UVB Stimulates the Expression of Endothelin B Receptor in Human Melanocytes via a Sequential Activation of the p38/MSK1/CREB/MITF Pathway Which Can Be Interrupted by a French Maritime Pine Bark Extract through a Direct Inactivation of MSK1

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Abstract

Melanogenesis is the physiological process by which melanin is synthesized to protect the skin from UV damage. While paracrine interactions between keratinocytes and melanocytes are crucial for regulating epidermal pigmentation, the endothelin (EDN)-endothelin B-receptor (EDNRB) interaction is one of the key linkages. In this study, we found that a single exposure of normal human melanocytes (NHMs) with UVB stimulates the expression of MITF and EDNRB at the transcriptional and translational levels. That stimulation can be abrogated by post-irradiation treatment with a French maritime pine bark extract (PBE). UVB stimulated the phosphorylation of p38 and c-jun N-terminal kinase (JNK), but not ERK, followed by the increased phosphorylation of MSK1 and CREB. The post-irradiation treatment with PBE did not affect the increased phosphorylation of p38 and JNK, but distinctly abrogated the phosphorylation of MSK1 and CREB. Post-irradiation treatment with the MSK1 inhibitor H89 significantly down-regulated the increased gene expression of MITF and EDNRB in UVB-exposed NHMs. Our findings indicate for the first time that the increased expression of MITF that leads to the up-regulation of melanocyte-specific proteins in UVB-exposed NHMs is mediated via activation of the p38/MSK1/CREB pathway but not the ERK/RSK/CREB pathway. The mode of action by PBE demonstrates that interrupting MSK1 activation is a new target for antioxidants including PBE which can serve as anti-pigmenting agents in a reactive oxygen species-depletion-independent manner.
Introduction

Melanogenesis is the physiological process by which melanin is synthesized in melanocytes located in the basal layer of the epidermis to protect the skin from UV irradiation. UVB-exposed keratinocytes secrete cytokines and growth factors, including endothelin (EDN) 1 [1–5], that stimulate cellular functions, especially proliferation and melanization, of adjacent melanocytes in the epidermis. The corresponding specific receptors are constitutively expressed by human melanocytes and the binding of cytokines and growth factors to their receptors transduces intracellular signals to initiate melanogenesis through specific signaling cascades [6]. On the other hand, UVB radiation directly induces the generation of reactive oxygen species (ROS) in epidermal keratinocytes and melanocytes and stimulates stress activated protein kinases, such as p38, c-jun N-terminal kinase (JNK) or extracellular regulated protein kinase (ERK) [7]. In UVB-exposed human melanocytes, the p38 pathway predominantly contributes to the increased expression of microphthalmia-associated transcription factor (MITF) [7], a master regulator of melanocyte functions, including differentiation [8–10], proliferation [11–14], survival [15, 16] and melanogenesis [17, 18]. MITF regulates the expression of many melanogenic enzymes, melanosome structural proteins, transporters and receptors, such as tyrosinase, tyrosinase-related protein 1 (TYRP1), dopachrome tautomerase (DCT), melanosomal protein 17 (PMEL17), melanoma antigen recognized by T-cells 1 (MART1) and endothelin B-receptor (EDNRB) [19].

EDN-EDNRB binding is one of the key paracrine interactions between keratinocytes and melanocytes that regulates skin pigmentation [1, 4, 20–22]. EDN1 is a vasoconstrictor peptide originally isolated from porcine endothelial cells [23]. We first reported that human keratinocytes produce a prepro-EDN1 and then convert it by metallo-proteinases including EDN-converting enzyme α, sequentially to big-EDN1 and EDN1, which is the final secretable form [1,20,24]. UVB-exposed human keratinocytes distinctly enhance the secretion of EDN1, which triggers adjacent melanocytes in the epidermis via EDNRB to stimulate melanin synthesis [1, 20]. EDNRB is a seven-transmembrane receptor coupled with G-protein that interacts equally with all forms of EDN, EDN-1, EDN-2 and EDN-3 [25]. Mutations of those genes causes Waardenburg Syndrome Type IV, which is an auditory-pigmentary syndrome characterized by hearing loss, abnormal pigmentation of skin, hair and eyes in association with Hirschsprung disease, which is a disorder that causes blockage of the intestine [26].

The role of the EDN-EDNRB interaction was reported to induce mitogenesis and melanogenesis in melanocytes [1, 4, 20–22]. Although EDN secretion from keratinocytes stimulated by UVB has been well investigated, little is known about EDNRB expression in UVB-exposed melanocytes. EDNRB expression has been shown to increase in the epidermis when human skin is exposed to solar-stimulated radiation or UVB radiation [27–29] and in skin with lentigo senilis [30]. The finding that a dominant-negative mutant of MITF reduces the expression of EDNRB in cultured melanocytes strongly suggested that EDNRB expression is predominantly regulated by MITF [31].

Skin pigmentation is a major factor that prevents the skin from UV-induced damage. Pigmented skin is unwanted by people who desire a lighter skin color, and many natural products have been utilized historically for cosmetic purposes in order to obtain a lighter skin appearance [32–33]. As depicted in Fig 1, the French maritime pine (Pinus maritima) bark extract (PBE) is a complex mixture of flavonoids, which contains 72.5% polyphenol (determined by Folin Denis method) including 5% procyanidin B1, 2.98% catechin, 0.23% epicatechin and about 60% (including the percentage of dimer) oligomeric proanthocyanidin (OPC) [34–38]. PBE has been used as a traditional medicine for scurvy by maritime Indians [37–38]. PBE has potent antioxidant properties [39–41] and oral administration of PBE has protective effects on
It was also reported that PBE by itself is highly effective in protecting the skin from UV irradiation [42]. Kim et al. demonstrated that PBE inhibits melanogenesis not via inhibition of tyrosinase but rather by inhibiting the autoxidation of melanin due to its antioxidant activity [41]. In a clinical study, oral administration of PBE at 40 or 100 mg daily for 12 weeks reduced the pigmentation of age spots [43].

