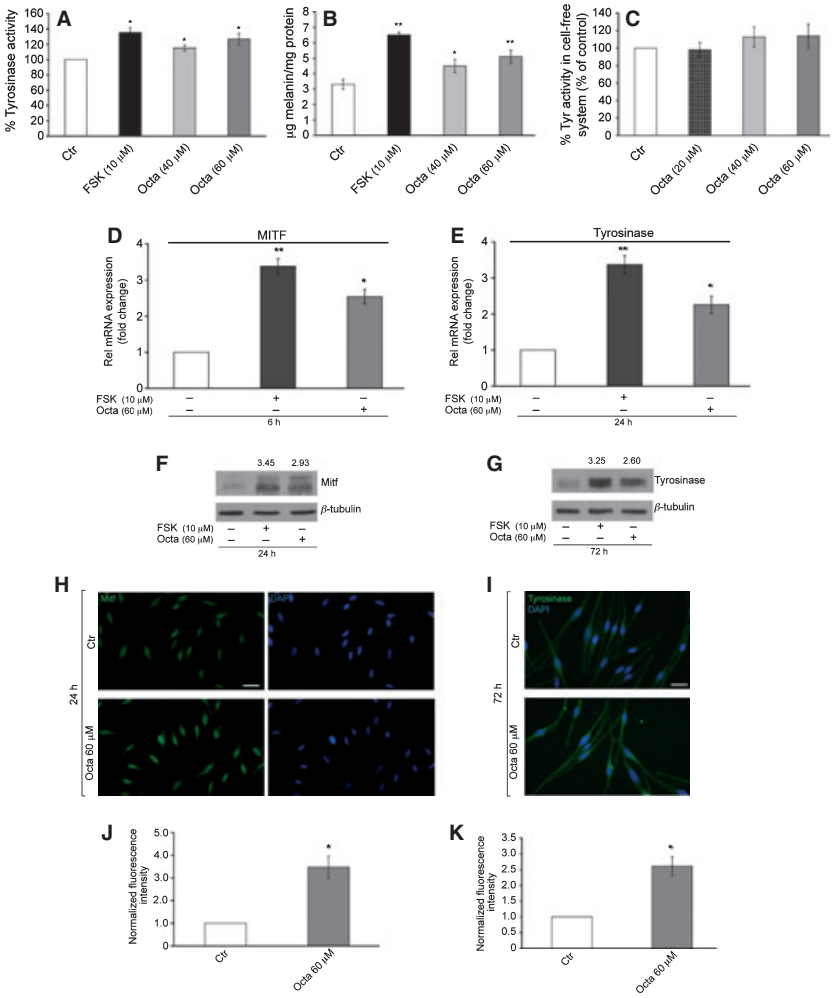

Figure 1. Effects of Octa on melanogenesis in NHMs. (A) Tyrosinase activity measured by L-DOPA oxidation using lysates obtained from NHMs treated for 72 h with Octa (40 and 60 μM). Results are expressed as percentage of the control cells. Forskolin (10 μM) was used as positive control. (B) Spectrophotometric analysis of intracellular melanin concentration of cell lysates after 5 days of treatment with Octa (40 and 60 μM). Results are expressed as μg melanin/mg protein. (C) Tyrosinase activity in cell-free system assayed by spectrophotometry using Octa standard solutions (20–60 μM). Results are expressed as percentage of the control cells. (D–E) Real-time RT-PCR was performed to measure the expression of MITF and tyrosinase mRNA after 6 and 24 h treatment with 60 μM Octa, respectively. *MITF* and *TYROSINASE* values are normalized to the expression of *GAPDH* and are expressed relative to untreated control cells. (F–G) Western blot analysis of MITF and tyrosinase expression after 24 and 72 h exposure to 60 μM Octa, respectively. Protein loading variations were determined by immunoblotting with an anti-β-tubulin antibody. Densitometric scanning of band intensities was used to quantify change of protein expression (control value taken as onefold in each case). Representative blots are shown. (H–K) Immunofluorescence analysis and corresponding quantitative image analysis of the fluorescence intensity of MITF and tyrosinase after 24 and 72 h exposure to 60 μM Octa, respectively. Cells were labelled with anti-MITF monoclonal antibody followed by FITC-conjugated secondary antibody and with anti-tyrosinase polyclonal antibody followed by Alexa-Fluor 488 secondary antibody. Nuclei were stained with DAPI. Scale bar: 20 μm. The data in the graphs are mean value ± SD of three independent experiments. *P < 0.01, **P < 0.001.

expression of melanogenesis-promoting proteins could be responsible for the enhanced melanin production. Therefore, we examined the mRNA and protein expression of both MITF, the 'master regulator of pigmentation' (Cheli et al., 2009; Vachtenheim and Borovanský, 2010) and tyrosinase (Slominski et al., 2004). A significant up-regulation of the mRNA levels of both genes was observed after 6 and 24 h exposure to 60 μM Octa, respectively (Figure 1D,E). Consistent with these quantitative real-time PCR results, a marked increase in MITF and tyrosinase expression was observed at the protein level by Western blot analysis at 24 and 72 h of treatment with 60 μM Octa, respectively (Figure 1F,G). The enhanced expression of MITF and tyrosinase proteins was further confirmed by immunofluorescence experiments. A high increase of MITF staining in the nuclei was evident in Octa-treated melanocytes in comparison with untreated cells (Figure 1H), as assessed by quantitative analysis of the fluorescence intensity (Figure 1J). Similarly, Octa led to a significant increase of

the tyrosinase signal in the cytoplasm of treated cells compared with the basal staining of control melanocytes (Figure 1I), as measured by image analysis (Figure 1K). These results indicate that Octa is able to increase the expression of melanogenesis-promoting proteins.

