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Pigmentation effects of solar simulated radiation as compared with UVA and UVB radiation

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Summary

Different wavelengths of ultraviolet (UV) radiation elicit different responses in the skin. UVA induces immediate tanning and persistent pigment darkening through oxidation of pre-existing melanin or melanogenic precursors, while UVB induces delayed tanning which takes several days or longer to develop and requires activation of melanocytes. We compared the effects of a two-week repetitive exposure of human skin to solar-simulated radiation (SSR), UVA or UVB at doses eliciting comparable levels of visible tanning and measured levels of melanins and melanin-related metabolites. Levels of eumelanin and pheomelanin were significantly higher in the order of SSR, UVB, UVA or unexposed control skin. Levels of free 5-*S*-cysteinyldopa (5SCD) were elevated about four-fold in SSR- or UVB-exposed skin compared with UVA-exposed or control skin. Levels of protein-bound form of 5SCD tended to be higher in SSR- or UVB-exposed skin than in UVA-exposed or control skin. Total levels of 5-hydroxy-6-methoxyindole-2-carboxylic acid (5H6MI2C) and 6H5MI2C were higher in SSR- than in UVB-exposed or control skin. These results show that SSR is more effective in promoting delayed tanning than UVB radiation alone, suggesting a synergistic effect of UVA radiation. Furthermore, free 5SCD may serve as a good marker of the effect of SSR and UVB.

Keywords

ultraviolet; solar radiation; melanin; eumelanin; pheomelanin; 5-*S*-cysteinyldopa; 5(6)-hydroxy-6 (5)-methoxyindole-2-carboxylic acid

Introduction

Ultraviolet (UV) radiation is one of the strongest stimuli that induce pigmentation in human skin (Miyamura et al., 2007). Exposure to UV radiation causes DNA damage, and thus the accumulation of melanin can be seen as an adaptive photoprotective response of melanocytes to prevent further DNA damage (de Winter et al., 2001). The UV portion of sunlight can be further split into UVA (320 nm-400 nm), UVB (280-320 nm) and UVC (<280 nm). Although both UVB and UVA lead to skin tanning following sun-exposure, they induce different pigmentary responses that are mainly defined by the time kinetics, *i.e.*, immediate pigment darkening and persistent pigment darkening as typical reactions to UVA as well as delayed tanning known to be induced by UVB in sunlight (Honingsmann, 2002).

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Our previous studies have clarified that delayed tanning is accompanied by an increase in melanin content (Miyamura et al., 2007; Tadokoro et al., 2003). However, the increase is only slight, less than two-fold (Hennessy et al., 2005; Tadokoro et al., 2003), and the observed several-fold increase in visible pigmentation is caused by additional contribution from changes in the distribution and particle size of melanosomes (Alauf et al., 2002a,b; Tadokoro et al., 2005).

We have also shown that serum levels of free 5-*S*-cysteinyldopa (5SCD), a pheomelanin precursor, increased shortly after UV radiation in human subjects while levels of free 6-hydroxy-5-methoxyindole-2-carboxylic acid (6H5MI2C), an O-methyl metabolite of the eumelanin precursor 5,6-dihydroxyindole-2-carboxylic acid (DHICA), increased later, suggesting that serum levels of 5SCD and 6H5MI2C may reflect the degrees of skin injury and pigmentation in the skin, respectively (Wakamatsu and Ito, 2006). In the skin, an isomer of 6H5MI2C, 5-hydroxy-6-methoxyindole-2-carboxylic acid (5H6MI2C), was found to be present in comparable amounts (Maeda and Hatao, 2004).

In the present study we characterized the wavelength-dependent differences of pigmentation in suction blisters of human epidermis after repeated *in vivo* irradiation over a two week period. Effects of solar simulated radiation (SSR) on the degree of pigmentation were compared with those elicited by UVB or by UVA. As indicators of pigmentation, we analyzed eumelanin, pheomelanin, DOPA, 5SCD, 5H6MI2C and 6H5MI2C. We also analyzed protein-bound forms of DOPA and 5SCD (Ito et al., 1983; Sutherland et al., 2003) to evaluate their significance as pigmentation markers. In contrast to many earlier studies investigating UV-induced pigmentation, we chose a repeated irradiation protocol to more closely simulate the outdoor tanning situation, while avoiding any erythema reactions.