Here we show that the expression of EDNRB is accentuated in UVB-exposed human melanocytes via activation of the p38/MSK1/CREB/MITF pathway where MSK1 activation is essentially responsible for CREB activation. Post-irradiation treatment with PBE does not affect p38 activation but can directly interrupt the UVB-induced activation of MSK1, which leads to abrogation of the UVB-induced up-regulation of melanocyte-specific proteins such as EDNRB.

Thus, it is anticipated that PBE can serve as an anti-pigmenting agent in a ROS depletion independent manner.

Materials and Methods

Materials

Anti-MITF (C5), anti-EDNRB (EPR7013), anti-CREB (48H2), anti-phospho-CREB (87G3), anti-β-actin (AC-15), anti-rabbit IgG HRP-conjugated and H89 dihydrochloride were purchased from Abcam (Cambridge, MA). Anti-mouse IgG HRP-conjugated was purchased from Jackson ImmunoResearch (West Grove, PA). Antibodies for MAPK and phosphorylated MAPK, the MAPK family sampler kit and the phospho-MAPK family sampler kit were.
purchased from Cell Signaling Technology (Beverly, MA). Antibodies for MSK1 (C27B2) and phosphorylated (S376 and T581) MSK1 were purchased from Cell Signaling Technology. For Real-time RT-PCR, primers for β-actin (Hs_ACTB_1_SG Quantitect Primer Assay; QT00095431), EDNRB (Hs_EDNRB_1_SG Quantitect Primer Assay; QT00014343) and MITF (Hs_MITF_1_SG Quantitect Primer Assay; QT00037737) were purchased from Qiagen (Hilden, Germany). PBE (Flavangenol) which obtained by hot water extraction method from French maritime pine (Pinus pinaster) bark was supplied by Toyo Shinyaku (Saga, Japan).

Melanocyte culture
Primary normal human epidermal melanocytes (NHMs) pooled from 250 individual human foreskins were purchased from Cell Systems (Kirkland, WA) and were maintained in Dermalife Ma culture medium (Lifeline Cell Technology, Walkersville, MD) supplemented with all of the supplements from the manufacturer.

UVB source
The UVB source employed in this study was a Phillips TL20W/12RS lamp (Phillips, Eindhoven, Holland). The energy exposed was measured using a UVX radiometer with a UVX-31 sensor (UVP Inc., San Gabriel, CA).

UVB irradiation and PBE treatment
NHMs were plated in 6-well plates at a density of 1×10^5 cells per well in complete medium. Twenty-four h later, NHMs were washed with warmed phosphate buffered saline (PBS) once and irradiated once with 60 mj/cm² UVB in a thin layer of warmed PBS, with the lid removed. Complete medium with or without the indicated concentration of PBE was added to the well immediately after the UVB irradiation and the plates were then cultured for the indicated periods. Non-irradiated NHMs were subjected to the identical procedure but without UVB irradiation. H89 treatment was carried out instead of PBE at the indicated concentration.

NHM viability
NHMs were plated in 96-well plates at a density of 1×10^4 cells per well in complete medium. Twenty-four h later, the medium was removed and NHMs were washed with warmed PBS once and irradiated once with the indicated energies of UVB with the lid removed. Complete medium with or without the indicated concentration of PBE was added to the well immediately after the UVB irradiation and the plates were then cultured for 24 h. Viable NHMs were determined by a colorimetric assay with a Cell counting kit 8 (Dojin Chemical, Kumamoto, Japan), according to the manufacturer’s protocol.

Real-time RT-PCR
Total RNAs from NHMs cultured for the indicated times were prepared using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Reverse transcription and Real-time PCR reaction were used with a QuantiTect Reverse Transcription kit and a Rotor-Gene SYBR PCR kit with the gene specific primer of β-actin as a reference and the gene of interest described in Materials section according to the manufacturer’s protocol. The Real-time PCR reaction and the signal detection were carried out with Rotor-Gene Q (Qiagen, Hilden, Germany) and data analyses were carried out with Rotor-Gene Q Series Software (Qiagen, Hilden, Germany).
Western blotting analysis

At the end of the culture, NHMs were washed twice with ice cold PBS and were lysed in RIPA buffer with the Halt Protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL). Amounts of total protein were quantitated using BCA protein reagent (Thermo Scientific, Rockford, IL). Total proteins (5 μg/lane) were denatured by heating at 95°C in Laemmli sample buffer (BioRad, Richmond, CA) for 5 min and loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels (BioRad, Richmond, CA). After electrophoresis, proteins were transferred onto Polyvinylidene difluoride (PVDF) membranes and were immunoblotted with appropriate primary and secondary antibodies. Immunoblotted proteins were visualized using an ECL substrate (BioRad, Richmond, CA) and were detected and analyzed by ChemiDoc XR+ System and Image Lab software (BioRad, Richmond, CA).

Statistical Analysis

All data are expressed as means ± SD (n = 3) unless noted otherwise. For pairwise comparisons, either Student’s t-test or Welch’s t-test was applied. For multiple comparisons, data were tested by one-way ANOVA, and subsequently using the Tukey or Dunnett multiple comparison test. P values less than 0.05 are considered statistically significant.

Results

Effect of PBE and UVB on the viability of NHMs

We examined the effect of UVB irradiation and/or PBE treatment on the viability of NHMs. While treatment with PBE slightly enhanced the cell viability at concentrations of 10–30 μg/ml, it did not decrease the cell viability at concentrations less than 60 μg/ml (Fig 2A). UVB irradiation had no affect on the viability of NHMs at energy doses less than 60 mJ/cm², but had a distinct effect on cell viability at a dose of 120 mJ/cm² (Fig 2B). The addition of PBE to UVB-exposed NHMs at a concentration of 30 μg/ml had no substantial influence on the viability of NHMs at energy doses of less than 60 mJ/cm².