Octa stimulates the cellular antioxidant defence

We had observed previously that parrodiene derivatives such as 2,4,6-octatrienol increase cell antioxidant defence in primary cultures of human dermal fibroblasts (Briganti et al., 2010). Therefore, we investigated whether exposure to Octa would also induce the antioxidant defence system in NHMs. To this end, we evaluated the biological antioxidant potential (BAP), an index of overall antioxidant status (Chen et al., 2009) and the expression and activity of catalase, the main enzyme responsible for the neutralization of hydrogen peroxide in melanogenically active cells (Maresca et al., 2008; Wood and Schallreuter, 2006; Yohn et al., 1991). Octa (60 μM) significantly increased BAP content after 24 and 48 h of treatment (Figure 2A). Octa also up-regulated the expression of catalase both at the mRNA and the protein level after 6 h and 24–48 h, respectively (Figure 2B,C). Octa also promoted the activity of this key reactive oxygen species (ROS) scavenging enzyme at 24 and 48 h (Figure 2D). Octa is thus able to induce the cellular antioxidant defence in NHMs.

Octa induces PPAR-γ mRNA expression and activation in NHMs

As RAR and PPAR-γ play a key role in the control of both melanogenesis and antioxidant defence (Lee et al., 2007; Okuno et al., 2010; Welsh et al., 1999; Wertz et al., 2005), we investigated the capability of Octa to activate nuclear receptor (NRs) signalling in NHMs. First, we monitored the ability of Octa to activate RAR responding element (RARE)-regulated signalling pathway activity in melanocytes. Octa did not appear to transactivate the RARE reporter or induce the expression of two target genes [retinoic acid 4-hydroxylase (CYP26) and cellular retinoic acid binding protein II (CRABPII); data not shown]. Instead, Octa activated another NR-dependent mechanism involving RXR and PPAR-γ signalling. The exposure to 40 and 60 μM Octa induced a dose-dependent increase of PPAR-γ mRNA expression at 6 h (Figure 3A). Ciglitazone (Cg, 10 μM), a known selective PPAR-γ agonist, was employed as positive control (Sertznig et al., 2008). Consistently, a luciferase assay using the pGL3-(Jwt)TKLuc reporter construct showed that Octa enhanced luciferase expression at 24 h in a dose-dependent manner (Figure 3B). To further confirm the capacity of Octa to induce PPAR-γ activation, we analysed the mRNA expression of adipocyte lipid binding protein (ALBP/aP-2), a PPAR-γ activation target gene. Octa dose-dependently increased aP-2 mRNA expression at 6 h (Figure 3C). These results demonstrate the ability of Octa to activate PPAR-γ signalling.

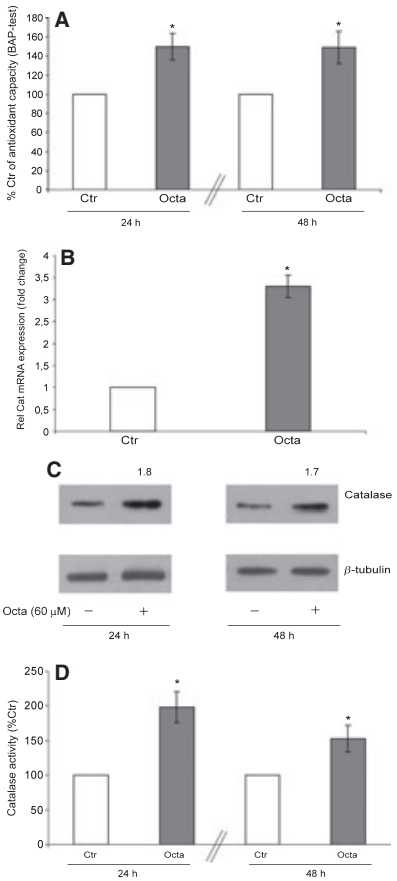

Figure 2. Effects of Octa on the cellular antioxidant defence in NHMs. (A) BAP-Test analysis on lysates obtained from NHMs treated for 24 and 48 h with 60 μM Octa. Results are expressed as percentage of the control cells. (B) Real-time RT-PCR was performed to measure the expression of catalase mRNA after 24 h of treatment with 60 μM Octa. *Catalase* values are normalized to the expression of *GAPDH* and are expressed relative to untreated control cells. (C) Western blot analysis of catalase expression after 24 and 48 h exposure to 60 μM Octa. β-Tubulin was used as loading control. Densitometric scanning of band intensities was used to quantify change of protein expression (control value taken as onefold in each case). Representative blots are shown. (D) Spectrophotometric analysis of catalase activity was determined in the cytosolic fraction as described in Materials and methods. Results are expressed as percentage of the control cells. The data in the graphs are mean value ± SD of three independent experiments. *$P < 0.01$.

Octa stimulates melanogenesis through PPAR-γ activation

To validate the hypothesis that Octa functions by PPAR-γ activation, we first compared the melanogenic response of NHMs to Octa or Cg. We found a similar induction of tyrosinase activity after 72 h treatment with Octa (60 μM) or Cg (10 μM) (Supporting Information Figure S2A). Moreover, a stronger induction of melanin content was observed in NHMs exposed to Octa (60 μM) than to Cg (10 μM) (Figure S2B). In parallel, we analysed the mRNA and protein expression of MITF and tyrosinase. A stronger up-regulation of the mRNA levels

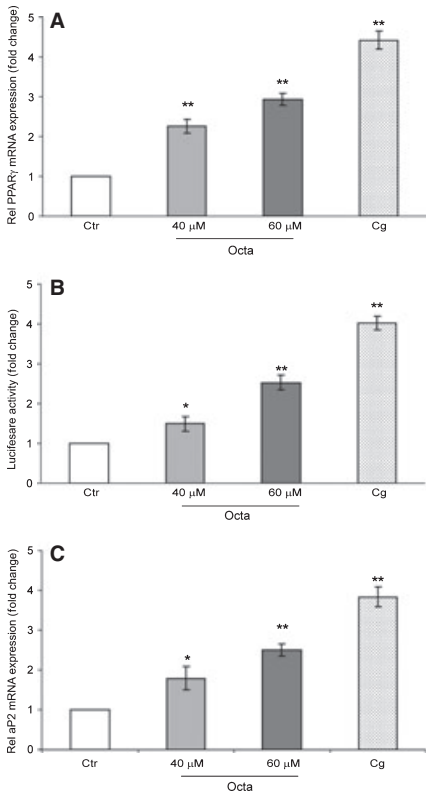

Figure 3. The effect of Octa on PPAR-γ activation in NHMs. (A) Real-time RT-PCR was performed to measure the expression of PPAR-γ mRNA after 6 h of treatment with 40 and 60 μM Octa. The level of PPAR-γ mRNA was normalized to the expression of *GAPDH* and is expressed relative to untreated control cells. Ciglitazone (10 μM) was used as a positive control. (B) Luciferase activity analysis of cells transfected with pGL3-(Jwt) 3TKLuc reporter construct. After 24 h of transfection, cells were treated with 40 and 60 μM Octa. The measurement of luciferase activity was carried out 24 h after treatment. The variability of transfection was normalized with renilla luciferase activity. (C) mRNA transcript level of *aP2* evaluated by real-time RT-PCR analysis in NHM treated with 40 and 60 μM for 6 h. *aP2* values are normalized to the expression of *GAPDH* and are expressed relative to untreated control cells. The data in the graphs are mean value ± SD of three independent experiments. *P < 0.05; **P < 0.01.

