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Ectoin: An Effective Natural Substance to Prevent UVA-Induced Premature Photoaging

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Key Words
Ectoin · UVA radiation · Second messenger release · Transcription factor AP-2 · Intercellular adhesion molecule-1 · Mitochondrial DNA mutation

Abstract
With the help of a new 'UVA stress model', it was shown that Ectoin protects the skin from the effects of UVA-induced cell damage in a number of different ways. Using cell cultures, high-performance thin-layer chromatography, gel electrophoresis mobility shift assays, reverse transcriptase polymerase chain reaction, ion exchange chromatography and UV spectroscopy, it was demonstrated that the UVA-induced second messenger release, transcription factor AP-2 activation, intercellular adhesion molecule-1 expression and mitochondrial DNA mutation could be prevented. The results obtained clearly demonstrate that Ectoin counteracts the effects of UVA-induced and accelerated skin aging at different cell levels.

Introduction

The UVA radiation inherent in sunlight can cause damage to human skin and it plays an important role in premature and accelerated skin aging. Photoaged skin shows increased wrinkling, thickness and laxity and a yellow hue with areas of brown pigmentation [1]. However, the natural substance Ectoin – from the group of compatible solutes – has now become available as a protection against skin aging. Ectoin is a neutral non-ionic, strong water-binding, organic molecule of low molecular weight [2] occurring in halophilic bacteria [2, 3], which grow under extreme conditions such as intensive sun irradiation, high temperatures and extreme dryness [2, 4]. In nature, these organisms protect themselves against these stresses by synthesising Ectoin [2, 5, 6]. In previous studies, it was demonstrated that Ectoin is able to protect biopolymers from external stress factors such as UV radiation. In this way, Ectoin prevents the Langerhans cells from being damaged or even destroyed. It also reduces the number of sunburn cells developed as a result of the human skin being exposed to UV radiation [7]. Ectoin does not absorb the UV radiation but protects the biopolymers by means of other still non-clarified mechanisms. However, it has been shown that Ectoin protects and stabilises the cell membrane of pretreated cells against the damaging effect of surfactants [7]. A molecular basis to stabilise biopolymers is seen in the kosmotropic nature of Ectoin, referring to the structure-forming ability in water [8]. Using a gel filtration method and near-infrared spectroscopy, it is demonstrated that Ectoin strongly influences surrounding water molecules [8].

Nowadays, Ectoin of high purity is produced in an industrial scale using biotechnological methods [9]. It is

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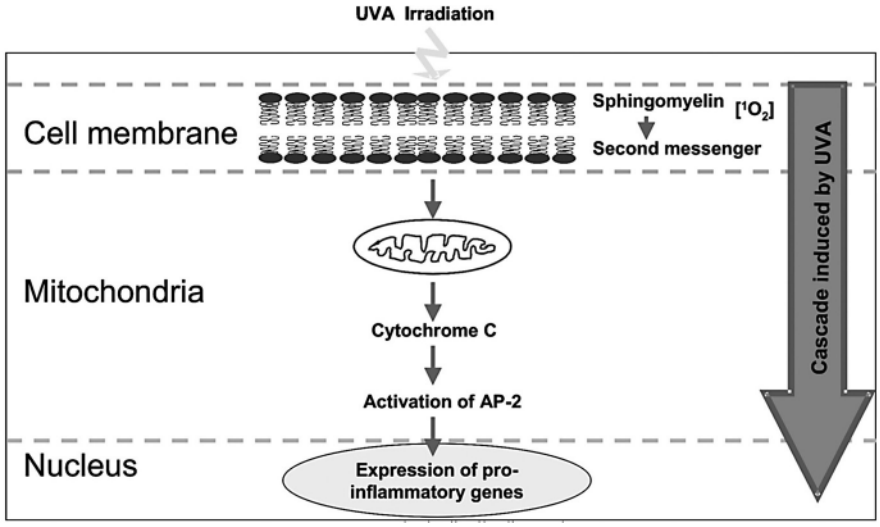

Fig. 1. The UVA stress model demonstrating the UVA-induced cascading effects in human skin cells and its relation to photoaging. Modified model of Grether-Beck et al. [17].

used in cosmetic products to protect the skin against damages induced by UV irradiation, especially UVA [10] and dry skin [11]. As amphoteric and very good water-soluble molecule, Ectoin can be applied in a wide range of different cosmetic formulations [12].