UVB stimulates the expression of EDNRB in NHMs, which is abrogated by post-irradiation treatment with PBE

We have already reported that EDNRB expression is increased by the exposure of human skin to UVB [29]. However, little was known about the biological mechanism(s) by which UVB irradiation stimulates EDNRB expression in the epidermis in vivo. Hence, we examined the effects of UVB irradiation at a dose of 60 mJ/cm² on the expression of EDNRB in NHMs. Real-time RT-PCR analysis revealed that the mRNA expression level of EDNRB was significantly increased by UVB irradiation (60 mJ/cm²) at 24 h but not at 6 or 12 h post-irradiation (Fig 3A). When added immediately after UVB irradiation at a dose of 60 mJ/cm², the enhanced expression of EDNRB mRNAs at 24 h post-irradiation was significantly abrogated by PBE at concentrations of 10, 20 and 30 μg/ml (Fig 3B). Western blotting analysis demonstrated that EDNRB protein levels were significantly increased at 24 h post-UVB irradiation (60 mJ/cm²), which was significantly abrogated by post-irradiation treatment with PBE at a concentration of 30 μg/ml (Fig 3C).
UVB stimulates MITF expression in NHMs, which is abrogated by post-irradiation treatment with PBE

Sato-Jin et al. reported that transfection of the dominant negative form of MITF suppressed the expression of EDNRB mRNA and suggested that EDNRB gene expression occurs downstream of MITF [31]. Therefore, we examined MITF expression when NHMs were exposed to UVB irradiation and then were treated with PBE. Real-time RT-PCR analysis revealed that, when exposed to UVB at 60 mJ/cm², the expression level of MITF mRNA was increased at 6, 12 and 24 h post-irradiation with a peak at 6 h post-irradiation (Fig 4A). When treated post-irradiation with PBE at a concentration of 30 μg/ml, the increased expression levels of MITF mRNA were significantly abrogated by PBE at 6 and 24 h post-irradiation. Further, the enhanced expression of MITF mRNA at 6 h post-irradiation was significantly abrogated by PBE at a concentration of 20 and 30 μg/ml in a fashion similar to those observed for the EDNRB mRNA expression (Fig 4B). Western blotting analysis demonstrated that the MITF protein level was significantly increased by UVB irradiation with 60 mJ/cm² at 12 h post-irradiation, which was significantly abrogated by post-irradiation treatment with PBE at a concentration of 30 μg/ml (Fig 4C).

CREB phosphorylation is attenuated by PBE

Cyclic AMP response element-binding protein (CREB) is a transcription factor regulating MITF gene expression, which is activated by phosphorylation in response to various signaling molecules [30]. Since the expression of MITF and its downstream target gene EDNRB was up-regulated by UVB irradiation at the transcriptional and translational levels, and that could be abrogated by post-irradiation treatment with PBE, we next determined if the phosphorylation of CREB is increased by UVB irradiation and/or whether the post-irradiation treatment with PBE can abrogate the CREB activation. Western blotting analysis using an antibody to phospho-CREB revealed that the phosphorylation level of CREB was significantly increased by UVB irradiation with 60 ml/cm² at 15 min post-irradiation, which was significantly abrogated by the post-irradiation treatment with PBE at a concentration of 30 μg/ml (Fig 5). These results indicate that the up-regulation of MITF protein level is mediated via CREB activation in
UVB-exposed NHMs and the post-irradiation treatment with PBE abrogates the CREB activation.

PBE interrupts the phosphorylation of MSK1 but not ERK, JNK and p38

We have already reported that in UVB-exposed NHMs, the generated ROS triggers p38 and JNK but not ERK activation, leading to their downstream target CREB activation predominantly via p38 activation [7]. Based on this evidence, we next determined which signaling molecule(s) upstream of CREB are attributable to the interruption of CREB phosphorylation by post-irradiation treatment with PBE. As expected, while UVB irradiation stimulated the phosphorylation of p38 and JNK but not of ERK, the post-irradiation treatment with PBE did not abrogate the increased phosphorylation of p38 and JNK, which suggests that the interruption of CREB phosphorylation by PBE is not attributable to its effect on p38 activation (Fig 6).

In UVB-exposed human primary keratinocytes, the activated p38 is known to stimulate nuclear kinase mitogen-and stress activated kinase (MSK1) which phosphorylates CREB and NFkBp65 in the nucleus during the NFkB signaling pathway [44]. Therefore, we next
determined if UVB radiation stimulates the phosphorylation of MSK1 in NHMs and/or if PBE can serve as an inactivator for MSK1 even when treated post-irradiation. Western blotting analyses revealed that the phosphorylation of Ser376 (Fig 7A) and Thr581 (Fig 7B) residues of MSK1 was significantly increased 15 min following UVB irradiation, which was significantly abrogated by PBE when treated post-irradiation at 30 μg/ml (Fig 7). This suggests that the interruption of CREB phosphorylation by PBE is attributable to its abrogating effect on MSK1 activation.

We next asked if the inhibition of MSK1 activation results in the down-regulated MITF and EDNRB expression in UVB-exposed NHMs. When the MSK1 inhibitor H89 was added to NHMs immediately after UVB irradiation, the increased expression level of MITF and EDNRB mRNA elicited by UVB irradiation was significantly abrogated by H89 (Fig 8). This suggests that the abrogation of UVB-stimulated expression of EDNRB via MITF transcription by the post-irradiation treatment with PBE is mediated via the interruption of MSK1 activation.

**Fig 4. Effect of treatment with UVB and/or PBE on MITF expression.** (A) Time course of MITF mRNA expression in NHMs treated without UVB in the absence of PBE, without UVB in the presence of 30 μg/ml PBE, with 60 mJ/cm² UVB in the absence of PBE, or with 60 mJ/cm² UVB in the presence of 30 μg/ml PBE and cultured for the indicated periods. (B) Dose dependency of PBE for MITF mRNA expression in NHMs at 6 h after treatment with or without 60 mJ/cm² UVB and 30 μg/ml PBE. Expression levels were detected by specific primers and antibodies for MITF and β-actin as the internal control. Error bars represent S.D. from triplicate experiments. *P < 0.05 and **P < 0.01 against NHMs UVB-irradiated in the absence of PBE, respectively.

