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Research paper

Photoprotection by dietary phenolics against melanogenesis induced by UVA through Nrf2-dependent antioxidant responses

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ABSTRACT

Dietary phenolics may play a protective role in UV-mediated skin pigmentation through their antioxidant and UV-absorbing actions. In this study, we investigated whether genetic silencing of Nrf2, regulating the transcription of antioxidant genes, affected melanogenesis in primary human epidermal melanocytes (HEMn) and B16F10 melanoma cells subjected to UVA (8 J/cm^2) exposure. Then, we explored the anti-melanogenic actions of phenolics; caffeic acid (CA) and ferulic acid (FA) providing partial UVA protection; quercetin (QU) and rutin (RU) providing strong UVA protection and; avobenzone (AV), an efficient UVA filter, in association with modulation of Nrf2-mediated antioxidant defenses in response to UVA insults in B16F10 cells. Upon oxidative insults, Nrf2 silencing promoted melanogenesis in both HEMn and B16F10 cells irradiated with UVA. Stimulation of melanogenesis by UVA correlated with increased ROS and oxidative DNA damage (8-OHdG), GSH depletion as well as a transient downregulation of Nrf2 nuclear translocation and of Nrf2-ARE signaling in B16F10 cells. All test compounds exerted anti-melanogenic effects with respect to their abilities to reverse UVA-mediated oxidative damage as well as downregulation of Nrf2 activity and its target antioxidants (GCLC, GST and NQO1) in B16F10 cells. In conclusion, defective Nrf2 may promote melanogenesis under UVA irradiation through oxidative stress mechanisms. Compounds with antioxidant and/or UVA absorption properties could protect against UVA-induced melanogenesis through indirect regulatory effect on Nrf2-ARE pathway.

1. Background

Oxidative stress induced by ultraviolet A (UVA) radiation has been recognized to play a crucial role in physiological and biological stress responses including dysregulation of melanogenesis in melanocytes and/or melanoma cells [1,2]. Whereas melanin production primarily regulated by tyrosinase plays a beneficial role in protecting the skin against damaging effects of UV radiation, excessive formation of melanin could be harmful, in particular following UV exposure [3,4].

UVA exposure has been demonstrated to play a crucial role in increased melanogenesis partly through induction of oxidative stress and impairment of antioxidant defense in melanocytes and/or melanoma cells [5,6], improvement of antioxidant defense system to cope with the overwhelmed oxidative stress could thus be one of effective and safe approaches to inhibit melanogenesis and photodamaged skin. Nuclear factor E2-related factor 2 (Nrf2), an important transcription factor controlling the antioxidant response in various tissues including the skin, has been reported to play a beneficial role in cellular function and integrity by protecting skin cells including melanocytes against oxidative insults particularly from UV exposure [7–12]. Attempts have thus been made to develop effective photoprotective agents targeting Nrf2.

Diet- and plant-derived phytochemicals have been proposed as good candidates for effective and safe photoprotective agents possibly due to their antioxidant and UV-absorbing properties [13,14]. Phytochemicals having antioxidant properties including caffeic acid (CA), ferulic acid (FA), quercetin (QU) and rutin (RU) found abundantly in plant-based diets and beverages as well as sunscreen agents have been reported to exert photoprotective and

Abbreviations: ARE, antioxidant response element; AV, avobenzone; CA, caffeic acid; CDNB, 1-chloro-2,4-dinitrobenzene; DCPIP, 2,6-dichloroindophenol; DMEM, dulbecco's modified Eagle medium; DPBS, dulbecco's phosphate buffered saline; DTNB, (5,5′-dithio-bis-2-(nitrobenzoic acid); FA, ferulic acid; γ-GCL, γ-glutamate cysteine ligase; γ-GCLC, γ-glutamate cysteine ligase catalytic subunit; γ-GCLM, γ-glutamate cysteine ligase modifier subunit; GSH, glutathione; GSSG, glutathione reductase; GST, glutathione S-transferase; H2DCFDA, non-fluorescent dichlorofluorescein; HEMn, primary human epidermal melanocytes; NQO1, NAD(P)H quinone oxidoreductase1; Nrf2, nuclear factor E2-related factor 2; 8-OHdG, 8-hydroxy-2'–deoxyguanosine; QU, quercetin; RNAi, RNA interference; ROS, reactive oxygen species; RU, rutin; siCtrl, non-silencing siRNA controls; siNrf2, siRNA against Nrf2; siRNA, small-interfering RNA; UVA, ultraviolet A

