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An EPR method for estimating activity of antioxidants in mouse skin using an anthralin-derived radical model

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Abstract

Inhibitory effects of intravenously or orally administered antioxidants on the anthralin-derived radical generated in skin (mainly in the epidermis) of living mice by ultraviolet-A (UVA) irradiation were estimated. Anthralin was applied to the dorsal skin of living mice and the mice were then exposed to UVA. The EPR signal intensity in skin tissue strips obtained from mice after anthralin-UVA treatment was measured by an X-band EPR spectrometer. Several common antioxidants such as ascorbate, glutathione and Trolox (a vitamin E analogue) intravenously administered to mice reduced anthralin-derived radical generation. Trolox showed the most prolonged and powerful effect. Intravenous injection of a clinically used cerebral neuroprotective drug, Edarabone (Radicut®), also showed depletion for the anthralin-derived radical. Oral administration of a commercialized nutritional supplement (a cocktail of 17 herbs and vitamins) also attenuated the anthralin-derived radical. The anthralin-UVA treatment model for antioxidant activity in the epidermis is a potentially feasible method to estimate activity of antioxidants in the body.

Keywords: *Electron paramagnetic resonance, electron spin resonance, anthralin-derived radical, antioxidant, epidermis*

Introduction

Skin is an important tissue to protect our body from exogenous invasions, such as physical stimulations, chemical or photochemical stimulations and heating. Photochemical stimulations can cause reactive oxygen species (ROS) in the skin [1] and give oxidative damage to the cell components. The accumulative effects of oxidative damage may finally cause skin fibrosis and/or skin cancer. Therefore, the skin itself must be protected from the ROS in order to prevent such damage. Estimation of antioxidative activity in the skin and the reinforcement of antioxidative activity by administering exogenous antioxidants are important to protect not only skin but also our body.

Electron paramagnetic resonance (EPR) spectroscopy can determine free radical species in biological samples and/or living animals non-invasively. Exogenously administered nitroxyl free radical species are used as redox probes to investigate free radical reactions *in vivo* and *in vitro* [2–6]. The nitroxyl radicals administered to an animal are distributed throughout the body rapidly. It is quite difficult to design an exogenous probe localized in a tissue or organ of interest, such as skin epidermis.

Administration of anthralin (1,8-dihydroxy-9-anthrone, also known as dithranol or cignolin) through mouse skin followed by ultraviolet-A (UVA) irradiation can generate anthralin-derived free radicals localized in the skin epidermis. Microscopic EPR spectral-spatial imaging showed that the anthralin-derived radicals were generated in the skin around 27 μm from the surface and EPR spectroscopy of histological samples showed that 85% of anthralin-derived radicals were obtained in the epidermis [7]. Therefore, the UVA induced anthralin-derived radicals are localized in the slightly deeper side of the epidermis.

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Hence, the combinations of antioxidants and the localized anthralin free radical can enable us to estimate the activity of antioxidants in the epidermis.

Anthralin is a medicine therapeutically used in the topical treatment of psoriasis [8]. Anthralin can be easily oxidized by alkaline oxidation and can be a primary radical, which gives a quintet EPR line [9]. The primary radical is converted to yield secondary multiple EPR line species, then finally a single broad EPR line species [9]. Photo-oxidation of anthralin in chloroform initially showed a triplet EPR signal, which then changed to a broad singlet line [9]. When anthralin is incubated with keratinocytes [9] and applied to excised pig skin [10] or UV-irradiated after painting on isolated mouse skin [9], generation of anthralin-derived free radical, which gives a broad singlet EPR line (g = 2.0036, linewidth = 0.6 mT), has been observed (Figure 1). The non-invasive detection of anthralin-derived free radicals in the skin of a live mouse using L-band EPR spectroscopy has also been reported [11]. The anthralin-derived free radicals in skin are not characterized chemically, although the broad singlet EPR line obtained in the skin is similar to the anthralin brown radical [9].