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Discussion

In this study, we found that a single exposure of NHMs by UVB stimulates EDNRB expression. Since the increased levels of EDNRB seem to respond to EDN1 to a greater extent than in unexposed NHMs, that finding suggests that UVB causes NHMs to become highly responsive to environmental stimuli such as EDN1 via an increased expression of the corresponding receptor, EDNRB. Consistently, we have already found that KIT ligand (KITL) up-regulates the expression of EDNRB in NHMs where the binding of 125I-labeled EDN1 to EDNRB increases significantly 2 days after incubation with KITL [29]. Similarly, we reported that a single exposure of NHMs with UVB stimulates expression of the KIT receptor, whose function was assessed by an increased phosphorylation following KITL stimulation [7]. Thus, it is likely that in addition to the increased production of melanogenic cytokines by UVB-exposed keratinocytes, EDNRB also plays a coordinated role in UVB-induced pigmentation by augmenting EDN1/EDNRB signaling through the accentuated function of EDNRB. In support of this, in UVB-exposed human skin where pigmentation is being stimulated, there is a significantly up-regulated expression level of EDNRB mRNA [29]. However, little is known about intracellular signaling mechanisms involved in the stimulation of EDNRB expression in UVB-exposed NHMs.

Anti-pigmentation agents have been developed as a target for various redox-sensitive biomolecules, including tyrosine hydroxylase or intracellular signaling intermediates during the melanogenesis cascade. Compounds including phytochemical agents or botanical extracts are adequate candidates for this purpose due to their distinct anti-oxidant properties [45,46]. In this study, we found that a French maritime PBE containing rich flavonoids including OPC [34–38] distinctly abrogates the increased expression of EDNRB at the transcriptional and translational levels following UVB radiation even when treated post-irradiation. PBE has a distinct antioxidant activity stronger than vitamin C and vitamin E as measured by lipid peroxidation of bovine retinal tissue [47]. Additionally, PBE possesses a potent scavenging activity for peroxynitrite (ONOO−), superoxide (•O2−) and nitric oxide (NO•), which play a central role in inhibiting the generation of these ROS. Further, PBE up-regulates the reduced-glutathione/oxidized-glutathione ratio [41]. Owing to these strong antioxidant properties, it was anticipated

![Figure 5: Effects of treatment with UVB and/or PBE on CREB phosphorylation.](image-url)

*Fig 5. Effects of treatment with UVB and/or PBE on CREB phosphorylation. CREB phosphorylation in NHMs at 15 min after treatment with or without 60 mJ/cm² UVB and/or 30 μg/ml PBE. Expression levels were detected by specific antibodies to non-phospho and phospho CREB. Error bars represent S.D. from triplicate experiments. *P<0.05 and **P<0.01 against NHMs UVB-irradiated in the absence of PBE, respectively.*

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that PBE has a potential to inhibit pigmentation by preventing the autoxidation of melanin [41]. Since PBE can behave as an antioxidant and a scavenger for ROS generated by UVB irradiation, its possible inhibitory effect on the increased expression of EDNRB could be accounted for by the depletion of generated ROS if treated pre-irradiation. However, our observation that post-irradiation treatment with PBE can also abrogate the increased EDNRB expression strongly suggests that PBE abrogates the up-regulation of EDNRB expression via an unknown novel signaling mechanism(s) in a ROS depletion-independent manner because the ROS lifetime is very short (e.g. lifetime of $\cdot O_2$ is 4 $\mu$s) [48], not sufficient to deplete the generated ROS when treated immediately after UVB radiation.

UVB exposure of human keratinocytes was reported to activate NFκB signaling by stimulating IKK kinase which phosphorylates IκB, causing NFκBp65 to transduce toward translocation into the nucleus during the signaling pathway downstream of the preceding p38 or JNK activation [28]. In contrast, UVB exposure of human melanocytes induces little or no activation of the NFκB pathway compared to the distinct activation of their upstream pathways such as p38 and JNK [49]. In melanocytes and melanoma, UVB has been shown to induce phosphorylation of the p38 and JNK/stress-activated protein kinase pathways, whereas NFκB remains at a constantly high expression level [50–53]. The activation of p38 or JNK following UVB radiation is

**Fig 6. Effects of treatment with UVB and/or PBE on phosphorylation of MAPKs.** The phosphorylation levels of p38 (A), JNK (B) and ERK1/2 (C) in NHMs at 15 min after treatment with or without 60 mJ/cm$^2$ UVB and/or 30 μg/ml PBE. Expression levels were detected by specific antibodies to non-phospho and phospho MAPKs. Error bars represent S.D. from triplicate experiments. *P<0.05 and **P<0.01 against NHMs UVB-irradiated in the absence of PBE, respectively.

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mediated by initial stress-activated protein kinases, which are activated by ROS via redox-interfering mechanisms involved in protein kinases as well as their conjugated protein phosphatases [54]. Owing to these mechanisms, many antioxidants can suppress UVB-induced cellular events by scavenging generated ROS when treated pre-irradiation. This evidence indicates that the hitherto reported inhibitory effects of antioxidants on the UVB-induced activation of IKKinase leading to the diminished nuclear translocation of NFκB [55–60] may occur via the abolishing effect on the activation of p38 or JNK due to the preceding ROS depletion by pre-treatment with antioxidants. Therefore, it is of considerable importance to determine the signaling mechanism(s) by which the post-irradiation treatment with PBE can abrogate the increased EDNRB expression.

![Fig 7. Effects of treatment with UVB and/or PBE on the phosphorylation of MSK1.](image)

The phosphorylation level of MSK1 at Ser376 residue (A) and Thr581 residue (B) at 15 min after treatment with or without 60 mJ/cm² UVB and/or 30 μg/ml PBE. Expression levels were detected by specific antibodies to non-phospho and phospho MSK1. Error bars represent S.D. from triplicate experiments. *P<0.05 against NHMs UVB-irradiated in the absence of PBE.