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depigmenting actions [15–21]. In this study, we therefore aimed to investigate antimelanogenic effects of compounds with different antioxidant and UVA blocking properties in correlation to UVA-mediated modulation of Nrf2-ARE signaling pathway and its downstream antioxidants including γ-glutamate cysteine ligase (γ-GCL), the rate-limiting enzyme for GSH synthesis, glutathione S-transferase (GST) and NAD(P)H quinone oxidoreductase 1 (NQO1). At first, we examined whether depletion of Nrf2 using small-interfering RNA-mediated silencing of Nrf2 affected UVA-induced melanogenesis in primary human epidermal melanocytes (HEMn) and B16F10 melanoma cells. In addition, UVA irradiation was suggested to induce photodamaged skin through activation of MAPK signaling in association with oxidative stress responses in various types of skin cells [22,23]. Thus, the role of MAPK signaling as upstream mediators that could regulate Nrf2 nuclear translocation in response to UVA irradiation was also evaluated in this study. Then, we explored the underlying mechanisms of dietary phenolics; CA and FA having ability to partially (approximately 30-50%) absorb UVA ray [21] (Supplementary Table 1); QU and RU having strong UVA absorption properties as well as; AV, an efficient UVA filter which does not possess antioxidant activity, in protecting B16F10 cells against UVA-induced melanogenesis in association with inhibition of oxidative stress and oxidative DNA damage (8-hydroxy-2'–deoxyguanosine; 8-OHdG) through modulation of Nrf2-ARE signaling and its downstream antioxidants.

2. Materials and methods

2.1. Cell cultures and treatment

Primary human epidermal melanocytes (HEMn) (Lonza, Basel, Switzerland) were grown in Medium 254 (#M-254-500) supplemented with human melanocyte growth supplement (HMGS) according to the manufacturer's instructions. B16F10 mouse melanoma cells (ATCC, Rockville, Md, USA), a gift from Assoc. Prof. Wajjwalku, Faculty of Veterinary Medicine, Kasetsart University, were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (100 units/ml)/streptomycin (100 μg/ml). All cells were maintained at 37 °C in a humidified air of 5% CO_2 (P_{CO2}=40 Torr) (a Forma Scientific CO_2 Water Jacketed Incubator).

To test whether cellular oxidative stress modulate melanogenesis, B16F10 cells were treated with H_2O_2 (up to 500 μM) without UVA irradiation for 30 min and with L-buthionine-(S,R)-sulfoximine; BSO (up to 500 μM), an inhibitor of gamma-glutamylcysteine synthetase, the first enzyme involved in GSH synthesis, for 24 h prior to UVA irradiation. To evalulate the effects of phenolics on melanogenesis induced by UVA through Nrf2-dependent antioxidant responses, cells were treated with test compounds (up to 30 μM) in Dulbecco's phosphate buffered saline (DPBS) for 30 min before exposure to a single dose of UVA radiation (8 J/cm^2) and, to achieve a UVA dose required, the UV intensity was evaluated as previously described [2,21]. The dose of UVA and concentrations of phenolics employed in this study were non-cytotoxic to both HEMn and B16F10 cells. To demonstrate an involvement of MAPK pathway in Nrf2 nuclear translocation, B16F10 cells were pretreated with 1 μM of specific ERK inhibitor (U0126), JNK inhibitor (SP600125) and p38 inhibitor (SB203580) in serum-free medium for 1 h prior to UVA (8 J/cm^2) irradiation and then harvested at 1 h after irradiation for western blot analysis of nuclear/cytosolic Nrf2 ratio. After UVA irradiation, cells were washed, further incubated in serum-free medium and harvested at different time points as indicated in Results. The UVA source was a xenon arc lamp (Dermalight ultrA1; Hoenle, Martinsried, Germany).