Results

Changes in levels of eumelanin and pheomelanin after UV radiation

Exposure to suberythemal doses of UV irradiation for two weeks caused a significant increase in levels of eumelanin by 52% or 32% over the control in the SSR- or UVB-treated groups, respectively, while UVA had no effect (Figure 1A). Similarly, levels of pheomelanin were increased by 73% or 23% after SSR and UVB, respectively, while no increase was observed in the UVA-treated group (Figure 1B). Interestingly, levels of both eumelanin and pheomelanin were significantly increased by 15% and 41%, respectively, in the SSR-treated group compared with the UVB-treated group.

Changes in levels of free and protein-bound DOPA and 5SCD and 5H6MI2C+6H5MI2C after UV radiation

Levels of free DOPA showed a significant 33% increase in the SSR-treated group compared with the control, but values in the four groups were rather variable (Figure 1C). On the other hand, levels of free 5SCD were significantly elevated 4.1-fold or 3.7-fold in the SSR- or UVB-treated groups, respectively, while UVA had little effect (Figure 1D). Levels of protein-bound DOPA did not show any significant changes (Figure 1E). Levels of protein-bound 5SCD tended to be higher in the SSR- or UVB-treated groups (Figure 1F). Levels of combined amounts of indoles, 5H6MI2C and 6H5MI2C, showed a significant increase (95%) in the SSR-treated group, but values in the four groups were rather variable (Figure 1G).

Discussion

Exposure of human skin to UV radiation results in a broad spectrum of cellular reactions, such as erythema, DNA damage, apoptosis, and tanning. These several types of cutaneous responses

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depend on the wavelength of the radiation (Miyamura et al., 2007). UVA radiation causes immediate pigment darkening and persistent pigment darkening, both of which are thought to result from oxidation and/or polymerization of existing melanin or melanogenic precursors (Routaboul et al., 1999; Maeda and Hatao, 2004). Reactive oxygen radicals are considered to be responsible for these processes. On the other hand, UVB radiation causes the delayed tanning reaction that takes several days or longer to develop (Young, 2006; Miyamura et al., 2007). The main mechanism of skin tanning caused by UVB is considered to be the production of melanin as a result of the increased activity of tyrosinase, although other factors, such as the redistribution and the particle size of melanosomes are important as well (Alaluf et al., 2002a, b; Tadokoro et al., 2005). Studies on the effects of SSR on skin pigmentation have been rather scarce, however, especially with respect to comparing effects elicited by UVA and/or UVB.

In the present study, SSR was found to increase levels of both eumelanin and pheomelanin. The increase was significant, but less than two-fold as has been observed in other studies using UVB (Tadokoro et al., 2003; Hennessy et al., 2005). Eumelanin accounted for more than 90% of total melanin both in control and in UV-irradiated skin, which is consistent with a previous study (Hennessy et al., 2005). Similar, but less pronounced increases in levels of eumelanin and pheomelanin were seen with UVB while UVA had no effect. Interestingly, SSR was significantly more effective in stimulating the production of both eumelanin and pheomelanin than was UVB. This suggests that UVB works synergistically with UVA to increase melanin production under our experimental protocol, *i.e.,* two-weeks of exposure at suberythemal doses. Thus, pigmentation in the skin appears to be a very complex process with a possible combination of the production of new melanin due to UVB and the oxidation of melanin precursors by UVA. It is remarkable that pheomelanin was increased by 73% after SSR exposure. A similar increase in pheomelanin level of the human epidermis following PUVA therapy was first reported by Thody et al. (1991).