Fuchs and Packer [9] found that alpha-tocopherol decreased radical formation in skin treated with anthralin, while Lambert et al. [12] reported no effect. The effect of antioxidants on the anthralin-derived radical generated in a skin strip is equivocal, since the previous experiments were done with excised skin. It is still obscure if orally or intravenously administered antioxidants are capable of reaching the skin epidermis and suppressing anthralin-derived radical generation in the skin epidermis (Figure 1).

In the present paper, the *in vivo* effects of several intravenously or orally administered antioxidants in the skin (mainly in the epidermis) were investigated. Utilization of the UVA-induced anthralin-derived radical as a localized free radical probe in skin epidermis is described.

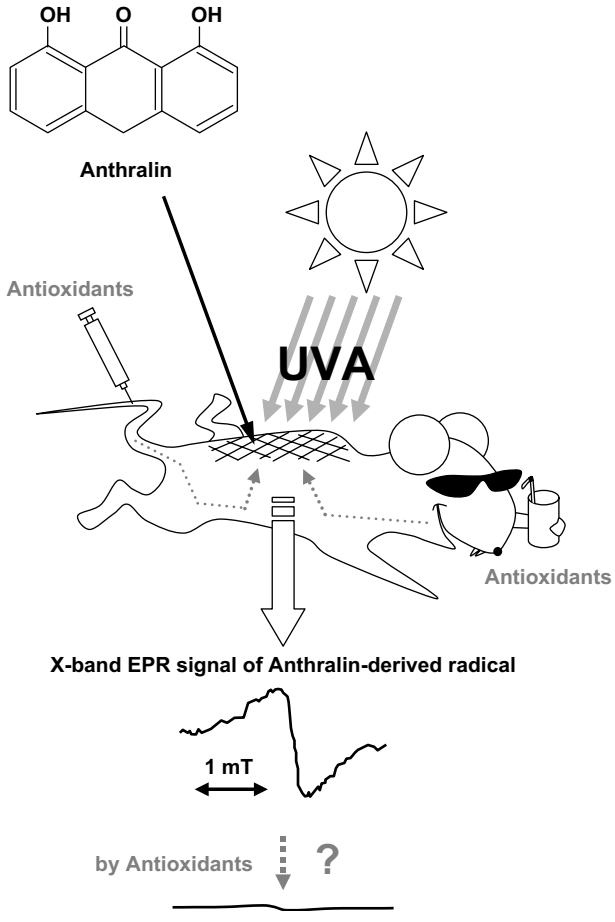

Figure 1. The experimental scheme. An acetone solution of anthralin was painted on the back of living mice and the mice were then exposed to UVA irradiation. The anthralin-derived radicals (g = 2.0036, linewidth = 0.6 mT) generated in the skin epidermis were measured by an X-band EPR spectrometer. The purpose of the experiment is to test if the orally or intravenously administered antioxidant can reach to epidermis and suppress the anthralin-derived radical generation.

Materials and methods

Chemicals

Anthralin was purchased from Aldrich Chemical Co. (Milwaukee, WI). Ascorbic acid (AsA), the reduced form of glutathione (GSH) and Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid) were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other reagents were reagent grade. Trolox was dissolved in 1 M sodium bicarbonate due to its poor solubility in water and then adjusted to pH 7.0 with 1 N HCl [13] (final concentration was 10 mM). The AsA and GSH was dissolved in water and adjusted to isotonic and pH 7 (final concentration was 10 mM). A clinically used cerebral neuroprotective drug, 3-methyl-1-phenyl-2-pyrazolin-5-one (Edaravone), was obtained from Mitsubishi Chemical Co. (Tokyo, Japan). Edaravone, also referred to as MCI-186 and Radicut®, was recently marketed for clinical use in Japan as a protective drug for cerebral ischemia and post-ischemic brain oedema [14–17]. An isotonic solution of Edaravone (3.0 mg/ml final concentration) was prepared by dissolving 30 mg of Edaravone into 0.5 ml of 1 N NaOH, adjusting pH 7 with 1 N HCl and being diluted to 10 mL with distilled water. A tonic-type nutritional supplement, which is available commercially as an oral liquid preparation (Table I), was used for invigoration and as a nutriment supplement in the treatment of physical fatigue and was a gift from Taisho Pharmaceutical Co. Ltd. (Saitama, Japan).

