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A novel mechanism of filaggrin induction and sunburn prevention by β-damascenone in Skh-1 mice

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ABSTRACT

Understanding how oral administration of aroma terpenes can prevent sunburn or skin cancer in mice could lead to more effective and safer ways of blocking sun damage to human skin. To establish sunburn preventive activity, female Skh-1 mice were given oral β-damascenone followed by irradiation with UVR from fluorescent 'sunlamps'. The following endpoints were evaluated versus controls at various times between 1 and 12 days after the terpene: whole genome gene expression and *in situ* immunohistochemistry of PCNA, keratin10, filaggrin and caspase 14, and sunburn was evaluated at 5 days. UVR-induced sunburn was prevented by a single oral β-damascenone dose as low as 20 μL (0.95 mg/g body weight). Microarray analysis showed sunburn prevention doses of β-damascenone up-regulated several types of cornification genes, including keratins 1 and 10, filaggrin, caspase 14, loricrin, hornerin and 6 late cornified envelope genes. Immunohistochemical studies of PCNA labeling showed that β-damascenone increased the proliferation rates of the following cell types: epidermal basal cells, follicular outer root sheath cells and sebaceous gland cells. Keratin 10 was not affected by β-damascenone in epidermis, and filaggrin and caspase 14 were increased in enlarged sebaceous glands. The thickness of the cornified envelope plus sebum layer nearly doubled within 1 day after administration of the β-damascenone and remained at or above double thickness for at least 12 days. β-Damascenone protected against sunburn by activating a sebaceous gland-based pathway that fortified and thickened the cornified envelope plus sebum layer in a way that previously has been observed to occur only in keratinocytes.

Introduction

This study investigates how the aroma terpene, β-damascenone, administered orally prevents UVR-induced erythema/sunburn. β-Damascenone is an example of terpenes found in citrus and other fruits at low concentrations. Terpenes, such as, perillyl alcohol and vitamin A acetate, block proliferation and/or increase apoptosis possibly through anti-oxidative properties (Burns et al., 2002, 2007a, 2007b; Gerhauser et al., 2009; Hakim et al., 2000; Horiki et al., 2000). The cornified envelope, the outermost non-living envelope of skin, functions primarily as a barrier preventing entry of noxious agents and retarding loss of water (Mildner et al., 2010). In addition to a lipid bilayer, the cornified envelope consists of a complex molecular matrix, including keratins and other proteins, such as, filaggrin and loricrin, that help to configure keratin filament elongation and cross-linking (Steven et al., 1990).

Loricrin, filaggrin and hornerin are synthesized and stored as 'granules' in the stratum granulosum of the keratinocytes before their degradation and incorporation into the fully cornified envelope (Candi et al., 2005; Greenberg et al., 1990; Nicotera and Melino, 2007). After liberation from the granulosum, filaggrin undergoes proteolytic degradation by exo- and endo-proteases, including caspase-14, into hygroscopic amino acids which help to hydrate the skin (Denecker et al., 2008). Filaggrin knockdown increases the skin sensitivity to ultraviolet radiation (UVR) and impairs the water barrier function in a human skin model (Mildner et al., 2010). Filaggrin mutations lead to a disturbed skin barrier and dry skin which are hallmarks of atopic dermatitis (Proksch et al., 2009). Loss-of-function mutations in the filaggrin gene also lead to reduced levels of natural moisturizing factors in the stratum corneum (Kezic et al., 2011). Caspase-14-deficient epidermis is characterized by an altered profilaggrin processing pattern, and skin of caspase-14-deficient mice shows increased sensitivity to the formation of cyclobutane pyrimidine dimers after UVR irradiation, leading to increased levels of UVR-induced apoptosis (Denecker et al., 2007).

The current study was designed (1) to establish the effectiveness of an ingested aroma terpene, β-damascenone, for protecting mouse skin against sunburn induced by UVR of a commercial sunlamp and (2) to quantify changes in cell proliferation rates utilizing proliferating cell

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Table 1
Thickness of epidermal layers at indicated times after end of oral administration of β-damascenone in Skh1 mice.

Row#	β-Damascenone amount (total)*	Sampling time (d)	Corn.+Sebum^ thickness (μ)	*p*-value@	Corn.+Sebum^ fold increase	Keratinocyte layer (μ)
1	Control (None)	5	16.1±2.5	–	1.00±0.22	57.6±4.9
2	20 μL×1 (20 μL)	1	35.8±15.5	≤0.05	2.22±0.46	62.7±24.2
3	10 μL×4 (40 μL)	1	27.4±10.4	N.S.	1.70±0.41	66.2±13.0
4	20 μL×4 (80 μL)	1	31.8±8.2	≤0.05	1.98±0.30	64.4±17.7
5	80 μL×1 (80 μL)	1	23.3±7.2	N.S.	1.45±0.35	70.4±10.6
6	20 μL×1 (20 μL)	5+	66.2±7.3	≤0.05	4.11±0.19	68.9±17.3
7	10 μL×4 (40 μL)	5+	36.2±6.1	≤0.05	2.25±0.23	72.0±14.0
8	20 μL×4 (80 μL)	5+	38.0±5.6	≤0.05	2.36±0.21	58.2±11.4
9	80 μL×1 (80 μL)	5	45.6±6.0	≤0.05	2.83±0.20	64.9±10.1
10	20 μL×1 (20 μL)	12	30.5±6.9	≤0.05	1.89±0.62	21.5±6.6
11	10 μL×4 (40 μL)	12	33.4±12.5	≤0.05	2.07±0.41	54.9±16.9
12	20 μL×4 (80 μL)	12	38.8±3.0	≤0.05	2.41±0.17	41.8±6.0