The four-fold increase in the level of free 5SCD after SSR or UVB exposure appears to reflect increases in tyrosinase activity. We have observed a strong increase in tyrosinase activity under similar conditions while little effect has been observed with UVA (Schlenz et al., 2005). This suggests the usefulness of free 5SCD as a marker of pigmentation in the skin. In fact, the production of 5SCD has been employed as an indicator of tyrosinase activity in vitro (Carstam et al., 1986). It should be noted that cysteinyldopas are the initial, stable products of melanogenesis regardless of whether melanocytes produce pheomelanin or eumelanin (Ito, 2003; Land and Riley, 2000). A similar, but much less pronounced increase of indoles (5H6MI2C + 6H5MI2C) was also observed after SSR exposure compared with control or UVB exposure. The production of these indoles in the epidermis has recently been reported (Maeda and Hatao, 2004).

The levels of protein-bound 5SCD tended to be higher in the SSR- or UVB-exposed groups compared with the control or UVA-exposed groups. We previously reported that tyrosinase is able to oxidize tyrosine residues in proteins to form DOPA and 5SCD residues (Ito et al., 1984). Thus, the increased production of protein-bound 5SCD may be related to the increase in tyrosinase activity in the SSR- or UVB-treated skin. Protein-bound 5SCD may also arise from reaction of cysteine residues in proteins with dopaquinone (Kato et al., 1986).

Finally, some implications of these findings to increased skin pigmentation need to be discussed. It is well known that serum levels of 5SCD rise in the summer, which may reflect skin injury rather than pigmentation (Wakamatsu and Ito, 2006). The present finding that levels of PB-5SCD and free 5SCD are in the same order suggests that PB-5SCD may not be a source of free 5SCD. Melanin precursors and metabolites, such as 5SCD, DHICA and 6H5MI2C, are considered to be photo-labile and play some roles in pigmentation (Maeda and Hatano, 2006;

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Schmitz et al., 1995). Even if this is the case, their roles may be marginal as levels of free 5SCD and indoles (5H6MI2C and 6H5MI2C) are less than 1 ng per blister roof, being less than two orders of magnitude lower than levels of eumelanin and pheomelanin.

Materials and methods

Volunteers

Five healthy volunteers (2 males and 3 females, aged between 32 and 63 years) with Fitzpatrick skin type II-III were included in this study. All gave written informed consent. The study was performed according to the guidelines of the International Conference on Harmonisation Good Clinical Practice and the ethical recommendations of the declaration of Helsinki. During the two week study period, the subjects did not take any drugs that might affect pigmentation, such as contraceptives (Dereure, 2001).

UV irradiation protocol and suction blister preparation

For UV irradiation, a solar simulator was used (Oriel solar simulator 1600W, Oriel Instruments, Stratford, CT, USA). For SSR, UV wavelengths below 290 nm were filtered out with an optical filter (WG320, Oriel Instruments). For UVA and UVB radiation, a “BC-Blocker” filter (cut-off: 320 nm, Oriel Instruments) and a custom-made filter combination (WG 320 + UG11 + bandpass 290-320, Tafelmayer, Rosenheim, Germany) were used, respectively. Visible light was minimized by a dichroit mirror. Doses were determined using an IL 1700 Research Radiometer (International Light, Newburyport, MA, USA).

Irradiations were given a total of ten times over two weeks on five consecutive days in the first and in the second weeks. The areas used for irradiation (control, UVA, UVB, SSR) were at the same relative location on the back of each subject and covered an area 2.5 cm in width and 7.5 cm in length each for the control and the irradiated areas. Before the start of the study, the minimal erythemal dose (MED) was determined for each volunteer using SSR, and is defined as the minimal dose yielding sharply demarked erythema after 20 hr. For SSR, irradiation doses of 0.4 MED in the first week and 0.5 MED in the second week were used (Schlenz et al., 2005; Miyamura et al., 2006). In order to obtain comparable visual tanning reactions for UVA and UVB radiation, adapted doses of UVA (2.3 times the UVA dose in the SSR irradiation) and UVB (1.1 times the UVB dose in the SSR irradiation) were used. These adapted doses of UVA and UVB were determined empirically. The skin pigmentation was scored with the L*a*b* system using a SpectroPen™ (Dr. Lange, Germany). The above doses of SSR, UVA and UVB gave comparable decreases in L-values (data not shown).