Animals and skin tissue preparation

Female hairless mice (HOS:HR-1, 5 weeks old), were supplied by Hoshino Experimental Animal Center

Table I. Constituents in the commercialized herbal nutritional supplement (in 50 mL).

Constituents	Amounts	Equivalent herbal/natural ingredients
Cistanche extract	151.6 mg	(≈ 500 mg as cistanchis herba)
Callopeptide extract	0.1 mL	(≈ 100 mg as phocae testis et penis)
Cervi parvum cornu tincture	1.08 mL	(≈ 300 mg as cervi parvum cornu)
Epimedium	100 mg	(≈ 1000 mg as epimedii herba)
Cordyceps extract	0.3 mL	(≈ 300 mg as cordyceps*)
Cnidium extract	600 mg	(≈ 300 mg as cnidium monnieri fructus)
Convolvulaceae extract	33 mg	(≈ 300 mg as cuscutae semen*)
Eucommia extract	0.3 mL	(≈ 300 mg as eucommiae cortex*)
Muirapuama extract-A	15 mg	(≈ 300 mg as muirapuama*)
Ginseng extract M	90 mg	(≈ 600 mg as ginseng radix*)
Dioscorea extract	0.3 mL	(≈ 300 mg as dioscoreae rhizoma*)
Agkistrodon tincture	1.25 mL	(≈ 250 mg as agkistrodon)
Cornus extract	0.5 mL	(≈ 500 mg as corni fructus*)
Poria extract-A	9.6 mg	(≈ 300 mg as poria)
Schisandra extract	0.3 mL	(≈ 300 mg as schisandrae fructus*)
Jiugancao extract	25 mg	(≈ 100 mg as glycyrrhizae radix*)
Rehmannia extract-A	150 mg	(≈ 300 mg as rehmanniae radix)
Taurin	500 mg	
Vitamin B_2	5 mg	
Vitamin B_6	5 mg	
Caffeine anhydrite	50 mg	

*Indicates herbal ingredients in which antioxidative activities have been reported. Other additives: white sugar, paraben, sodium benzoate, polyoxy hydrogenated castor oil, povidone, citric acid, sodium citrate, fragrances, ethylvanillin, L-menthol, alcohol (< 1.5 mL).

(Fukuoka, Japan) and kept on a standard diet (MF, Oriental Yeast Co. Ltd., Osaka, Japan) with free access to food and water. An aliquot (500 μL) of an acetone solution of anthralin (50 mM) was applied to an ~ 3 × 1.5 cm oval area of the dorsal skin of mice. The mice were moved to a cage specially arranged for UVA irradiation and kept in the cage with access to water and food *ad libitum*. UVA irradiation was started soon after all of the treated mice were placed in the cage. The mice were illuminated at a distance of 40 cm from the skin with an UVA lump (National FL15 BL-B; wavelength range 300–420 nm with a maximum at 352 nm). The irradiation power was estimated to be 10 mJ/cm^2/h with an actinometer (UV Checker, Yamashita Denso Co., City, Japan). To avoid severe inflammation of the skin, which would disturb preparation of the skin strip for EPR spectroscopic analysis, a relatively weak irradiation power was chosen for this study. The unusual long UVA irradiation time (i.e. beyond 12 h) in some experiments was for the purpose of making comparisons. After UVA exposure for various time periods, mice were sacrificed by cervical dislocation and the skin tissue separated immediately. After the subcutaneous fat had been removed by scraping the dermal side with soft tissue paper, the skin was cut exactly into a 2.0 × 0.3 cm strip and then mounted in an EPR tissue cell (LTC-10, Labotec Co.). This sample size was arranged for the special Teflon sample stage, which was designed for skin imaging experiments, as reported previously [7]. The same stencil plate was used to make the skin strip, while imaging experiments were not carried out in this paper. All procedures and animal care were approved by the Committee on the Ethics of Animal Experiments, Graduate School of Pharmaceutical Sciences, Kyushu University, Japan, and conducted according to the Guidelines for Animal Experiments of the Graduate School of Pharmaceutical Science, Kyushu University, Japan.