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![Fig 8. Effect of H89 on the gene expression of MITF and EDNRB in NHMs exposed to UVB.](image)

The indicated concentration of H89 was added into the medium immediately after UVB irradiation and cells were cultured for 6h (for MITF, A) or 24 h (for EDNRB, B). Total mRNAs were purified and Real-time RT-PCR was carried out with MITF or EDNRB primer and β-actin primer as the internal control. Error bars represent S.D. from triplicate experiments. *P<0.05 and **P<0.01 against NHMs UVB-irradiated in the absence of H89, respectively.

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We have already reported that EDNRB gene expression occurs downstream of the melanocyte-master transcription factor MITF [31]. Consistently, in this study, the gene and protein expression levels of MITF are significantly up-regulated by UVB radiation, and can be significantly abrogated by the post-irradiation treatment with PBE. This suggests that the up-regulated EDNRB expression by UVB radiation is mainly associated with the increased protein expression level of MITF and the abrogating effect of PBE on the increased expression of EDNRB is mainly attributed to the down-regulated level of MITF protein.

In NHMs, at the terminal point of the EDN1-triggered intracellular signaling cascade, the gene expression levels of melanocyte-specific proteins including EDNRB are strictly associated with the steady state levels of MITF protein. The MITF gene expression level is positively regulated by the levels of activated (phosphorylated) CREB in association with other transcription factors including SOX10, PAX3, lymphoid-enhancing factor-1 (LEF-1) and T cell factor (TCF) [61,62]. Therefore, the abrogating effect of PBE on the up-regulated expression of MITF led us to determine whether CREB phosphorylation is stimulated by UVB radiation and whether PBE abrogates this stimulation. As expected, UVB exposure of human melanocytes distinctly stimulates the phosphorylation of CREB, which is abolished by the post-irradiation treatment with PBE. This suggests that the abrogating effect of PBE on the up-regulated protein expression of MITF is mainly attributed to the interruption of CREB activation. Therefore, we next determined how the CREB is activated by UVB radiation in human melanocytes.

In our previous similar study focusing on KIT receptor expression in UVB-exposed human melanocytes, the inhibition of p38 activation by its inhibitor SB203580 results in the complete abrogation of both the up-regulated phosphorylation of CREB and the increased gene expression levels of MITF up to the non-stimulated control levels [7]. This suggests that the increased phosphorylation of CREB by UVB irradiation is mediated predominantly via the activation of p38 but not the cyclic AMP/PKA pathway. In this study, in agreement with our results and another study [7,49,63], UVB exposure of human melanocytes significantly stimulates the phosphorylation of p38 and JNK but not of ERK, whereas the increased phosphorylation of p38 and JNK is not abrogated by the post-irradiation treatment with PBE. Since p38 cannot directly phosphorylate CREB, these findings strongly suggest that the post-irradiation treatment with PBE affects signaling intermediates capable of phosphorylating CREB, which occur downstream of p38 activation. There are at least four protein kinases that have a distinct ability to phosphorylate CREB, protein kinase A (PKA), p90 ribosomal protein S6 kinase (p90RSK), MAPK-activated protein kinase-2 (MK2) and MSK1. MSK1 has a Km value much lower than the other 3 kinases, all of which are distinctly activated by p38 or ERK [64,65]. Therefore, we next determined whether MSK1 is activated by UVB radiation in human melanocytes and whether the post-irradiation treatment with PBE can abrogate the MSK1 activation.

MSK1 is generally expressed in epidermal keratinocytes and, as shown in Fig 9, is activated by p38 MAPK or the ERK p44/42 MAPKs through phosphorylation of either Thr581 or Ser360 [64,66,67]. The phosphorylation of Ser360 is an essential requirement for MSK1 activation [68]. Further, Ser376 is auto-phosphorylated as a result of the phosphorylation at Ser360 and Thr581 by either ERK1/2 or p38 MAPK activation [64,66,67]. However, little is known about the role of MSK1 in the signaling pathways leading to melanogenesis in human melanocytes. Western blotting analysis of MSK1 activation revealed that the phosphorylation of MSK1 at Thr581 and Ser376 was distinctly accentuated by UVB radiation, and could be significantly abrogated by the post-irradiation treatment with PBE. Since in this study ERK is not activated by UVB irradiation and PBE has no affect on ERK phosphorylation, the above findings strongly suggest that MSK1 is a signaling target of PBE, leading to the abrogation of CREB activation in UVB-exposed human melanocytes when treated post-irradiation. In this study, we also corroborated that the MSK1 inhibitor H89 significantly abrogates the increased gene
expression level of MITF and EDNRB even when treated post-irradiation. This strongly indicates that MSK1 inhibition leads to the attenuated expression of MITF and EDNRB. Although the abrogated expression of MITF and EDNRB may also be attributable to the inhibition of cAMP-dependent PKA by H89, this possibility can be ruled out by the fact that the activation of CREB in UVB-exposed human melanocytes is mediated predominantly via the activation of p38 but not the cyclic AMP/PKA pathway [7], an indication that H89 treatment could not abrogate the UVB-stimulated expression of MITF and EDNRB via an interruption of the cAMP/PKA pathway. This is the first report showing that the MSK1 activation is essentially involved in the CREB activation in UVB-exposed human melanocytes and an antioxidant can directly interrupt UVB-induced MSK1 activation, which leads to the abrogation of UVB-induced up-regulation of melanocyte-specific proteins such as EDNRB.

In conclusion, as shown in Fig 9, our findings indicate for the first time that the increased expression of MITF leading to the up-regulation of the melanocyte-specific protein EDNRB in UVB-exposed human melanocytes is mediated via the activation of the p38/MSK1/CREB pathway but not of the ERK/RSK/CREB pathway. The mode of action by PBE demonstrates that the interruption of MSK1 activation is a new target for antioxidants including PBE which can
serve as anti-pigmenting agents in UVB-melanosis. This study provides a deep insight into understanding of signaling mechanisms involved in UVB-accentuated expression of melanocyte-specific proteins as well as the regulatory role of redox-sensitive MSK1 in the UVB-activated melanogenic signaling pathway in human melanocytes.