of both genes was observed in NHMs exposed to 60 μM Octa than to Cg (Figure S2C,D). Consistent with the quantitative real-time PCR results, a greater increase in MITF and tyrosinase protein levels was observed after treatment with 60 μM Octa (Figure S2E,F). On the basis of these results, we then asked whether the pigmentary effects observed in Octa-treated NHMs were specifically due to PPAR-γ activation. To do so, we evaluated tyrosinase activity, melanin content as well as MITF mRNA and protein expression in the presence of GW9662, a specific and selective PPAR-γ inhibitor (Leesnitzer et al., 2002). GW9662 (3 μM) was able to reverse the increase of tyrosinase activity and melanin content (Figure 4A B) as well as the up-regulation of MITF mRNA and protein (Figure 4C,D) induced by 60 μM Octa.

To corroborate these observations independently, NHMs were transiently transfected with siRNA for PPAR-γ (siPPAR-γ) or control (siCtr). Quantitative real-time PCR analysis confirmed that 6 h after nucleofection, PPAR-γ mRNA was significantly reduced in NHMs transfected with siPPAR-γ (Figure 5A). A parallel immunofluorescence analysis demonstrated that PPAR-γ protein expression was efficiently decreased in siPPAR-γ-cells compared with siCtr-melanocytes at 24 h after transfection (Figure 5A, insert). We also examined the effect of PPAR-γ silencing on tyrosinase activity. Octa significantly promoted the enzymatic activity in siCtr-cells, as expected, whereas it failed to induce it in siPPARγ-NHMs (Figure 5B). In addition, Octa failed to up-regulate *MITF* in PPAR-γ-deficient NHMs (Figure 5C). This result was confirmed at protein level by immunofluorescence analysis performed on siCtr- and siPPAR-γ-cells treated with Octa for 24 h. Quantitative measurement of the fluorescence intensity showed that Octa induced a significative increase of MITF signal in siCtr melanocytes but did not up-regulate MITF in PPAR-γ-silenced NHMs (Figure 5D,E). This conclusively documents the critical involvement of PPAR-γ in the Octa-mediated stimulation of pigmentation in NHMs.

Octa also promotes antioxidant defence via PPAR-γ activation

Finally, we wished to elucidate whether the enhanced cellular antioxidant defence observed in Octa-treated NHMs could be attributed to the same nuclear receptor-mediated signalling mechanism. We first compared the antioxidant response of NHMs to Octa or Cg. As regards the effect of Cg on catalase mRNA expression, no significant changes were produced after a short treatment period (6 h) (Figure S2H); a 48-h exposure was needed to produce a 1.7-fold induction. The treatment with Cg (10 μM) up to 48 h did not appear to influence either BAP (Figure S2G) or the protein expression and activity of catalase (Figure S2I,J), contrary to the effects induced by Octa (60 μM).We then examined catalase expression in siCtr and siPPAR-γ melanocytes. As expected, Octa significantly increased catalase mRNA expression in siCtr cells but failed to up-regulate catalase in PPAR-γ-silenced NHMs (Figure 5F). This result was confirmed at the protein level by Western blot analysis performed on siCtr and siPPAR-γ cells treated with Octa for 24 h (Figure 5G). The results indicate that PPAR-γ-mediated signalling is critically involved in the Octa-mediated activation of the cellular antioxidant defence in NHMs.

Octa stimulates epidermal pigmentation of organ-cultured human scalp skin

To confirm our results on an in vivo model, we switched to normal human epidermis studies, using the serum-free organ culture of full-thickness human scalp skin (Lu et al., 2007).We first treated NHMs with doses of Octa

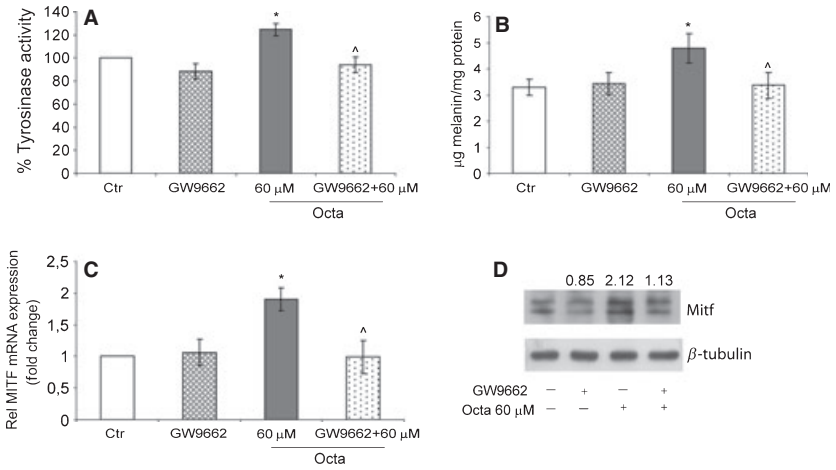

Figure 4. The effect of PPAR-γ inhibition on melanogenesis in NHMs. (A) Tyrosinase activity measured by L-DOPA oxidation using lysates obtained from NHM treated for 72 h with 60 μM Octa and/or 3 μM GW9662. Results are expressed as percentage of the control cells. (B) Spectrophotometric analysis of intracellular melanin concentration of cell lysates after 5 days of treatment with 60 μM Octa in the presence of 3 μM GW9662. Results are expressed as μg melanin/mg protein. (C) Real-time RT-PCR was performed to measure the expression of MITF mRNA after 6 h treatment with 60 μM Octa alone or in combination with 3 μM GW9662. *MITF* values are normalized against the expression of *GAPDH* and are expressed relative to untreated control cells. (D) Western blot analysis of MITF after 24 h exposure to 60 μM Octa and/or 3 μM GW9662. β-Tubulin was used as loading control. Densitometric scanning of band intensities was used to quantify change of protein expression (control value taken as onefold in each case). A representative blot is shown. The data in the graphs are mean ± SD of three independent experiments. *P < 0.01 relative to Ctr; ^P < 0.001 relative to Octa-treated cells.