Ascorbic acid (AsA) and α-Tocopherol (α-Toc) contents in the skin

AsA concentration in the tissue homogenate was determined by a HPLC method [18]. The mouse skin was homogenized in a 5% metaphosphoric acid solution. The skin homogenate was centrifuged at 4°C at 3000 × g for 5 min. The supernatant was again centrifuged at 4°C at 10 000 × g for 60 min. An aliquot (20 μL) of final supernatant was subjected to the HPLC. Conditions of HPLC were follows: Column was TSKgel ODS-80Ts (4.6 mm i.d. × 250 mm length, TOSOH, Tokyo, Japan). The mobile phase was water at pH 2.2 (sulphuric acid). The flow rate = 1.0 mL/min and the detector was a UV-8020 (TOSOH) operated at 254 nm wavelength.

α-Toc concentration in the tissue homogenate was also determined by a HPLC method [19]. The mouse skin was chopped by cithers in 500 μL of an aqueous buffer solution containing 130 mM NaCl, 5 mM D-glucose, 1 mM EDTA-2Na and 10 mM $NaH_2PO_4/2H_2O$. Then 500 μL of the buffer, 50 μL of 10 mg/ml BHT and 1 mL of 0.1 M SDS solution was added and then homogenized. Two millilitres of a mixture of ethanol and isopropanol (95:5) was added to homogenate and vortexed. Then, 2 mL of hexane

was added and vortexed. The homogenate was centrifuged at 4°C at 1000 × g for 10 min. The supernatant (hexane) was moved to a microtube and dried under N_2 gas flow. The residue was again dissolved in 500 μL of methanol and 20 μL of the methanol solution was subjected to HPLC. The HPLC conditions were as follows: Column was Nova-Pak C_{18} (4.6 mm i.d. × 250 mm length, Waters, Milford, MA), the mobile phase was methanol, flow rate = 1.0 mL/min, the detector was Waters 474 Fluorescence Detector with an excitation wavelength of 290 nm and a detection wavelength of 325 nm.

EPR measurement

The EPR tissue cell containing the skin was placed in an X-band TE-mode cavity of an EPR spectrometer (JEOL JES-RE1X) and the spectra were recorded at room temperature using the following settings: Microwave power = 5 mW (9.4 GHz), modulation amplitude = 0.5 mT, modulation frequency = 100 kHz and the time constant = 0.1 s.

Experimental scheme and dose of antioxidants

Torolox, AsA, GSH solution or the corresponding vehicle was intravenously injected from the tail vein of the mice with doses of 0.02, 0.2 or 2.0 mmol/kg antioxidants 5 min before starting UVA irradiation. UVA irradiation was carried out by several periods. Edaravone or the corresponding vehicle was intravenously administered to mice 5 min before or 5 h after starting UVA exposure. Doses of Edaravone were 1 or 3 mg/kg (6 or 17 μmol/kg). Mice were irradiated by UVA for 10 h. The commercialized herbal nutritional supplement was administered orally to mice with a dose of 10 μL/g/day every day for 3 days before our experiment. The period of UVA irradiation was 5 h.

Statistical test

The statistical differences were estimated with alternative Student's or Welch's *T*-test. The suitable test for the data was automatically selected according to the variance of the data. Grades of significance were estimated by $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Results and discussion

Figure 2 shows the time course of EPR signal intensity of anthralin-derived radical in the skin during UVA irradiation. The EPR signal intensity increased with increasing UVA irradiation time and reached a plateau level after 12 h.

Figure 3 shows the time course of endogenous AsA and α-Toc contents in the mice skin during the anthralin-UVA treatment. AsA contents immediately