Notes: Each group contained 3 mice; total of 36 mice.
± = Standard error of the mean.
* x1 = once only, x4 = daily for 4 days.
^ Corn. + Sebum = Cornified envelope + surface sebum layer.
@ Student's t test versus control; N.S., not significant.
+ Sunburn evaluation performed.

nuclear antigen (PCNA) and changes in the expression of cornification genes, including keratin 10, filaggrin, and caspase 14 in the epidermis and sebaceous glands.

Materials and methods

Mice. Albino, female Skh-1 mice were used in all experiments. The Skh-1 strain has been extensively used in cancer chemoprevention studies (Burns et al., 2008; Rossman et al., 2001). The mice were housed 3 per cage and fed *ad libitum* a commercial grain-based diet certified to be adequate in all essential nutrients (Ralston Purina Inc., St. Louis, MO USA).

Oral application of terpene. The test terpene, β-damascenone, was obtained as a 99% pure compound from Biokeys for Flavors, LLC, Norwood, NJ, USA. Typically the treatment consisted of 10 or 20 μL of pure β-damascenone administered *per os* by a feeding tube attached to a micropipette. Thirty six mice were used for a dose–response experiment, where β-damascenone was administered either once only or daily for 4 consecutive days as outlined in the column 2 of Table 1. Histopathology of dorsal skin samples was performed at 1, 5, and 12 days after the final β-damascenone dose to quantify tissue responses in the absence of UVR exposure.

UVR exposure of mice. For sunburn protective activity of β-damascenone, 16 mice were grouped as UVB alone (3 mice) and β-damascenone + UVB (13 mice) for different doses of β-damascenone as specified in Table 2. For positional restraint during UVR exposures, the mice were anesthetized with i.p. nembutal at a dose of 35 mg/kg body weight and placed in boxes configured to allow a rectangular (2×4 cm) region of dorsal skin to be exposed to UVR. The UVR was generated by of a bank of four parallel Westinghouse fluorescent sun lamps (FS-20). The lamps were centered 24 cm above the skin surface. The dose rate of UVB was 0.20 kJ/m^2 per minute as measured with a calibrated International Light 1400A digital radiometer/photometer equipped with a SEL240 UVB-1 detector (International Light, Inc., Wilmington, MA USA). A dose of 1.5 kJ/m^2 of UVB was utilized to produce sunburn. The UVB exposures occurred at 5th day after the final β- damascenone dose and sunburn was evaluated at the peak response 4–6 days after UVB. All experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee of New York University School of Medicine.

Statistical analysis of sunburn. Sunburn was evaluated in 4 equal, contiguous subregions (2.0 cm×1.0 cm) running anterior to posterior along the spinal axis within the UVB-exposed 2.0 cm×4.0 dorsal skin region of each mouse. Sunburn assessment was performed at day 5 of β-damascenone of 20 μL×4, 10 μL×4 and 20 μL×4. Sunburn (erythema) in a subregion qualified for inclusion in the 'with sunburn' column in Table 2. *p*-values were obtained by using Fisher's one-sided, exact test for 2×2 contingency tables, based on the assumption of no correlation between subregions.

Gene expression experiment. Gene expression microarray analysis was performed in samples obtained from control or β-damascenone-treated mice by utilizing the Affymetrix mouse whole genome microarray chip (Affy 430_2) containing about 39,000 transcripts with 45,101 probe sets. The mRNA was extracted from a 0.1 g samples of mouse skin treated with or without β-damascenone by using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and 200 μg of total RNA was used for the isolation of poly A + mRNA by using Oligotex mRNA mini columns (QIAGEN, Valencia, CA, USA). The final centrifugation was performed after the incubation with 1/10 volume of 3.0 M NaOAc, 2.5 volumes of

Table 2
β-Damascenone-induced sunburn protection in Skh-1 mice.

Group	Treatment		Subregions with sunburn	Subregions with no sunburn	Total subregions (# mice)	*p*-value (one-tail)	
	UVB	Damas.				Value	Significance
1	1.5 kJ/m^2	None	8	4	12 (3)	–	–
2	1.5 kJ/m^2	10 μL×1	5	7	12 (3)	0.219	Not significant
3	1.5 kJ/m^2	10 μL×4	2	6	8 (2)	0.068	Borderline
4	1.5 kJ/m^2	20 μL×1	2	10	12 (3)	0.013	High
5	1.5 kJ/m^2	20 μL×4	1	19	20 (5)	0.001	Very high

Note 1: Damas., β-damascenone; indicated amounts of β-damascenone were administered *per os* by feeding tube either 4 consecutive days (x4) or once only (x1). Ultraviolet radiation (UVB) generated by a commercial sunlamp was applied to a 2.0×4.0 cm region of dorsal skin at 5th day after the final β-damascenone application, and the sunburn response was evaluated 4–6 days later.