between 0.7 and 3.6 μM for 72 h to evaluate whether lower doses for a longer period of treatment were capable of inducing pigmentation. The exposure to 0.7 and 1.8 μM Octa did not affect tyrosinase mRNA expression at 48 h, but a dose-dependent increase was clearly observed at 72 h. GW9662 (3 μM) was able to reverse this induction (Figure 6A), further confirming the involvement of PPAR-γ in the Octa-mediated stimulation of pigmentation in NHMs.

In a first pilot experiment on full-thickness human scalp skin, we dissolved Octa (0.7–3.6 μM) in the culture medium, thus imitating a systemic mode of administration, and investigated the effects of Octa on melanogenesis by quantitative Masson–Fontana histochemistry (Ito et al., 2005). Using this method, all three tested Octa doses significantly stimulated intra-epidermal melanin synthesis in situ after 6 days (Figure 6B). Quantitative melanin histochemistry was independently confirmed by quantitative gp100 immunohistomorphometry (Figure 6C), which indicates that Octa actually stimulated the generation and transfer of melanosomes from intra-epidermal melanocytes to human epidermal keratinocytes in situ (Singh et al., 2008). In a second pilot experiment, we treated human skin samples from a different female patient by topical application of Octa (72–360 μM in polyethylene glycol 6000) in skin organ culture for 4 days. Again, all three test doses significantly stimulated intra-epidermal melanin synthesis (Figure 6D) relative to vehicle control skin. These findings strongly supported our working hypothesis that parrodienes such as Octa not only are pigment molecules themselves but can also stimulate human epidermal melanogenesis upon topical and systemic application.

Discussion

Here, we provide the first conclusive evidence that the parrodiene Octa operates, via PPAR-γ activation, as a novel promoter of melanogenesis and antioxidant defence in normal human melanocytes in situ and in vitro. Although Octa does not stimulate NHM proliferation or directly interact with tyrosinase, Octa induces MITF and tyrosinase expression, tyrosinase activity and melanin synthesis. Furthermore, Octa promotes the biological antioxidant potential, an index of overall antioxidant status, and the expression and activity of catalase. That Octa stimulates epidermal pigmentation not only in cultured melanocytes but also in normal human scalp skin (and this after both topical and 'systemic' administration) strongly suggests that Octa deserves to be systematically examined for possible clinical applications, namely as a novel skin photoprotectant candidate.

UV exposure is a known key culprit for the development of skin cancer and a key factor in the acceleration of the photoaging (González et al., 2008; Nakanishi et al., 2009; Pinnell, 2003; Wang et al., 2010). Pigmentation is the main photoprotective mechanism in the skin and epidemiological evidence indicates that the incidence of skin cancer, including melanoma, inversely correlates with skin pigmentation (Abdel-Malek et al., 2010; Bishop and Bishop, 2005). Due to the importance of the tanning responses in protecting human skin from the harmful