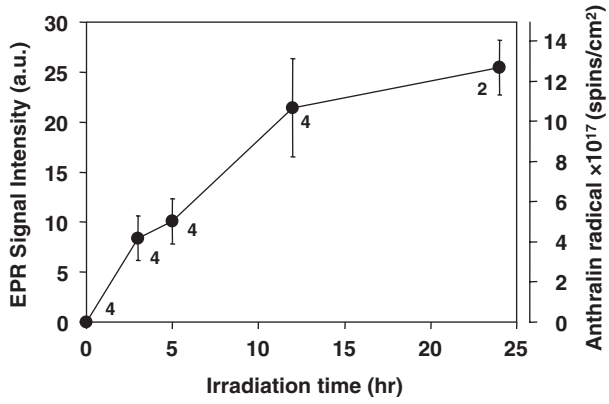

Figure 2. Time course of anthralin-derived radical generation in epidermis by UVA irradiation. EPR signal intensity of anthralin-derived radical caused in mouse skin after anthralin-UVA treatment was measured. The mice were exposed to UVA irradiation (10 mJ/cm^2/h) for 3, 5, 12 and 24 h. The skin tissue was separated from the mice and the X-band (9.4 GHz) EPR spectra were recorded by the following conditions; microwave power 5 mW, modulation amplitude 0.5 mT, modulation frequency 100 kHz and time constant 0.1 s. The dots and bars indicated with the dots showed mean ± SD. Numbers indicated by the dots show the number of mice. Skin sample preparations and EPR measurements are duplicated for each mouse.

decreased after the start of UVA irradiation and then kept at a lower level (Figure 3A). AsA contents also decreased with anthralin alone, while the decrease was lesser than that for the anthralin-UVA treatment. AsA contents looked slightly decreased by UVA irradiation alone, while no significance was observed. These decreasing profiles obtained with three different treatments looked parallel, but levels are anthralin-UVA > anthralin > UVA. α-Toc contents gradually decreased during the 24 h anthralin-UVA treatment period (Figure 3B). α-Toc contents looked slightly decreased by anthralin alone, while no significance was observed. UVA irradiation alone did not affect α-Toc contents for up to 5 h, while α-Toc contents decreased after 5 h. α-Toc contents 24 h after starting UVA irradiation were significantly lower than the normal level. The effect of UVA to α-Toc was different from that to AsA. AsA may trap any radicals unselectively, while α-Toc may only trap organic free radicals, such as lipid radicals. α-Toc does not trap primary anthralin radical, however can inhibit the formation of anthralin-derived radical in skin treated with anthralin [11]. It is possible that lipophilic α-Toc is localized in the lipidic region and consumed by lipid peroxidation products formed in the lipidic region during the delay just before starting UVA irradiation. Actually, TBARS level in the skin began to increase after 5 h irradiation of UVA (data not shown).

The treatment with anthralin alone can result in half the amount of anthralin-derived radicals even in the dark (data not shown). UVA can exaggerate this effect. The consumption of AsA and α-Toc was also accelerated by UVA irradiation. It was reported that the