Author Contributions
Conceived and designed the experiments: HT GI. Performed the experiments: HT AM SK MT. Analyzed the data: HT AM SK. Contributed reagents/materials/analysis tools: KY KT. Wrote the paper: HT GI.

References
1. Imokawa G, Yada Y, Miyagishi M. Endothelins secreted from human keratinocytes are intrinsic mitogens for human melanocytes. J Biol Chem. 1992; 267: 24675–24680. PMID:1280264
2. Tada A, Suzuki I, Im S, Davis MB, Cornelius J, Babcock G, et al. Endothelin-1 is a paracrine growth factor that modulates melanogenesis of human melanocytes and participates in their responses to ultraviolet radiation. Cell Growth Differ. 1998; 9: 575–584. PMID:9690625
3. Yohn JJ, Morelli JG, Walck SJ, Rundell KB, Norris DA, Zamora MR. Cultured human keratinocytes synthesize and secrete endothelin-1. J Invest Dermatol. 1993; 100: 23–26. PMID:8423387
4. Yada Y, Higuchi K, Imokawa G. Effects of endothelins on signal transduction and proliferation in human melanocytes. J Biol Chem (1991) 266: 18352–18357. PMID:1917960
5. Imokawa G, Yada Y, Kimura M. Signaling mechanisms of endothelin-induced mitogenesis and melanogenesis in human melanocytes. Biochem J. 1996; 314: 305–312. PMID:8660299
6. Tada A, Pereira E, Beitner-Johnson D, Kavanagh R, Abdel-Malek ZA. Mitogen- and ultraviolet-B-induced signaling pathways in normal human melanocytes. J Invest Dermatol. 2002; 118: 316–322. PMID:11841550
7. Mizutani Y, Hayashi N, Kawashima M, Imokawa GA. single UVB exposure increases the expression of functional KIT in human melanocytes by up-regulating MITF expression through the phosphorylation of p38/CREB. Arch Dermatol Res. 2010; 302: 283–294. doi: 10.1007/s00403-009-1007-x PMID:19937254
8. Hou L, Panthier JJ, Armheiter H. Signalizing and transcriptional regulation in the neural crest-derived melanocyte lineage: interactions between KIT and MITF. Development. 2000; 127: 5379–5389. PMID:11076759
9. Hou L, Armheiter H, Pavan WJ. Interspecies difference in the regulation of melanocyte development by SOX10 and MITF. Proc Natl Acad Sci U S A. 2006; 103: 9081–9085. PMID:16757562
10. Yang R, Zheng Y, Li L, Liu S, Burrows M, Wei Z, et al. Direct conversion of mouse and human fibroblasts to functional melanocytes by defined factors. Nat Commun. 2014; 5: 5807. doi: 10.1038/ncomms6807 PMID:25510211
11. Carreira S, Liu B, Goding CR. The gene encoding the T-box factor Tbx2 is a target for the microphthalmia-associated transcription factor in melanocytes. J Biol Chem. 2000; 275: 21920–21927. PMID:10709222
12. Vance KW, Carreira S, Brosch G, Goding CR. Tbx2 is overexpressed and plays an important role in maintaining proliferation and suppression of senescence in Cancer. Res. 2005; 65: 2260–2268. PMID:15781639
13. Du J, Widlund HR, Horstmann MA, Ramaswamy S, Ross K, Huber WE, et al. Critical role of CDK2 for melanoma growth linked to its melanocyte-specific transcriptional regulation by MITF. Cancer Cell. 2004; 6: 565–576. PMID:15607961
14. Carreira S, Goodall J, Aksan I, La Rocca SA, Galibert MD, Denat L, et al. Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. Nature. 2005; 433: 764–769. PMID:15716956
15. McGill GG, Horstmann M, Widlund HR, Du J, Motyckaova G, Nishimura EK, et al. Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. Cell. 2002; 109: 707–718. PMID:12086670
16. Hornyak TJ, Jiang S, Guzmán EA, Scissors BN, Tuchinda C, He H, et al. Mitf dosage as a primary determinant of melanocyte survival after ultraviolet irradiation. Pigment Cell Melanoma Res. 2009; 22: 307–318. doi: 10.1111/j.1755-148X.2009.00551.x PMID:19192212

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PLOS ONE | DOI:10.1371/journal.pone.0128678 June 1, 2015 14 / 17
17. Ganss R, Schütz G, Beermann F. The mouse tyrosinase gene. Promoter modulation by positive and negative regulatory elements. J Biol Chem. 1994; 269: 29806–29816. PMID: 7961973

18. Yasumoto K, Yokoyama K, Shibata K, Tomita Y, Shibahara S. Microphthalmia-associated transcription factor as a regulator for melanocyte-specific transcription of the human tyrosinase gene. Mol Cell Biol. 1994; 14: 8058–8070. PMID: 7969144

19. Vachterenheim J, Borovansky J. "Transcription physiology" of pigment formation in melanocytes: central role of MITF. Exp Dermatol. 2010; 19: 617–627. doi: 10.1111/j.1600-0625.2009.01053.x PMID: 20201954

20. Imokawa G, Miyagishi M, Yada Y. Endothelin-1 as a new melanogen: coordinated expression of its gene and the tyrosinase gene in UVB-exposed human epidermis. J Invest Dermatol. 1995; 105: 32–37. PMID: 7615973

21. Imokawa G, Kobayashi T, Miyagishi M, Higashi K, Yada Y. The role of endothelin-1 in epidermal hyperpigmentation and signaling mechanisms of mitogenesis and melanogenesis. J Cell Physiol. 1997; 10: 218–228. PMID: 9263329

22. Hyter S, Coleman DJ, Ganguli-Indra G, Merril GF, Ma S, Yanagisawa M, et al. Endothelin-1 is a transcriptional target of p63 in epidermal keratinocytes and regulates ultraviolet-induced melanocyte homeostasis. Pigment Cell Melanoma Res. 2013; 26: 247–258. doi: 10.1111/pcmr.12063 PMID: 23279852

23. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature. 1988; 332: 411–415. PMID: 2451132