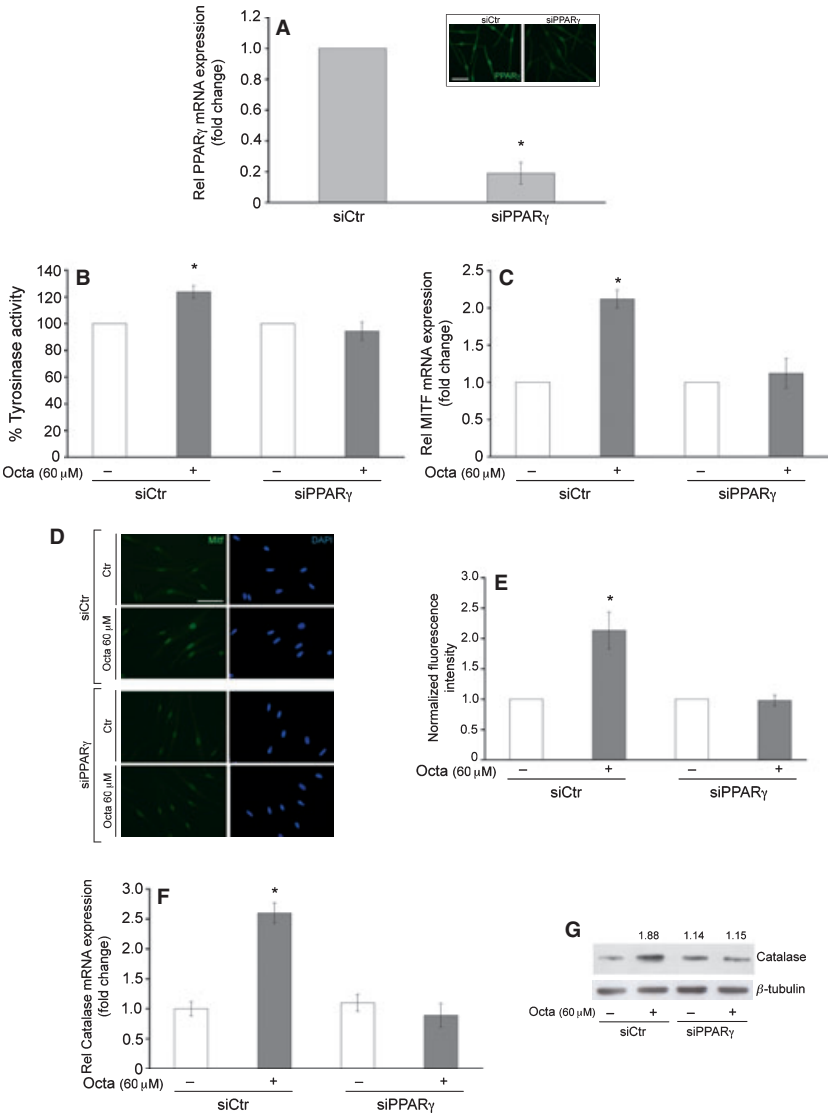

Figure 5. The effect of PPAR-γ silencing on melanogenesis and catalase expression in NHMs. (A) NHMs were transfected with siRNA specific for PPAR-γ (siPPAR-γ) or non-specific siRNA (siCtr). PPAR-γ level was evaluated by real-time RT-PCR and immunofluorescence analysis using an anti-PPAR-γ monoclonal antibody followed by FITC-conjugated secondary antibody (insert). Scale bar: 50 μm. (B) Tyrosinase activity measured by L-DOPA oxidation using lysates obtained from NHMs transfected with siPPAR-γ or siCtr and treated for 72 h with 60 μM Octa. (C) MITF mRNA expression evaluated by real-time RT-PCR in NHMs transfected with siPPAR-γ and siCtr and treated for 6 h with 60 μM Octa. *MITF* values were normalized against the expression of *GAPDH* and are expressed relative to untreated control cells. (D) Immunofluorescence staining with MITF monoclonal antibody followed by FITC-conjugated secondary antibody in NHMs transfected with siPPAR-γ and siCtr and treated for 24 h with 60 μM Octa. Cell nuclei are visualized with DAPI. Scale bar: 50 μm (E) Quantitative analysis of the corresponding fluorescence intensity. (F) The level of catalase mRNA in NHMs transfected with siPPAR-γ or siCtr and exposed to 60 μM Octa for 6 h was normalized to the expression of *GAPDH* and is expressed relative to untreated control cells. (G) Western blot analysis of catalase expression after 24 h exposure to 60 μM Octa in NHMs transfected with siPPAR-γ and siCtr. β-Tubulin was used as loading control. Densitometric scanning of band intensities was used to quantify change of protein expression (control value taken as onefold in each case). Representative blot is shown. The data in the graphs are mean value ± SD of three independent experiments. *$P < 0.01$.

effects of UV radiation, skin cancer prevention strategies are aimed at understanding the regulation of melanin synthesis to improve the pigmentary response in human skin (Abdel-Malek et al., 2010; D'Orazio et al., 2006).

Among possible systems contributing to skin photoprotection, a key role is played by 'photoadaptive responses', characterized by reduction of DNA damage, accelerated removal of UV-induced photoproducts, and up-modulation of the antioxidant defence system. In this view, the constitutive antioxidant defence system can influence the ability of skin cells to counteract UV-induced oxidative stress.

Long-term photoprotection with well-tolerated agents is thus critical to avoid UV-damage. Photoprotective