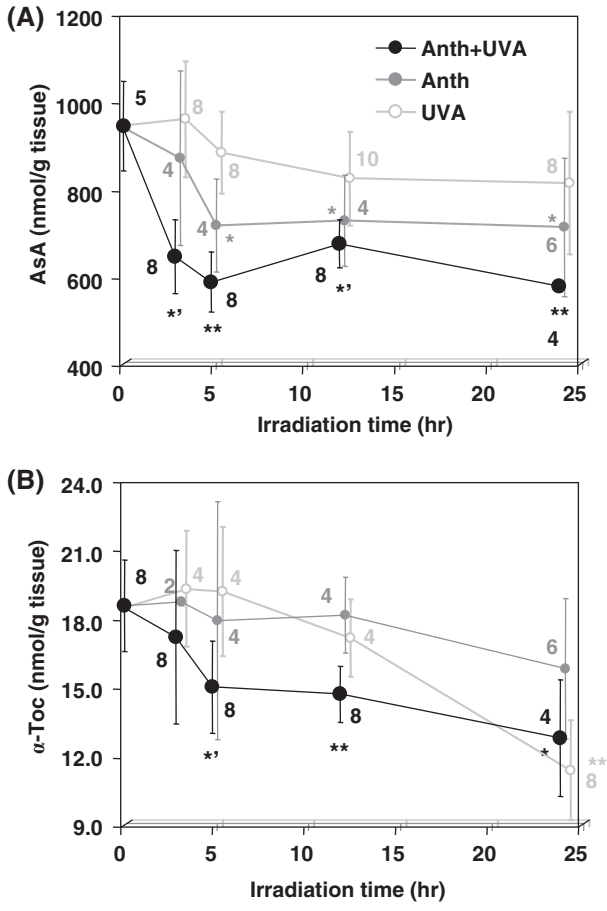

Figure 3. Effect of anthralin, UVA and anthralin-UVA treatments to endogenous AsA and α-Toc in mouse skin. Time course of (A) AsA and (B) α-Toc during treatments. The mice were exposed to UVA, anthralin or anthralin-UVA treatment for 3, 5, 12 and 24 h. The skin tissue was separated from the mice and AsA and α-Toc in the skin was measured by HPLC based methods. The circles and bars indicate mean ± SD. White, gray and black circles indicate UVA, anthralin or anthralin-UVA treatment. Numbers indicated by the dots show the number of mice. Skin sample preparations and EPR measurements are duplicated for each mouse. Significance to the control level (at 0 h) was indicated by *, *, and ** as $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

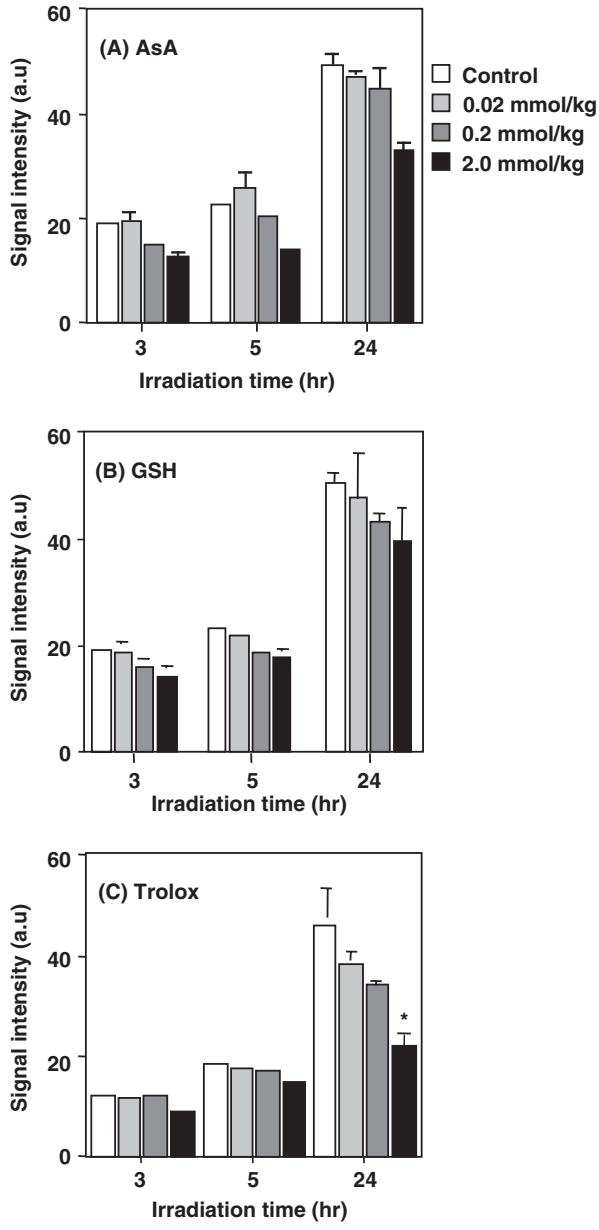

Figure 4. Effect of intravenous injection of AsA, GSH and Trolox on the generation of anthralin-derived radical in epidermis of living mice. EPR signal intensity of anthralin-derived radical caused after anthralin-UVA treatment was suppressed by intravenous administration of (A) AsA, (B) GSH and (C) Trolox dose-dependently. The antioxidant solutions were intravenously administered to the mice by several doses 5 min before starting UVA irradiation. The mice were exposed to UVA irradiation (10 mJ/cm^2/h) for 3, 5 and 24 h. The skin tissue was separated from the mice and the X-band (9.4 GHz) EPR spectra were recorded by the following conditions; microwave power 5 mW, modulation amplitude 0.5 mT, modulation frequency 100 kHz and time constant 0.1 s. The columns and bars indicated with the columns show mean ± SD. White, light gray, dark gray and black column indicate 0 (vehicle), 0.02, 0.2 and 2 mmol/kg doses, respectively. The number of mice used for the experiment was eight for 3 and 5 h irradiation and nine for 24 h irradiation. Significance to the control (vehicle) was indicated by * as $p < 0.05$.