This investigation focuses on the protection against the UVA-induced cell damage shown by a 'UVA stress model' (fig. 1). In several previous investigations with Ectoin, the whole sun spectrum was used and results on the anti-photoaging benefits for the human skin were published [7, 10, 11]. However, for these new investigations, the UVA radiation has been chosen, because the cis- and trans-acting genetic elements responsible for gene induction by UVC or UVB radiation have been well characterised [13]. In contrast, the mechanisms by which UVA radiation induces transcriptional activation of human genes are poorly understood and most likely differ from those induced by short-wavelength UV radiation, because they are involved in different photobiology effects [14]. UVA radiation is the major component of the UV solar spectrum that reaches the earth. The amount of UVA radiation reaching the surface of the earth is 20 times greater than UVB radiation [15]. The capacity of UVA radiation and/or singlet oxygen to induce human gene expression through activation of AP-2 indicates a previously unrecognized role of this transcriptional factor in the mammalian stress response. UVA radiation is thought to play a pivotal role in photoaging, in the pathogenesis of the most frequent photodermatosis, i.e., polymorphous light eruption and photocarcinogenesis [16].

UVA radiation initiates the gene-regulatory mechanisms in human keratinocytes that are responsible for the damage caused by such radiation. It has been shown that the formation of singlet oxygen [$^1O_2$] in the membrane of UVA-irradiated keratinocytes releases ceramides that, as second messengers, initiate an UVA-induced activation of the transcription factor AP-2 and expression of some pro-inflammatory genes [17, 18]. In this particular case, the regulation of transcription factor AP-2 for UVA-induced gene expression, e.g. of pro-inflammatory genes such as intercellular adhesion molecule-1 (ICAM-1), is of primary importance [17, 18]. In this cell cascade, the released ceramides act on mitochondria and induce the release of cytochrome C from the mitochondria into the cytoplasm of the cell. The subsequent interaction of cytochrome C with transcription factor AP-2 leads to the activation of AP-2 via redox regulation, AP-2 is oxidized, whereas cytochrome C is being reduced. Oxidized AP-2 (AP-2β) has a greatly enhanced capacity to bind to AP-2-responsive elements in the promoter region of UVA-inducible genes [17, 18]. In addition to UVA-induced ICAM-1 expression, matrix metalloproteinases were activated and cause wrinkles by degradation of the extracellular matrix proteins. The measured parameters of the used UVA stress model show that Ectoin inhibits the UVA-induced cascade and consequently the cell aging process in keratinocytes and fibroblasts.

UVA-induced mutation of mitochondrial DNA is also of decisive importance in the premature aging of skin [19, 20]. The spherical mitochondrial DNA is located near the inner mitochondrial membrane. This means that it could be subject to increased external stress that may result in mutation of mitochondrial DNA. This, in turn, is important in the development of degenerative disease and in the

aging of cells and tissues. UVA radiation induces mutation of mitochondrial DNA and causes skin damage. It has been shown that repetitive UVA irradiation of human fibroblasts can lead to both time- and dose-dependent mutation of mitochondrial DNA [19, 20].

Materials and Methods

RonaCare®Ectoin (Merck KGaA, Darmstadt, Germany) is a pure (>96%) and natural substance isolated from *Halomonas elongata* by biotechnological methods. It is a fine white powder with a high aqueous solubility.

Second Messenger Release

Long-term cultured normal human keratinocytes, which were either untreated or had been pretreated for 24 h with different concentrations of Ectoin, were exposed in vitro to a single dose of 30 J/cm^2 UVA radiation within the 340- to 400-nm range (UVA_1-Sellas 2000, Lamp-System Dr. Sellmeier, Sellas GmbH, Gevelsberg, Germany). This dose had previously been shown to induce the signaling cascade described above without affecting cell viability [17]. Cells were harvested 1 h after irradiation, which is at the maximum of UVA radiation-induced second messenger formation. Lipid extracts based on 500 μg of protein were prepared after lipid extraction according to Bradford [21] and Folch et al. [22] and mild alkaline hydrolysis. The lower phase of the extract by Folch et al. including the released ceramide (second messenger) of the cell membrane was evaporated. Lipids including the ceramides were dissolved in 2:1 (v/v) chloroform/methanol and resolved by analytical sequential high-performance thin-layer chromatography using a CAMAG AMD-2 instrument (Muttenz, Switzerland). The measured quantities of this second messenger (ceramides split off membranal sphingomyelin) indicate the protection potential of Ectoin against UVA-induced cell damage. For more details, see Grether-Beck et al. [17].