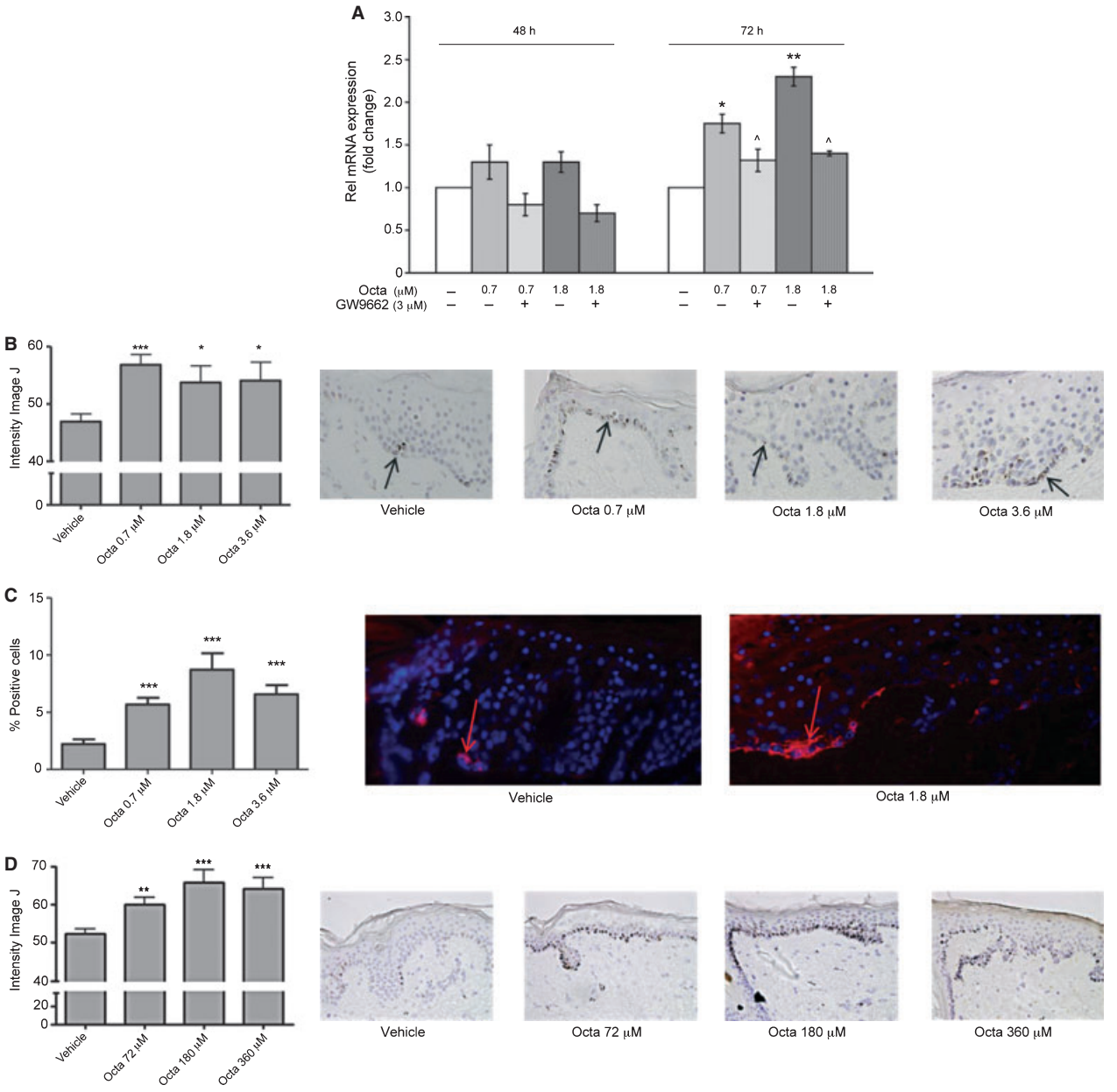

Figure 6. Effects of Octa on skin pigmentation in organ-cultured human scalp skin (A) Real-time RT-PCR was performed to measure the expression of tyrosinase mRNA after 48 and 72 h treatment with 0.7 and 1.8 μM Octa, alone or in combination with 3 μM GW9662. *TYROSINASE* values are normalized to the expression of *GAPDH* and are expressed relative to untreated control cells. *P < 0.05, **P < 0.01 relative to Ctr; ^P < 0.01 relative to Octa-treated cells. (B) All three doses of Octa tested (0.7–3.6 μM) stimulated skin pigmentation in organ-cultured human scalp skin as assessed by IMAGE J software. Black arrows point to melanin pigment in epidermal cells. (C) Octa application increased the number of NKI/beteb cells in epidermal cells. Red arrows demonstrate dendritic melanocytes. (D) All three doses of Octa tested (72–360 μM) stimulated skin pigmentation in organ-cultured human scalp skin as assessed by IMAGE J software in a topical application assay on skin sections from an additional female patient. *P < 0.05, **P < 0.01;***P < 0.001; mean ± SD.

agents can be classified according to their main mechanism of action. Some of them absorb or deflect UV photons (sunscreens), whereas others prevent the deleterious effects of UV exposure. Topical antioxidants, such as vitamin C, vitamin E and beta-carotene, can contribute to reduce UV-related damage of the skin. In addition, there are new natural substances under investigations, such as flavonoids and green tea polyphenols (González et al., 2008; Pinnell, 2003; Wang et al., 2010). Against this background, Octa is a particularly attractive novel 'natural' candidate photoprotectant on several levels, as it appears to target several steps of the melanogenic process, including MITF and tyrosinase expression, tyrosinase activity and melanin synthesis, as

well as activating the endogenous antioxidant defence, in particular catalase.

Considering that Octa shares some structural features with retinoids and carotenoids, we investigated whether some NRs can play a role in Octa-mediated observed effects. Several NRs, such as RAR, RXR, and PPARs, in fact, have been shown to regulate melanocyte proliferation, differentiation and melanogenesis (Ho et al., 1992; Kang et al., 2004; Watabe et al., 2002; Welsh et al., 1999; Yoshimura et al., 2001). In particular, the PPAR-γ agonists, such as ciglitazone, promote melanogenesis in NHMs through the induction of tyrosinase activity and the up-regulation of tyrosinase and MITF proteins (Grabacka et al., 2008; Kang et al., 2004; Lee et al., 2007; Sertznig et al., 2008). We recently discovered the existence of a link between α-MSH and PPAR-γ activation in inducing melanocyte differentiation and pigmentation (Maresca et al., 2010). In this study, we showed that Octa failed to activate RAR but was able to induce the expression of PPAR-γ and aP-2, a PPAR-γ activation target gene, suggesting that the activation of this NR is imputable to a direct binding or to a mechanism involving retinoid X receptor, an obligate heterodimeric partner of PPAR-γ. RXR, in fact, can control the function of many other NRs, thus integrating upstream signals into co-ordinated responses (Yang et al., 2000; Ziouzenkova and Plutzky, 2008). Moreover, Octa promotes pigmentation analogously to ciglitazone, a known PPAR-γ-selective agonist (Lee et al., 2007). PPAR-γ antagonist GW9662 or PPAR-γ silencing were able to completely abrogate the melanogenic effect of Octa, clearly indicating that activation of PPAR-γ is needed for the pigmentary effects of Octa.

Considering the possible mechanism of PPAR-γ-mediated melanocyte differentiation, a connection with MITF could be hypothesized. MITF promoter is unlikely to be a canonical transcriptional target of PPAR-γ because no PPAR response element (PPRE) has been identified. PPAR-γ is able to influence gene expression through interactions with other transcription factors or signalling proteins, such as the WNT/beta-catenin (β-catenin), which is involved in MITF-induced melanocyte development and differentiation (Bellei et al., 2011; Grabacka et al., 2008; Liu et al., 2006; Schepsky et al., 2006; Takeda et al., 2000). The induction of MITF protein in response to ciglitazone is in agreement with a previous study on human melanocytes (Lee et al., 2007). However, this result is in contrast to a recent report showing that the same agonist down-regulated MITF levels in melanoma cells apparently independently of PPAR-γ activation (Botton et al., 2011). In any case, melanoma cell lines and NHMs have shown different features and responses to PPAR agonist (Botton et al., 2011; Lee et al., 2007).

Several experimental and epidemiological data suggest that an optimal photoprotection can be achieved only by a combined action of pigmentary response and activation of physiological systems able to reduce UV damage. In this view, reduction of oxidative stress can contribute to counteract UV harmful effects (Kokot et al., 2009; Song et al., 2009; Svobodová and Vostálová, 2010).

Parrodienes exert antioxidant and anti-inflammatory activity and inhibit cell membrane lipoperoxidation (Morelli et al., 2003; Pini et al., 2004). As the current human melanocyte data confirm previously obtained results on fibroblasts (Briganti et al., 2010), this suggests that Octa promotes cellular antioxidant defence in cell populations of very different embryonal origin. We focused on catalase because this was described as the main enzyme responsible for hydrogen peroxide decomposition into melanocytes (Yohn et al., 1991). Therefore, it is particularly interesting and important that Octa induces catalase expression and activity. We provided evidence that activation of PPAR-γ by Octa was functionally relevant for the induction of catalase expression, as the use of a specific PPAR-γ siRNA abolishes this effect. PPAR-γ regulates the expression of catalase via functional PPREs identified in its promoter and exerts protective effects in several tissues by reducing oxidative stress when stimulated by pioglitazone or rosiglitazone (Girnun et al., 2002; Okuno et al., 2010).