For preparation of cell lysate, cells were harvested and resuspended in lysis buffer consisted of 50 mM Tris-HCl, 10 mM ethylene diaminetetraacetic acid (EDTA), 1% (*v*/*v*) Triton X-100, phenylmethylsulfonyl fluoride (PMSF) (100 mg/ml) and pepstatin A (1 mg/ml) in DMSO and leupeptin (1 mg/ml) in H_2O, pH 6.8. The lysed cells were centrifuged at 10,000 rpm for 10 min at 4 °C and the total lysates were collected and either assayed immediately or stored frozen at −80 °C.

2.2. Silencing of Nrf2 via RNA interference (RNAi)

A combination of four gene-specific small-interfering RNA (siRNA) against human Nrf2 (NM_006164) was used (FlexiTube GeneSolution GS4780 for NFE2L2, Qiagen; Cat.#:1027416). HEMn and B16F10 cells were transfected with 5 nM siRNA against Nrf2 (siNrf2) or equal molar non-silencing siRNA controls (siCtrl, Qiagen; Cat.#:1022076) for 48 h. These siRNAs were earlier complexed with liposome carrier (HiPerFect Transfection Reagent, Qiagen; Cat.#: 301705) at 0.08 μL/ng siRNA concentration by incubating mixture for 5–10 min at room temperature in serum-free culture medium. At 48 h post-transfection, cells were then washed with DPBS and subjected to UVA irradiation, following which melanin content, tyrosinase activity and protein were determined. Cells appeared normal morphologically and did not differ from untransfected cells in cell viability. At 48 h post-transfection, all siRNAs were verified to ensure achieving functional and specific silencing by evaluating mRNA and protein levels of Nrf2 and known Nrf2 target genes including GCLC, GCLM, GST and NQO1 before employment in all experiments. To evaluate melanogenic response of Nrf2-depleted cells to UVA irradiation, HEMn and B16F10 cells transfected with Nrf2-siRNA or non-silencing negative control siRNA (siCtrl) were irradiated with 8 J/cm^2 of UVA and harvested at 1 h post-irradiation for determination of melanin content and tyrosinase activity and at 24 h post-irradiation for tyrosinase protein expression.

2.3. Melanin content assay

An evaluation of melanin production was performed as described previously [19]. Cells were harvested at 1 h after UV radiation (8 J/cm^2) and the cell pellets were solubilized in 1 N NaOH for 1 h to dissolve melanin, which was then measusred spectrophotometrically at 475 nm. The melanin content (μg/mg protein) was calculated by comparison to a standard curve derived using synthetic melanin.

2.4. Tyrosinase activity assay

The rate of L-DOPA oxidation was measured to assess cellular tyrosinase activity at 1 h following exposure to a UVA dose of 8 J/cm^2. The assay was performed as previously described by Shin et al. [12]. Briefly, 20 mM L-DOPA used as the substrates was added to each lysate in a 96-well plate and absorbance of dopachrome formation was measured spectrophotometrically at 475 nm every 10 min for 1 h at 37 °C by a spectrophotometer. The tyrosinase activity (unit/mg protein) was calculated by comparison to a standard curve using tyrosinase (2034 U/mg).

2.5. Measurement of intracellular glutathione content

GSH level was spectrophotometrically measured using glutathione reductase (GR): (5,5′-dithio-bis-2-(nitrobenzoic acid) (DTNB) enzymatic recycling method following the kit protocol from Sigma-Aldrich (MO, US). The assay is based on conversion of glutathione disulfide (GSSG) to GSH by GR in the presence of NADPH and GSH oxidation by the sulfhydryl reagent DTNB to