AP-2 Activation

The activated transcription factor AP-2 was determined by gel electrophoresis mobility shift assays. The gel electrophoresis mobility shift assays of nuclear extracts were prepared from UVA-irradiated (30 J/cm^2) cultured human keratinocytes. The binding of nuclear extracts to a double-stranded, radiolabelled oligonucleotide containing an AP-2 consensus sequence deduced from the sequence of the human ICAM-1 promoter was studied in the presence and absence of an unlabelled competitor. One hour after UVA exposition, cells were harvested and the nucleus extract was prepared according to Dignam et al. [23] and incubated with the consensus oligonucleotide sequence. The AP-2 consensus sequence was deduced from the ICAM-1 promoter [24]. Specificity controls were performed as described by Grether-Beck et al. [17].

ICAM-1 Expression

The total RNA was isolated using RNeasy Total RNA Kits (Qiagen, Hilden, Germany). Expression of ICAM-1 was measured by differential reverse transcriptase polymerase chain reaction (RT-PCR) using the RT-PCR Core Kit (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany) and a specific primer pair for ICAM-1 based on the cDNA sequence published by Staunton et al. [25]. Expression was normalized on the expression of the housekeeping gene β-actin using the primer pair. The sequence of the housekeeping gene has been published by Vandekerckhove and Weber [26] and Ponte et al. [27]. Semi-quantitative analysis of the RT-PCR products was carried out using ion exchange chromatography connected to an online UV spectrophotometer (A260 nm) [28, 29]. This method is also described in more detail by Grether-Beck et al. [17].

Mitochondrial DNA Mutation

Primary human dermal fibroblasts were exposed 3 times per day to sublethal doses of UVA radiation (8 J/cm^2) for 12 days (36 times). Cells were subdivided into equal parts with one aliquot stored at –80°C until extraction of mitochondrial DNA and the second sample was plated to a 10-cm culture dish for culture and irradiation. The generation of mitochondrial DNA mutations was assessed as formation of a large-scale deletion from mitochondrial DNA called the 'common deletion', which had previously been shown to be increased in photoaged human skin and to be inducible by UVA radiation in vitro and in vivo [19, 20]. Assessment of the formation of the common deletion in UVA-irradiated fibroblasts was performed using a semi-quantitative PCR technique as described in detail by Berneburg et al. [19, 20].

Results and Discussion

The studies showed that Ectoin produced an effect on UVA radiation-induced signaling in human keratinocytes and fibroblasts.

Ectoin and UVA Radiation-Induced Second Messenger Release

The data of three independent experiments are summarized in figure 2. It appears that 1 m*M* Ectoin completely prevents UVA radiation-induced second messenger formation.

Ectoin and UVA Radiation-Induced AP-2 Activation

Exposure of dermal keratinocytes to UVA radiation activates the transcription factor AP-2 by enhancing the binding capacity to the promoter region of UVA-inducible pro-inflammatory genes. The results in figure 3 demonstrate that the induction of transcription factor AP-2 after UVA exposure can be suppressed significantly by pretreatment of cells with 1 m*M* Ectoin.

Protection against UV-Induced ICAM-1 Expression

The effect of Ectoin on UVA radiation-induced ICAM-1 expression was investigated. Data from two independent experiments are shown in figure 4. UVA radiation induces an increase in ICAM-1 expression. Pretreatment of keratinocytes with 1 m*M* Ectoin almost completely cancels out UVA radiation-induced ICAM-1 induction at all measured time points.

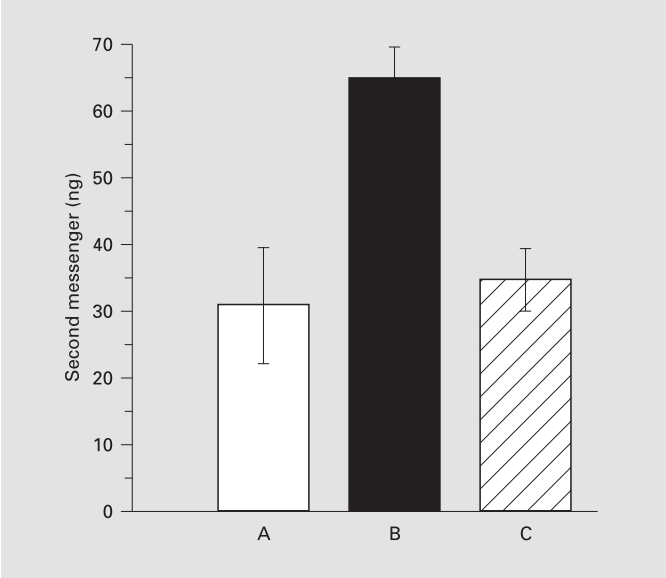

Fig. 2. UVA-induced second messenger (ceramides split off membranal sphingomyelin) release (ng) as a function of different treatments from three independent experiments: A = untreated and non-irradiated control, B = untreated, irradiated with 30 J/cm^2 and C = pretreated for 24 h with 1 m*M* Ectoin and irradiated with 30 J/cm^2. Data given as mean ± SD of released second messenger.