We showed a relevant up-modulation of catalase mRNA expression only after 48 h of treatment with ciglitazone, according to previous results (Girnun et al., 2002). Moreover, ciglitazone did not change catalase protein expression and/or activity up to 48 h, whereas Octa was able to influence cell antioxidant response significantly starting from 6 h of treatment, indicating different kinetics for the two molecules. Ligands with various degrees of agonism, in fact, act differently on the transcriptional machinery that modulates PPAR-γ expression (Choi et al., 2010; Costa et al., 2010).

In summary, we show that the parrodiene 2,4,6-octatrienoic acid holds major promise as a promoter of melanogenesis and antioxidant defence in normal human melanocytes in vitro and in situ and deserves to be fully explored as a novel candidate skin photoprotectant. This compound possesses different strong points: (i) its natural origin, which can support a physiological interference with cellular pathways modulating UV skin response; (ii) a biologically fascinating mechanism of action involving PPAR-γ activation as a possible 'sensor' able to activate different aspects of endogenous skin protection; and (iii) the evidence that treatment with this molecule is free, both in vitro and in vivo (Briganti et al., 2010; Giuliani et al., 2010), from long-term side effects.

Methods

Cell culture and treatments

NHMs, derived from neonatal foreskins after mechanical dissection of skin biopsies, were maintained in M254 medium with Human Melanocyte Growth Supplements (Invitrogen, Life Sciences, Milan,

Italy) and antibiotics (Gibco, Life Technologies Italia, Milan, Italy) in a humidified atmosphere containing 5% CO_2 at 37°C. During the experiments, the culture medium was deprived of bovine pituitary extract (BPE) and phorbol 12-myristate 13-acetate (PMA). Cells were plated and 24 h later were stimulated with chemicals in fresh medium. Octa (kindly supplied by Giuliani Pharma SpA, Milan, Italy) was used at doses of 0.7–100 μM. GW9662 (Sigma-Aldrich Srl, Milan, Italy), a potent and irreversible antagonist of PPAR-γ, was used at the dose of 3 μM. Ciglitazone (Cg) (Sigma-Aldrich), a specific agonist of PPAR-γ, was employed at the dose of 10 μM. Forskolin (FSK) (Sigma-Aldrich), a cAMP-stimulating agent, was used at the dose of 10 μM.

Tyrosinase assay

Tyrosinase activity was estimated by measuring the rate of L-DOPA (3,4-dihydroxyphenylalanine) oxidation as previously described (Bellei et al., 2011). Briefly, cells were treated with different concentrations of Octa (40–60 μM) and Cg (10 μM) for 72 h. The cells were solubilized with 1% Triton ×100 and lysates were clarified by centrifugation at 10 000 *g* for 10 min. After protein quantification by Bradford reagent (Sigma-Aldrich) and adjustment of protein concentration with lysis buffer, 80 μl aliquots of each lysate (each containing the same amount of protein) were placed in the wells of a 96-well plate, with 20 μl of 5 mM L-DOPA. Absorbance was measured spectrophotometrically at 475 nm following a 20-min incubation period at 37°C. Measurement was repeated five times. To perform mushroom tyrosinase assay, 300 μl of 2.5 mM L-DOPA solution, 50 μl of DMSO with or without Octa (20–60 μM), and 1.1 ml of 0.1 M phosphate buffer (pH 6.8) were mixed. The mixture was preincubated at 25°C for 10 min before 50 μl of 1380 units/ml tyrosinase in aqueous solution was added, and the reaction was monitored by the OD of 475 nm. A control reaction was conducted with DMSO alone. The percentage of activity of tyrosinase was calculated as follows: $100 - [(A - B)/A - 100]$, where A represents the difference in the absorbance of the control sample between an incubation time of 0.5 and 1.0 min, and B represents the difference in the absorbance of the test sample over the same incubation period. The results represent the average of three independent experiments performed in triplicate.

Melanin content determination

Cells were treated with increasing concentrations of Octa (40–60 μM) and Cg (10 μM) for 5 days. For intracellular melanin content determination, cell pellets were dissolved in 1 M NaOH and incubated at 60°C for 1 h. Total melanin in the cell suspension was determined with a μQUANT spectrophotometer (BIOTEK Instruments Inc., Winooski, VT, USA) by reading the absorbance at 405 nm. Melanin content was calculated by interpolating the results with a standard curve, generated by the absorbance of known concentrations of synthetic melanin. The results were normalized by protein levels in each sample. The results represent the average of three independent experiments performed in triplicate.

Real-time RT-PCR

Total RNA was isolated using an RNeasy® Mini kit (Qiagen, Hilden, Germany). Following DNAse I treatment, cDNA was synthesized using oligo-dT primers and ImProm-II™ Reverse Transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Real-time RT-PCR was performed in a total volume of 15 μl with SYBR Green PCR Master Mix (Bio-Rad Laboratories Srl, Milan, Italy) and 200 nM concentration of each primer. Real-time PCR protocol and primer sequences for PPAR-γ, Mitf, tyrosinase, catalase, aP2 and GAPDH are available in Supporting Information Data S1. The results represent the average of three independent experiments performed in triplicate.

Western blot analysis

Cells were lysed in denaturing conditions supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). Equal amounts of protein (30 μg) were resolved on acrylamide SDS-PAGE, transferred onto nitrocellulose membrane (Amersham Biosciences, Milan, Italy) and then treated overnight at 4°C with anti-tyrosinase antibody (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-MITF (C5) antibody (1:500; Abcam Inc., Cambridge, MA, USA) or anti-catalase antibody (1:1000; Sigma-Aldrich). For tyrosinase detection, a secondary bovine anti-goat IgG HRP-conjugated antibody (1:1000; Santa Cruz Biotechnology) was used. A secondary goat anti-mouse IgG HRP-conjugated antibody (1:3000; Santa Cruz Biotechnology) was used for MITF and catalase. Antibody complexes were visualized using ECL (Santa Cruz Biotechnology). A subsequent hybridization with anti-β-tubulin (1:10 000; Sigma-Aldrich) was used as a loading control. Protein levels were quantified by measuring the optical densities of specific bands using a GS-800 Calibrated Image Densitometer (Bio-Rad). Western blot assays were representative of at least three independent experiments.