Suction blister samples (7 mm in diameter), five in each area, were taken 5 days after the last irradiation by initially applying a negative pressure of 180 mbar followed by 320 mbar after 30 min to the respective areas. Blister roofs were removed with a sterilized skin surgery set and were cryoconserved for later analysis. One blister roof from each area was used for the determination of melanins, one for the determination of free and protein-bound catechols, and one for the determination of indoles. The remaining two blister roofs from each area were saved for other analyses (unpublished).

Concentrations of melanin and melanin-related metabolites were calculated on the basis of suction blister roof (ng or nmol per one suction blister). Each suction blister had an area of 38 mm^2 and weighed 1.56 mg on the average. We adopted this calculation to avoid the influence by the changed thickness of epidermis (Hennessy et al., 2005) and unavoidable errors in measuring mg quantity of wet samples. Weights of blister samples did not differ significantly among the four groups with averages ± SEM being control 1.53 ± 0.08 mg, UVA 1.55 ± 0.11 mg, UVB 1.54 ± 0.10 mg, and SSR 1.63 ± 0.09 mg for the 20 samples used for this study.

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Determination of eumelanin and pheomelanin

Determination of eumelanin and pheomelanin in suction blister samples were carried out as described previously (Ito and Wakamatsu, 1994; Wakamatsu et al., 2002). The two classes of melanin were quantified by chemical degradation of eumelanin by acidic permanganate oxidation to form pyrrole-2,3,5-tricarboxylic acid (PTCA) and hydriodic acid reductive hydrolysis of pheomelanin to form 4-amino-3-hydroxyphenylalanine (4-AHP). Each blister top was homogenized with 400 μL water and a 100 μL aliquot of each homogenate was subjected to the melanin assays, the eumelanin assay being performed in duplicate. Contents of these specific degradation products were determined using HPLC assays, and were converted to eumelanin contents by multiplying with factors of 160 and 9, respectively (Wakamatsu and Ito, 2002).

Determination of free DOPA and 5SCD and protein-bound DOPA and 5SCD

Determination of free DOPA and 5SCD in suction blister roofs was performed using HPLC with electrochemical detection, as previously reported for serum samples (Wakamatsu and Ito, 1994). Briefly, each blister roof was homogenized with 400 μL 0.4 M perchloric acid and a 100 μL aliquot was treated with alumina after dilution with 400 μL 1% EDTA.2Na.

Protein-bound DOPA and 5SCD were then analyzed by the method of Sutherland et al. (2003) with some modifications. The pellet remaining after the perchloric acid extraction (see above) was washed with 400 μL 0.4 M perchloric acid and was centrifuged, after which each pellet was washed with chloroform:methanol (1:1, v/v) by vortex-mixing for 1 min. The following steps were described in our recent paper (Murakami et al., 2007). In brief, protein-bound DOPA and 5SCD were hydrolyzed in 6 M HCl containing 5% thioglycolic acid and 1% phenol, and DOPA and 5SCD liberated were extracted by alumina adsorption at pH 8.6 and analyzed by HPLC.

Determination of 5H6MI2C and 6H5MI2C

Determination of 5H6MI2C and 6H5MI2C was performed using HPLC as previously reported (Wakamatsu et al., 1991), but using a fluorescence detector, JASCO FP-2020, instead of the originally described electrochemical detector. The excitation wavelength was 315 nm and the emission wavelength was 420 nm. Each blister roof was homogenized with 400 μL ice-cooled 0.4 M perchloric acid:methanol, 80:20 (v/v), and was ultrasonicated for 30 sec. An 80 μL aliquot of each extract was immediately injected into the HPLC system. Care was taken to process each sample quickly to minimize the decomposition of the indoles due to photo-induced oxidation (Maeda and Hatao, 2004).

Statistical analysis

Statistical testing was carried out using JMP 5.0 for Macintosh (SAS Institute, Tokyo, Japan, http://www.jmp.com/japan/corp/index.shtml). Differences were analysed for statistical significance using student's paired *t*-test.

