“Ectoine”; A new candidate for skin whitening: Inhibitory effects on the incorporation of melanosomes into keratinocytes and the proliferation of melanocytes after UVB irradiation.

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Introduction

Melanin is synthesized and stored in melanosomes (MS) in melanocytes. During the maturation of MS, they are carried to the tips of the melanocyte dendrites and are finally transferred to keratinocytes through a phagocytosis fashion. In the visual recognition of skin pigmentation, the dispersion of MS in the epidermis is essential. Therefore, suppression of MS transfer into keratinocytes is one approach to prevent skin pigmentation. On the other hand, the number and activity of melanocytes in the basal cell layers of the epidermis is a critical factor in the regulation of skin pigmentation. UV light is known to stimulate the proliferation and activation of melanocytes and the transfer of MS to keratinocytes due to the secretion of various paracrine factors from skin cells such as keratinocytes and fibroblasts. In addition, oxidative stress generated by UV exposure has been reported to stimulate the secretion of those paracrine factors.

Ectoine is a cyclic amino acid produced by bacteria that live in an environment with a high concentration of salts. Also, it has been reported that ectoine promotes the intracellular synthesis of Heat shock protein 70 (Hsp 70), which possesses anti-oxidative effects in cells\textsuperscript{1}). These facts led us to expect that ectoine would suppress and improve skin pigmentation through its inhibition of melanocyte proliferation and MS transfer into keratinocytes initiated by UVB irradiation. Thus, we examined the effects of ectoine on UV-induced skin pigmentation focusing on the proliferation of melanocytes and the transfer of MS to keratinocytes.

Methods

Melanocyte proliferation after exposure to UVB

HaCaT keratinocytes were treated with ectoine for 24 hours and then were irradiated with UVB. After UVB irradiation, cells were cultured in Medium-254 (GIBCO) supplemented with 0.5% FBS and 3 µg/mL heparin from Human Melanocyte Growth Supplement (HMGS; KURABO). After 48 hours of cultivation, the culture medium was collected as the conditioned medium (CM). The proliferation rate of Normal Human Epidermal Melanocytes (NHEMs) cultured with the CM was then measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.
Transfer of Pseudo-MS into keratinocytes with exposure to UVB and H$_2$O$_2$

Fluorescent beads (FluoSpheres$^\text{TM}$ carboxylate-modified 0.2 µm, blue, Invitrogen) were used as pseudo-MS. In order to prepare the CM, HaCaT keratinocytes pretreated with ectoine for 24 hours were exposed to UVB at 40 mJ/cm$^2$ and H$_2$O$_2$ (500 µM) for 1 hour. Cells were then cultured for 24 hours in the CM containing fluorescent beads, and the uptake of fluorescent beads into the cells was evaluated by measuring the fluorescence intensity of cells (Ex: 365 nm, Em: 415 nm). The cells were lysed with PBS containing 0.5% Triton X-100, and amounts of protein were quantified using the BCA protein assay kit (Thermo Scientific). The uptake index is expressed as fluorescence intensity per µg protein.

MS transfer by the CM of HaCaT cells exposed to H$_2$O$_2$

HaCaT keratinocytes were treated with ectoine for 24 hours, exposed by H$_2$O$_2$ for 1 hour and then were cultured with fresh DMEM without FBS in order to prepare the CM. HaCaT keratinocytes prepared in other dishes were cultured with the CM containing MS that had been isolated from pretreated B16 melanoma cells for 24 hours. To visualize MS transfer to HaCaT cells, MS were stained with the Fontana-Masson Staining method.

Results

Inhibitory effects of ectoine on NHEM proliferation with UVB Irradiation

The proliferation rate of NHEMs cultured with the CM from UVB-exposed keratinocytes was increased significantly. Meanwhile, pre-treatment of HaCaT cells with ectoine abolished the proliferation rate of NHEMs cultured with the CM from UVB-exposed keratinocytes in a dose-dependent manner. (Fig. 1)

![Fig. 1 Ectoine Inhibition of Normal Human Epidermal Melanocyte Growth](image-url)
Inhibitory effects of Ectoine on Pseudo-MS transfer into HaCaT keratinocytes by CM exposed to UVB and H$_2$O$_2$

The transfer of fluorescent beads into HaCaT cells was increased by the CM of 40 mJ/cm$^2$ UVB-exposed and H$_2$O$_2$-exposed cells. In these conditions, CMs prepared from UVB-exposed and H$_2$O$_2$-exposed HaCaT cells pretreated with ectoine showed reduced transfer of MS in a treatment dose-dependent manner. (Fig. 2, 3)

![Graph showing inhibitory effects of Ectoine on Pseudo-MS transfer into HaCaT keratinocytes by CM exposed to UVB and H$_2$O$_2$.](image1)

**Fig.2** The uptake of fluorescent beads as pseudo-MS into keratinocytes was suppressed by ectoine.

![Graph showing examination for Ectoine Inhibition on Pseudo-MS Phagocytosis with H$_2$O$_2$ Exposure](image2)

**Fig.3** Examination for Ectoine Inhibition on Pseudo-MS Phagocytosis with H$_2$O$_2$ Exposure
Suppressive effects of ectoine on MS transfer stimulated by H₂O₂ treatment

The transfer of MS isolated from B16 melanoma cells was increased by CM prepared from H₂O₂-exposed HaCaT keratinocytes. The elevation was abolished by pretreatment with ectoine (Fig. 4).

![Fig. 4 Examination for Ectoine Inhibition on MS Phagocytosis with H₂O₂ Exposure](image)

Fontana-Masson Stained MS Isolated from B16 Melanomcells a and Incorporated by H₂O₂-exposed HaCaT Keratinocytes and Impact of Ectoine Addition (0 mM, 100 mM) on the Uptake of MS

Discussion

Skin pigmentation is regulated by several factors. First, the activation of melanin synthesis involving increased numbers of melanocytes due to the proliferation and activation of potentials for melanin synthesis in each melanocyte. Second, the importance of MS diffusion in the epidermis has recently been proposed to be responsible for pigmentated spots on the skin. The proliferation of NHEMs is regulated by soluble factors secreted from UVB-exposed keratinocytes. Existing studies have demonstrated that IL-1α, endothelin-1, prostaglandin E₂ (PGE₂) and α-melanocyte stimulating hormone (α-MSH) activate the proliferation of melanocytes and the synthesis of tyrosinase and its related proteins. Among those soluble factors, it is known that PGE₂ and α-MSH are induced by excess oxidative stress. In addition, it has been reported that α-MSH accelerates the transfer of MS into keratinocytes through its binding to the melanocortin-1 receptor (MC1R). Our previous study showed that ectoine reduced intracellular ROS levels and also decomposition of H₂O₂ penetrated extracellularly. Concomitantly, since ectoine induced Hsp70, which has an anti-oxidative property, the
characteristics of ectoine was expected to suppress skin pigmentation initiated by oxidative stress. In this study, we demonstrated that ectoine reduces melanocyte stimulation and MS transfer into keratinocytes stimulated by UVB and H$_2$O$_2$.

Considering these facts, we propose that the elevation of intracellular antioxidative capabilities by ectoine has these effects due to the removal of oxidative stress. We conclude that ectoine is a promising candidate for a skin lightening ingredient.

Reference