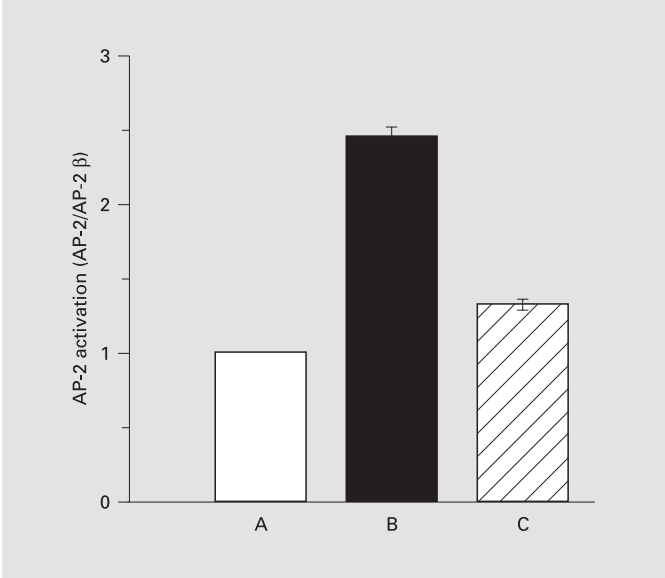

Fig. 3. UVA-induced transcription factor AP-2 activation to AP-2β form with enhanced binding capacity to the promoter of UVA-inducible genes. The amount of the activated transcription factor AP-2 in relation to the inactivated AP-2β and the housekeeping gene β-actin is shown on the y-axis in arbitrary units (n = 2): A = untreated and non-irradiated control, B = untreated, irradiated with 30 J/cm^2 and C = pretreated for 24 h with 1 m*M* Ectoin, irradiated with 30 J/cm^2. Data given as mean ± SD of activated transcription factor AP-2.

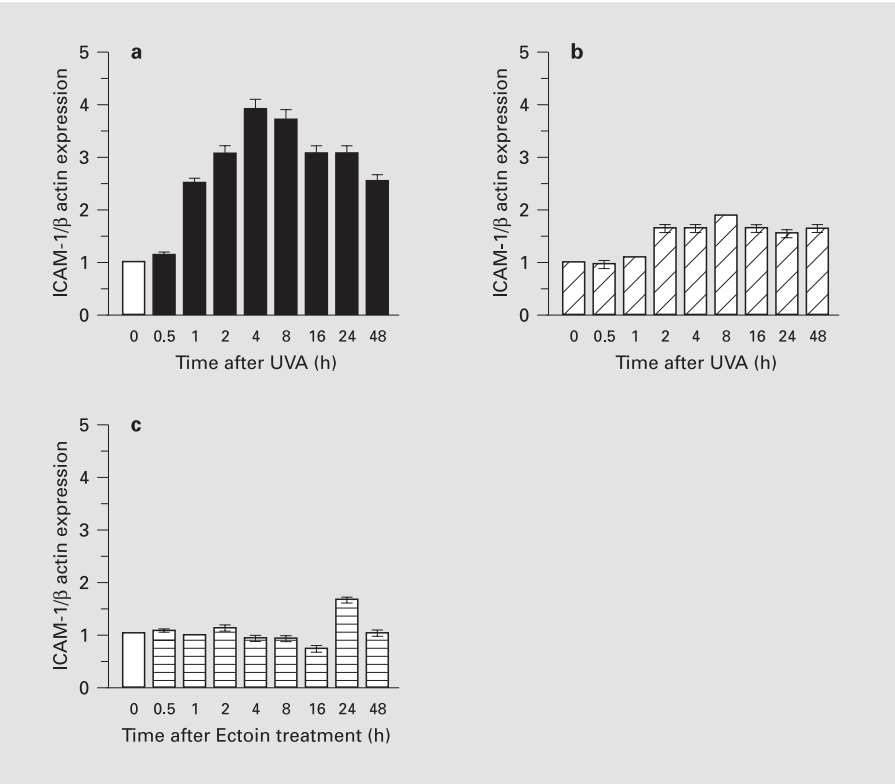

Fig. 4. UVA-induced ICAM-1 mRNA expression in comparison with the inhibition of these expressions by Ectoin pretreatment. ICAM-1 mRNA expression was assessed by differential RT-PCR in comparison to the housekeeping gene β-actin. **a** Untreated and irradiated with 30 J/cm^2. **b** Treated for 24 h with 1 m*M* Ectoin and irradiated with 30 J/cm^2. **c** Treated for 24 h with 1 m*M* Ectoin, non-irradiated. Data from two experiments are summarized in this diagram. Data given as mean ± SD of ICAM-1 mRNA expression.