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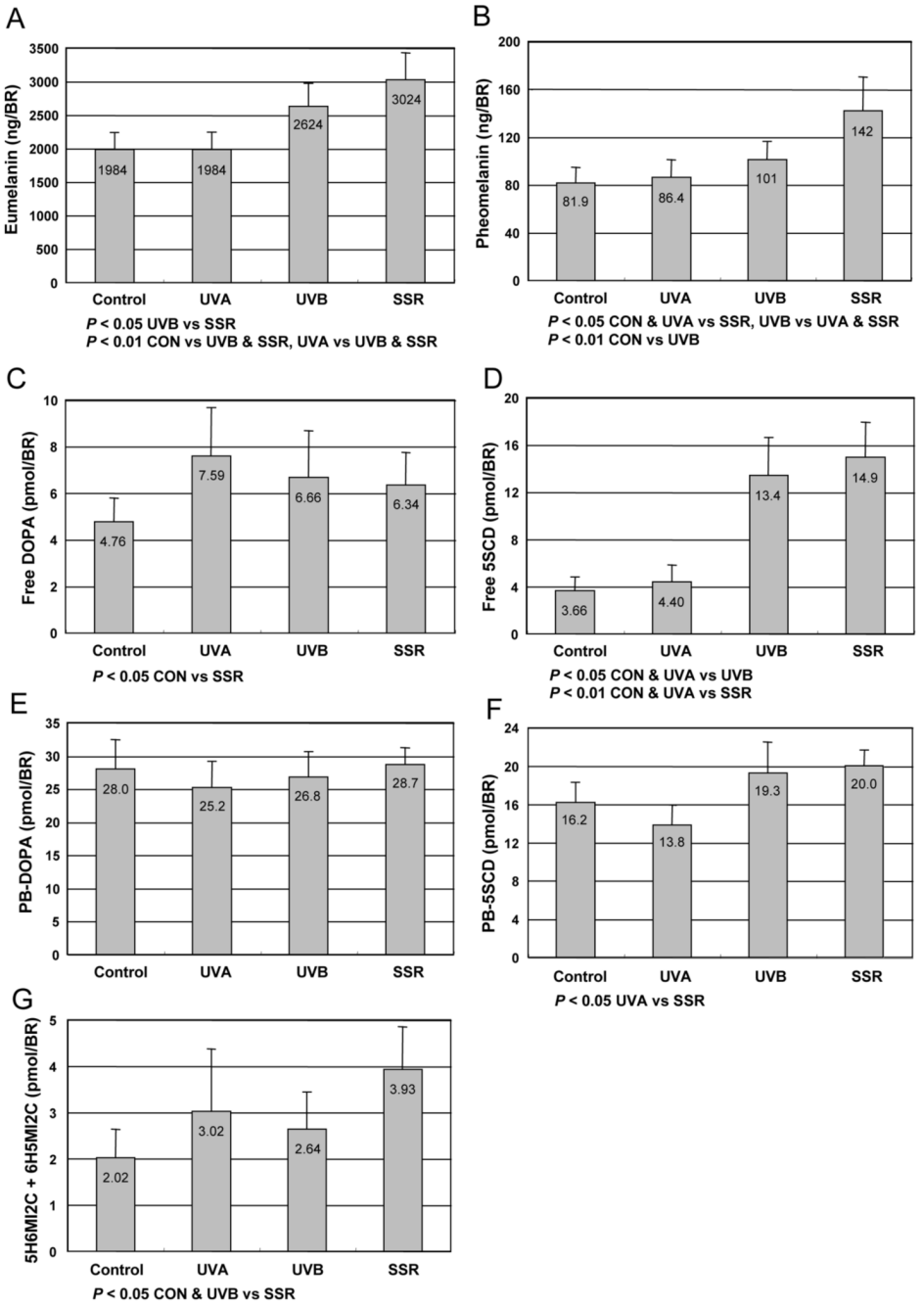

Figure 1.

Changes in levels of (A) eumelanin, (B) pheomelanin, (C) free DOPA, (D) free 5SCD, (E) protein-bound (PB) DOPA, (F) protein-bound (PB) 5SCD, and (G) 5H6MI2C+6H5MI2C in suction blister roofs. Data are expressed as per blister roof (BR) and are means for five subjects with standard errors of the mean (SEM). CONT denotes control.