radical formation by photo-oxidation of anthralin in organic solvents was inhibited under nitrogen and was enhanced by higher oxygen concentration [9]. Therefore, oxygen radicals may have an important role to generate anthralin-derived radicals when the anthralin was treated on the skin. UVA can induce ROS generation in skin [1]. Formation of ROS through auto-oxidation of anthralin was also reported [20,21]. The consumption of AsA and α-Toc when the mice were treated with anthraline alone may be due to ROS generation through auto-oxidation of anthralin.

In the next step, several hydrophylic antioxidants, i.e. AsA, GSH and Trolox, were administered to mice and the effect on anthralin-derived radical generation in mice skin was tested. Intravenous administrations of AsA, GSH or Trolox were tested to investigate whether they could suppress the formation of anthralin-derived radicals in the skin of living mice (Figure 4). The suppression of anthralin-derived radical generation was observed in all doses and periods of UVA exposure. However, the effects of the

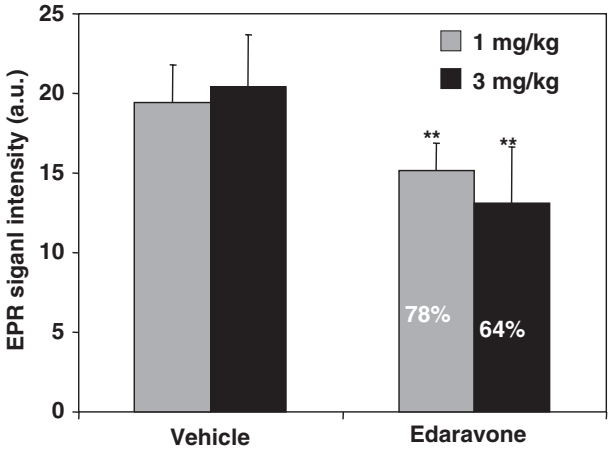

Figure 5. Effects of intravenous injection of Edaravone on EPR signal intensity of epidermal anthralin-derived radical. The solution of Edaravone (3.0 mg/mL of final concentration) was intravenously administered into the female hairless mice (HOS:HR-1, 5 weeks old) 5 min before starting UVA exposure. Same volume of vehicle was administered to the vehicle group. The mice were exposed to UVA irradiation (10 mJ/cm^2/h) for 10 h with access to water and food *ad libitum*. The EPR spectrum in the skin sample was measured under the following conditions; microwave power 5 mW, microwave frequency 9.4 GHz, modulation amplitude 0.5 mT, modulation frequency 100 kHz and time constant 0.1 s. Column and bar indicates mean ± SD of 10 mice. Numbers in the bars indicate the percentage of mean signal intensity compared with the corresponding vehicle group. Significance to the control was indicated by ** as $p < 0.001$.