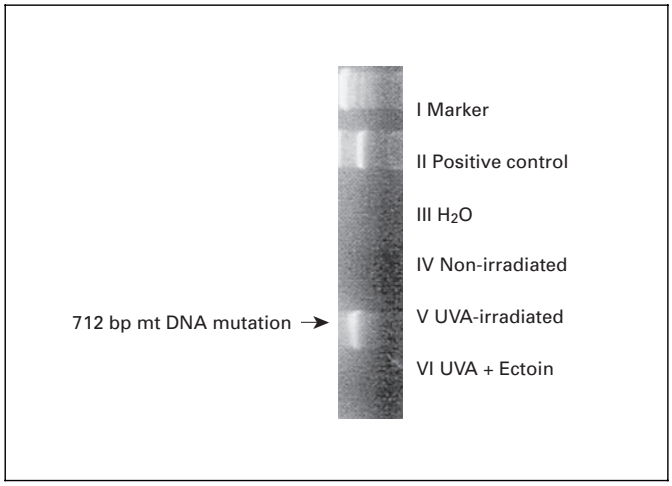

Fig. 5. UVA-induced mitochondrial (mt) DNA mutation. One of two experiments (n = 2) is depicted showing a representative agarose gel with mRNA marker (I), PCR amplifications of representative products representing the common deletion (II), H_2O (III), being non-irradiated (IV), UVA-irradiated (V), and UVA-irradiated (with 8 J/cm^2, 3 times a day, for 4 consecutive days over 3 weeks) and pretreated with 1 m*M* Ectoin over 24 h (VI).

Block of Increased UVA-Induced Mitochondrial DNA Damage in Human Dermal Fibroblasts

Human dermal fibroblasts were irradiated with 8 J/cm^2 of UVA 36 times (see fig. 5). The total cellular DNA was extracted and subjected to PCR, amplifying either the reference fragment representing the total mitochondrial genome or the common deletion. The positive control was a sample of a patient with a disease known to be caused by common deletion. Irradiated control cells (V) were treated identically with regard to medium change and passaging. Ectoin has an effect on the UVA radiation-induced formation of mitochondrial DNA mutations. It appears that UVA radiation is capable of inducing the 'common deletion' as a marker mutation for large-scale DNA deletions in human dermal fibroblasts. In both experiments, pretreatment of cells with 1 m*M* Ectoin was sufficient to completely inhibit UVA radiation-induced mitocondrial DNA mutagenesis.

These studies demonstrate in figure 5 that Ectoin has the capacity to inhibit UVA radiation-induced signal transduction in human keratinocytes in vitro as well as UVA radiation-induced formation of mitochondrial DNA mutations in cultured human dermal fibroblasts.

UVA radiation-induced second messenger generation and AP-2 activation is crucial for keratinocytes for increasing the expression of pro-inflammatory genes such as ICAM-1. Ectoin was also found to inhibit UVA radiation-induced ICAM-1 expression. These studies demonstrate that Ectoin is capable of preventing UVA radiation-induced gene expression in vitro in human keratinocytes. This property indicates that it could – in theory – be used to protect human skin against detrimental effects caused by UVA radiation-induced gene expression including photodermatosis (e.g. polymorphous light eruption) and photoaging. Current mechanistic pathological concepts of photoaging comprise the UV-induced activation of transcription factors AP-1 and NF-κB and the resulting expression of matrix metalloproteinases in human skin [30].

These studies also indicate that Ectoin has the potential to protect mitochondria of human dermal fibroblasts in vitro against UVA radiation-induced mutagenesis. It is known that UVA-induced generation of the common deletion is accompanied by a marked up-regulation of the expression of matrix metalloproteinase-1 [31] followed by skin wrinkle formation [32]. The experimental results presented here, in previous publications [7] and by Lehmann [10] could contribute to demonstrate the potential of Ectoin in providing protection against the photoaging of human skin cells in vitro. Whether this is due to a direct effect on mitochondrial DNA or to indirect effects that are initiated at the level of the cell membrane, which subsequently leads to the formation of mitochondrial DNA mutation, remains to be determined.

Conclusion

By employing different and independent assay systems, the studies reported here demonstrate that Ectoin has the potential to provide protection against the undesired effects exerted by UVA radiation on human skin cells in vitro.

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