24. Hachiya A, Kobayashi T, Takeya Y, Imokawa G. Biochemical characterization of endothelin-converting enzyme-1alpha in cultured skin-derived cells and its postulated role in the stimulation of melanogenesis in human epidermis. J Biol Chem. 2002; 277: 5395–5403. doi: 10.1074/jbc.M105874200 PMID: 11723113

25. Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, et al. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature. 1990; 348: 732–735. PMID: 2175397

26. Read AP, Newton VE. Waardenburg syndrome. J Med Genet. 1997; 34: 656–665. PMID: 9279758

27. Brenner M, Coelho SG, Beer JZ, Miller SA, Wolber R, Smuda C, et al. Long-lasting molecular changes in human skin after repetitive in situ UV irradiation. J Invest Dermatol. 2009; 129: 1002–1011. doi: 10.1038/jid.2008.325 PMID: 18946495

28. Choi W, Miyamura Y, Wolber R, Smuda C, Reinhold W, Liu H, et al. Regulation of human skin pigmentation in situ by repetitive UV exposure: molecular characterization of responses to UVA and/or UVB. J Invest Dermatol. 2010; 130: 1685–1696. doi: 10.1038/jid.2010.5 PMID: 20147966

29. Hachiya A, Kobayashi A, Yoshida Y, Kitahara T, Takeya Y, Imokawa G. Biphasic expression of two paracrine melanogenic cytokines, stem cell factor and endothelin-1, in ultraviolet B-induced human melanogenesis. Am J Pathol. 2004; 165: 2099–2109. PMID: 15579452

30. Kadono S, Manaka I, Kawashima M, Kobayashi T, Imokawa G. The role of the epidermal endothelin cascade in the hyperpigmentation mechanism of lentigo senilis. J Invest Dermatol. 2001; 116: 571–577. PMID: 11286625

31. Sato-Jin K, Nishimura EK, Akasaka E, Huber W, Nakano H, Miller A, et al. Epistatic connections between microphthalmia-associated transcription factor and endothelin signaling in Waardenburg syndrome and other pigmentary disorders. FASEB J. 2008; 22: 1155–1168. PMID: 18093992

32. Smit N, Vicanova J, Pavel S. The hunt for natural skin whitening agents. Int J Mol Sci. 2009; 10: 5326–5349. doi: 10.3390/ijms10125326 PMID: 20054473

33. Li EPH, Hyun JM, Belk RW, Kimura J, Bahl S. Skin lightening and beauty in four Asian cultures. Adv Consum Res. 2008; 35: 444–449.

34. Shimada T, Tokuhara D, Tsubata M, Kamiya T, Kamiya-Sameshima M, Nagamine R, et al. Flavanogenol (pine bark extract) and its major component procyanidin B1 enhance fatty acid oxidation in fat-loaded models. Eur J Pharmacol. 2012; 677: 147–153. doi: 10.1016/j.ejphar.2011.12.034 PMID: 22227333

35. Ohkita M, Kiso Y, Matsumura Y. Pharmacology in health foods: improvement of vascular endothelial function by French maritime pine bark extract (Flavangenol). J Pharmacol Sci. 2011; 115: 461–465. PMID: 21436602

36. Yoshida A, Yoshino F, Tsubata M, Ikeguchi M, Nakamura T, Lee MC. Direct assessment by electron spin resonance spectroscopy of the antioxidant effects of French maritime pine bark extract in the maxillofacial region of hairless mice. J Clin Biochem Nutr. 2011; 49: 79–86. doi: 10.3164/jcbn.10-103 PMID: 21980222

37. Rohdewald P. A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology. Int J Clin Pharmacol Ther. 2002; 40: 158–168. PMID: 11996210
38. Maimoona A, Naeem I, Saddique Z, Jameel K. A review on biological, nutraceutical and clinical aspects of French maritime pine bark extract. J Ethnopharmacol. 2011; 133: 261–277. doi:10.1016/j.jep.2010.10.041 PMID: 21044675

39. Packer L, Rimbach G, Virgili F. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (Pinus maritima) bark, pycnogenol. Free Radic Biol Med. 1999; 27: 704–724. PMID: 10490291

40. Nelson AB, Lau BH, Ide N, Rong Y. Pycnogenol inhibits macrophage oxidative burst, lipoprotein oxidation, and hydroxyl radical-induced DNA damage. Drug Dev Ind Pharm. 1998; 24: 139–144. PMID: 15605443

41. Kim YJ, Kang KS, Yokozawa T. The anti-melanogenic effect of pycnogenol by its anti-oxidative actions. Food Chem Toxicol. 2008; 46: 2466–2471. doi: 10.1016/j.fct.2008.04.002 PMID: 18482785

42. Saliou C, Rimbach G, Moini H, McLaughlin L, Hosseini S, Lee J, et al. Solar ultraviolet-induced erythema in human skin and nuclear factor-kappa-B-dependent gene expression in keratinocytes are modulated by a French maritime pine bark extract. Free Radic Biol Med. 2001; 30: 154–160. PMID: 11163532

43. Furumura M, Sato N, Kusaba N, Takagaki K, Nakayama J. Oral administration of French maritime pine bark extract (Flavangenol) improves clinical symptoms in photoaged facial skin. Clin Interv Aging. 2012; 7: 275–286. doi: 10.2147/CIA.S33165 PMID: 22956863

44. Terazawa S, Nakajima H, Shingo M, Niwano T, Imokawa G. Astaxanthin attenuates the UVB-induced secretion of prostaglandin E2 and interleukin-8 in human keratinocytes by interrupting MSK1 phosphorylation in a ROS depletion-independent manner. Exp Dermatol. 2012; 21 Suppl 1: 11–17. doi: 10.1111/j.1600-0625.2012.01496.x PMID: 22626465

45. Zhu W, Gao J. The use of botanical extracts as topical skin-lightening agents for the improvement of skin pigmentation disorders J Invest Dermatol Symp Proc. 2008; 13: 20–24.