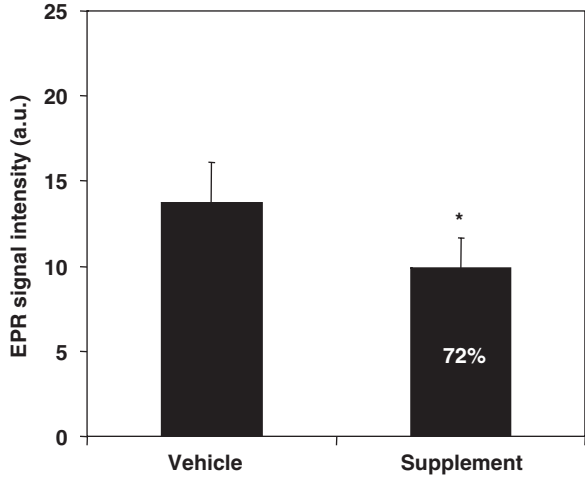

Figure 6. Effects of commercialized tonic-type nutritional supplement on EPR signal intensity of epidermal anthralin-derived radical. The nutritional supplement was orally administered to the mice every day with the dose of 10 μL/g/day from 3 days before irradiation. The mice were exposed to UVA irradiation (10 mJ/cm^2/h) for 5 h with access to water and food *ad libitum*. The EPR spectrum in the skin sample was measured under the following conditions; microwave power 5 mW, microwave frequency 9.4 GHz, modulation amplitude 0.5 mT, modulation frequency 100 kHz and time constant 0.1 s. Column and bar indicates mean ± SD of five mice. Number in the bar indicates the percentage of the mean signal intensity to the vehicle group. Significance to the control was indicated by * as $p < 0.05$.

suppressing anthralin-derived radical generation were different among the antioxidants. Trolox showed the strongest suppression of anthralin-derived radical generation after 24 h UV exposure. The intravenous 2 mmol/kg dose of Trolox suppressed the intensity of anthralin-derived radical to be 50% of the control group. This result indicates that the effect of Trolox on suppressing radical generation in the skin under UV exposure is persistent. These results suggested that Trolox shows a better inhibitory effect on the generation of anthralin-derived radicals in the skin and that the effect had been observed even 24 h after intravenous injection.

Figure 5 demonstrates the EPR signal intensities obtained under several dose conditions of clinically used free radical scavenger, Edaravon. The result showed inhibitory effects of those anti-oxidative manufactures to the epidermal anthralin-derived radical generation. A clinically used drug, Edaravone, is a protective drug for cerebral ischemia and post-ischemic brain oedema [14,17]. Edaravone is a potent scavenger of hydroxyl radicals and has the ability to inhibit lipoxygenase metabolism [15,16]. Edaravone (6 or 17 μmol/kg) was intravenously administered to mice before or during 10 h of UVA exposure. Both doses of Edaravone showed distinct suppressant effects on anthralin-derived radical generation in the mouse skin. The effect was significant even when 17 μmol/kg of Edaravone was administered 5 h after starting UVA exposure (data not shown). The intravenous injection of 17 μmol/kg of Edaravone was estimated to have almost the same activity as 2 mmol/kg of Trolox. These results indicate the strong antioxidant activity of Edaravone toward radical generation in mouse skin.

Figure 6 demonstrates the EPR signal intensities obtained under a dose of commercialized herbal nutritional supplement. This nutritional supplement contains extracts of 17 herbs, taurin, vitamin B_2, vitamin B_6 and caffeine. The contents of the commercially available herbal nutritional supplement are summarized in Table I. Antioxidative activities are reported in nine of the herbs, i.e. cordyceps [22–24], cuscutae semen [25], eucommiae cortex [26–28], muirapuama [29,30], ginseng radix [31,32], dioscoreae rhizoma [33–35], corni fructus [36,37], schisandrae fructus [38,39] and glycyrrhizae radix (licorice) [40,41]. The nutritional supplement administered orally to mice at a dose of 10 μL/g/day for 3 days significantly suppressed the generation of anthralin-derived radical in the epidermis after UVA exposure, although the dose used in this experiment was higher than a preferable human dose (50 mL/day for adults). Orally administered antioxidants, AsA and GSH, also suppressed anthralin-derived radical generation in mouse skin (data not shown). The result shows a potent antioxidant effect of orally administered herbal nutritional supplement on radical generation in skin by UVA exposure.

Conclusion

In conclusion, the amount of anthralin-derived radical generated in the skin by the anthralin-UVA treatment increased with increasing irradiation time. The amounts of both intrinsic AsA and α-tocopherol in the mouse skin were reduced by the anthralin-UVA treatment. The amount of anthralin-derived radical generated in the skin was suppressed by intravenous administration of the antioxidants, such as AsA, GSH or Trolox. Intravenous injection of a clinically used cerebral neuroprotective drug Edarabone (Radicut®) exhibited the suppression of anthralin-derived radical generation in the skin. Oral administration of a commercialized nutritional supplement also showed an antioxidative effect in the skin. Therefore, the anthralin-UVA treatment procedure is a useful experimental model to demonstrate the seeping antioxidative effect of intravenously and/or orally administered antioxidants to the skin.

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