Immunofluorescence analysis

Cells were fixed with cold methanol for 4 min at −20°C or with 4% paraformaldehyde for 30 min at 25°C and then incubated for 1 h with the following primary antibodies: anti-Ki67 polyclonal antibody (1:50; Zymed Laboratories Inc., San Francisco, CA, USA), anti-MITF monoclonal antibody (1:200; Santa Cruz Biotechnology), anti-tyrosinase polyclonal antibody (1:50; Santa Cruz Biotechnology) and anti PPAR-γ monoclonal antibody (1:50; Santa Cruz Biotechnology). Primary antibodies were visualized using anti-rabbit IgG-Alexa Fluor 594 (1:500; Invitrogen), anti-mouse IgG-FITC (1:200; Millipore, Chemicon, Temecula, CA, USA) and anti-goat IgG-Alexa Fluor 488 (1:500). Nuclei were labelled with 4′,6′-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Fluorescence signals were analyzed by recording stained images using a CCD camera (Zeiss, Oberkochen, Germany). Ki67-labelled cells for each treatment were quantified by evaluating the number of NHMs showing positively stained nuclei observed in 10 different fields and expressed as percentage of the control cells. Quantitative analysis of MITF and tyrosinase fluorescence intensity was performed using the AxioVision 4.7.1 software (Zeiss). For each condition, 12 digital images were taken from different microscopic fields and results are expressed as fluorescence intensity mean value ± SD relative to control. Three distinct experiments were analyzed.

Biological antioxidant potential (BAP) assay

Cells were treated with 60 μM Octa and 10 μM Cg for 24 and 48 h. BAP was measured using an assay kit (Diacron Srl, Grosseto, Italy), which is based on the ability of a solution, containing a source of ferric (Fe^{3+}) ions, to decolour when Fe^{3+} ions are reduced to ferrous ions (Fe^{2+}). Cell lysate is dissolved in $FeCl_3$ with a chromogenic substrate (Benzie and Strain, 1996). After 5 min, antioxidant potential was evaluated by a photometric assay (at 593 nm) of the intensity of decolourization. The data were determined by interpolating the absorbance on a calibration curve obtained with Trolox (30–1000 μM). Results of three independent determinations performed in triplicate are expressed as medium percentage of variation (±SD) compared with control values of untreated cells.

Catalase activity

Cells were treated with 60 μM Octa and 10 μM Cg for 24 and 48 h. Catalase activity was determined on the supernatants using a Lambda 25 UV/Vis spectrophotometer (Perkin-Elmer, London, UK), as previously described (Maresca et al., 2008). Standard curves were obtained by using scalar units of bovine catalase (Sigma-Aldrich). One unit of catalase is defined as the amount that degrades 1 mM H_2O_2 per min at 25°C. Units were normalized for the content of protein in the supernatant of cell lysate. Results of three independent determinations performed in triplicate are expressed as medium percentage of variation (±SD) compared with control values of untreated cells.

Luciferase assay

NHM were transfected with pGL3-(Jwt) 3TKLuc reporter construct (Rocchi et al., 2001) using the Amaxa® human melanocyte Nucleofector kit (Lonza, Basel, Switzerland) according to the manufacturer's instructions. Twenty-four hours after treatment with Octa (40–60 μM) and Cg (10 μM), cells were harvested in 100 μl lysis buffer and 20 μl of the extract was assayed for luciferase activity using Promega's Dual Luciferase (Promega) according to the manufacturer's protocol. The renilla luciferase plasmid was also transfected as an internal control for monitoring transfection efficiency and for normalizing the firefly luciferase activity. The luciferase activity was expressed as fold of the activity obtained in cells treated with the different molecules divided by luciferase activity from non-stimulated cells. The results represent the average of three independent experiments performed in triplicate.

RNA interference experiments

For the RNA interference experiments, NHMs were transfected with 100 pmol (h) siRNA specific for PPAR-γ (sc-29455; Santa Cruz Biotechnology). An equivalent amount of non-specific siRNA (sc-44234; Santa Cruz Biotechnology) was used as a negative control. Cells were transfected using the Amaxa® human melanocyte Nucleofector kit (Lonza) according to manufacturer's instructions using the NHEM-Neo Amaxa Nucleofector kit (program U-016). To ensure identical siRNA efficiency among the plates, cells were transfected together in a single cuvette and plated immediately after nucleofection. Twenty-four hours following transfection, 60 μM Octa was added to some samples in agreement with the experimental design. Three independent experiments were performed in duplicate.

Skin sample organ culture and pigmentation evaluation

Human scalp skin sample was obtained after written informed consent from two healthy females, undergoing routine face-lift surgery, and following the Institutional Research Ethics Committee permission. Full-thickness organ cultures were prepared as previously described (Lu et al., 2007). For the first experiment, samples were treated for 6 days with Octa or vehicle dissolved in the culture medium (0.7–3.6 μM), with culture medium change at days 1 and 4. For the second experiment, samples were treated for 4 days with a topical application of Octa (72–360 μM) or vehicle, with daily change of medium and application of the test compound or vehicle. At the end of the treatment period, skin samples were embedded and prepared for immunohistochemistry. Masson–Fontana histochemistry was used for visualizing melanin pigment as previously described (Ito et al., 2005). Melanin was stained as brown dots and the degree of pigmentation was assessed by quantitative histomorphometry in defined reference regions in the epidermis using NIH image software (National Institutes of Health, Bethesda, MD, USA) (Bodó et al., 2007). Immunostaining for the sensitive, melanin-independent melanocyte marker NKI/beteb, which recognizes gp100 and allows identification of melanosomes (Singh et al., 2008), was performed with a mouse anti-NKI/beteb antibody (1:20; Monosan, Uden, the Netherlands) followed by rhodamine-labelled goat anti-mouse antibody (1:200, Alexa Fluor, Eugene, OR, USA) and counterstained with 4,6-diamino-2-phenylindole as previously described (Meyer et al., 2009). The number of clearly visible NKI/beteb positive cells were quantified in relation to the number of cells in the photographed section, and presented as percent of the total number of cells.

Statistical analysis

Statistical significance was assessed using paired Student's t-test. The minimal level of significance was $P = 0.05$.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effects of Octa on metabolism and proliferation in NHMs.

Figure S2. Comparison of the effects exerted by Octa and Cg on melanogenesis and antioxidant defence in NHMs.

Data S1. Materials and methods.

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