46. Smit N, Vicanova J, Pavel S. The hunt for natural skin whitening agents. Int J Mol Sci. 2009; 10: 5326–5349. doi: 10.3390/ijms10125326 PMID: 20054473

47. Chida M, Suzuki K, Nakanishi-Ueda T, Ueda T, Yasuhara H, Koide R, et al. In vitro testing of antioxidants and biochemical end-points in bovine retinal tissue. Ophthalmic Res. 1999; 31: 407–415. PMID: 10474069

48. Redmond RW, Kochevar IE. Spatially resolved cellular responses to singlet oxygen. Photochem Photobiol. 2006; 82: 1178–1186. PMID: 16740059

49. Muthusamy V, Piva TJ. UVB-stimulated TNFα release from human melanocyte and melanoma cells is mediated by p38 MAPK. Int J Mol Sci. 2013; 14: 17029–17054. doi: 10.3390/ijms140817029 PMID: 23965971

50. Abdel-Malek ZA, Kadekaro AL, Swope VB. Stepping up melanocytes to the challenge of UV exposure. Pigment Cell Melanoma Res. 2010; 23: 171–186. doi: 10.1111/j.1755-148X.2010.00679.x PMID: 20128873

51. Liu J, Yang D, Minemoto Y, Leitges M, Rosner MR, Lin A. NF-κB is required for UV induced JNK activation via induction of PKCd. Mol Cell. 2006; 21: 467–480. PMID: 16483929

52. Yanase H, Ando H, Horikawa M, Watanabe M, Mori T, Matsuda N. Possible involvement of ERK1/2 in UV-A-induced melanogenesis in cultured normal human epidermal melanocytes. Pigment Cell Res. 2001; 14: 103–109. PMID: 11310789

53. Tada A, Pereira E, Beilner-Johnson D, Kavanagh R, Abdel-Malek ZA. Mitogen- and ultraviolet-B-induced signaling pathways in normal human melanocytes. J Invest Dermatol. 2002; 118: 316–322. PMID: 11841550

54. Matsuzawa A, Ichijo H, Redox control of cell fate by MAP kinase: physiological roles of ASK1–MAP kinase pathway in stress signaling. Biochim Biophys Acta. 2008; 1780, 463–473. doi: 10.1016/j.bbamcr.2007.12.011 PMID: 18206122

55. Lee SJ, Bai SK, Lee KS, Namkoong S, Na HJ, Ha KS, et al. Astaxanthin inhibits nitric oxide production and inflammatory gene expression by suppressing I(kappa)B-dependent NF-kappaB activation. Mol Cell. 2003; 16, 97–105.

56. Suzuki Y, Ohgami K, Shiratori K, Jin X, Ilieva I, Koyama Y, et al. Suppressive effects of astaxanthin against rat endotoxin-induced uveitis by inhibiting the NF-kappaB signaling pathway. Exp Eye Res. 2006; 82, 275–281. PMID: 16126197

57. Hur S, Lee YS, Yoo H, Yang JH, Kim TY. Homoisoflavanone inhibits UVB-induced skin inflammation through reduced cyclooxygenase-2 expression and NF-kappaB nuclear localization. J Dermatol Sci. 2010; 59: 163–169. doi: 10.1016/j.jdermsci.2010.07.001 PMID: 20724116
58. Afaq F, Adhami VM, Ahmad N, Mukhtar H. Inhibition of ultraviolet B-mediated activation of nuclear factor κB in normal human epidermal keratinocytes by green tea Constituent (-)-epigallocatechin-3-gallate. Oncogene. 2003; 22: 1035–1044. PMID: 12592390

59. Vicentini FT, He T, Shao Y, Fonseca MJ, Verri WA Jr, Fisher GJ, et al. Quercetin inhibits UV irradiation-induced inflammatory cytokine production in primary human keratinocytes by suppressing NF-κB pathway. J Dermatol Sci. 2011; 61: 162–168. doi: 10.1016/j.jdermsci.2011.01.002 PMID: 21282043

60. Adhami VM, Afaq F, Ahmad N. Suppression of Ultraviolet B exposure-mediated activation of NF-KB in normal human keratinocytes by resveratrol. Neoplasia 5: 74–82. PMID: 12659672

61. Wan P, Hu Y, He L. Regulation of melanocyte pivotal transcription factor MITF by some other transcription factors. Mol Cell Biochem. 2011; 354: 241–246. doi: 10.1007/s11010-011-0823-4 PMID: 21519923

62. Potterf SB, Furumura M, Dunn KJ, Arnheiter H, Pavan WJ. Transcription factor hierarchy in Waardenburg syndrome: regulation of MITF expression by SOX10 and PAX3. Hum Genet. 2000; 107: 1–6. PMID: 10982026

63. Kim DS, Kim SY, Lee JE, Kwon SB, Joo YH, Youn SW, et al. Sphingosine-1-phosphate-induced ERK activation protects human melanocytes from UVB-induced apoptosis. Arch Pharm Res. 2003; 26:739–746. PMID: 14560924

64. Deak M, Clifton AD, Lucocq LM, Alessi DR. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. EMBO J. 1998; 17: 4426–4441. PMID: 9687510

65. Wiggin GR, Soloaga A, Foster JM, Murray-Tait V, Cohen P, Arthur JS. MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF1 in fibroblasts. Mol Cell Biol. 2002; 22: 2871–2881. PMID: 11909979

66. McCoy CE, Campbell DG, Deak M, Bloomberg GB, Arthur JSE. MSK1 activity is controlled by multiple phosphorylation sites. Biochem J. 2005; 387: 507–517. PMID: 15568999

67. McCoy CE, Macdonald A, Morrice NA, Campbell DG, Deak M, Toth R, et al. Identification of novel phosphorylation sites in MSK1 by precursor on scanning MS. Biochem J. 2007; 402: 491–501. PMID: 17117922

68. Cherestensen CA, Sturgill TW. Characterization of the p90 ribosomal S6 kinase carboxyl-terminal domain as a protein kinase. J Biol Chem. 2002; 277: 27733–27741. PMID: 12016217