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Stimulation of endogenous antioxidant enzymes

ABSTRACT

Skin ageing is induced by an excess of reactive chemical species caused by ultraviolet exposure, by pollutants or as adverse effects of normal cellular metabolism. Protection of the skin is mediated by the transcription factor Nrf2 which activates the expression of antioxidant and detoxification enzymes. Nrf2 is normally repressed by binding to Keap1 in the cytoplasm. In response to chemical stress, Nrf2 is released from Keap1 to migrate into the nucleus for activation of gene expression.

The peptide acetyl-DEETGEF which comprises the binding sequence of Nrf2 to Keap1, can be used to disrupt the complex and this way to stimulate Nrf2 activity to enhance protection against reactive chemical species. For better skin uptake, the peptide was incorporated into solid lipid nanoparticles.

A cream containing the peptide ingredient applied to skin explants was indeed found to up-regulate the expression of Nrf2-regulated genes. In skin explants exposed to UV, the peptide strongly inhibited the formation of sunburn cells and depletion of Langerhans cells. In a clinical study, the cream with the peptide ingredient showed a significant, placebo-controlled protection against UVA-induced DNA damage.

It is well known that lifestyle factors such as sun bathing or smoking and environmental factors such as pollution accelerate skin ageing. The common mechanism involved is based on the formation of reactive chemical species. But this is also the principal mechanism in normal, chronological skin ageing because the energy metabolism in mitochondria continuously produces reactive chemical byproducts. Reactive chemical species are free radicals containing unpaired electrons such as superoxide, hydroxyl or nitric oxide but also non-radical derivatives of oxygen and nitrogen such as hydrogen peroxide. These reactive chemical species finally lead to DNA damage and the formation of oxidised lipids and proteins. The cells in our tissues normally react with antioxidant molecules and an up-regulated expression of antioxidant and detoxification enzymes. This cellular protection system can however not cope with an excess of detrimental chemical species related to unhealthy lifestyle factors. In addition, there is an age-related decline in cellular protection.

Topically applied antioxidants like vitamins C and E can neutralise reactive chemical species but the vitamins are consumed after one reaction only. The better strategy would be to up-regulate the expression of antioxidant and detoxification enzymes which neutralise electrophiles and replenish used cellular antioxidants such as glutathione. The induction of protective enzymes in response to reactive chemical stress is regulated at the transcriptional level (Fig. 1). These cytoprotective enzymes are characterised by a specific gene sequence, called antioxidant response element (ARE). The expression of these proteins is regulated by the transcription factor Nrf2 that binds to the ARE site in the promoter regulatory sequence.[1,2] Nrf2 induces the expression of a number of genes involved in protecting cells against free radicals and oxidative stress-comprising genes involved in glutathione synthesis, protein thiol homeostasis and phase II antioxidant enzymes such as heme oxygenase 1(HO-1) and NAD(P)H dehydrogenase quinone 1 (NQO1).[3]

Under basal conditions, Nrf2 is repressed in the cytoplasm by binding to Keap1 (Fig. 1). In response to toxic chemicals and oxidative stress, the Nrf2-Keap1 complex is disrupted and Nrf2 translocates into the cellular nucleus to activate gene expression. When bound to Keap1, Nrf2 is constantly degraded because Keap1 targets Nrf2 for ubiquitination and degradation by the proteasome.[4] Disruption of the Nrf2-Keap1 complex to avoid proteasomal degradation allows nuclear translocation and expression of ARE-regulated genes. Figure 2 shows the interaction between Nrf2 and Keap1 at the molecular level. The 'ETGE' amino acid motif in the Neh2 domain of Nrf2 is responsible for high-affinity interaction with

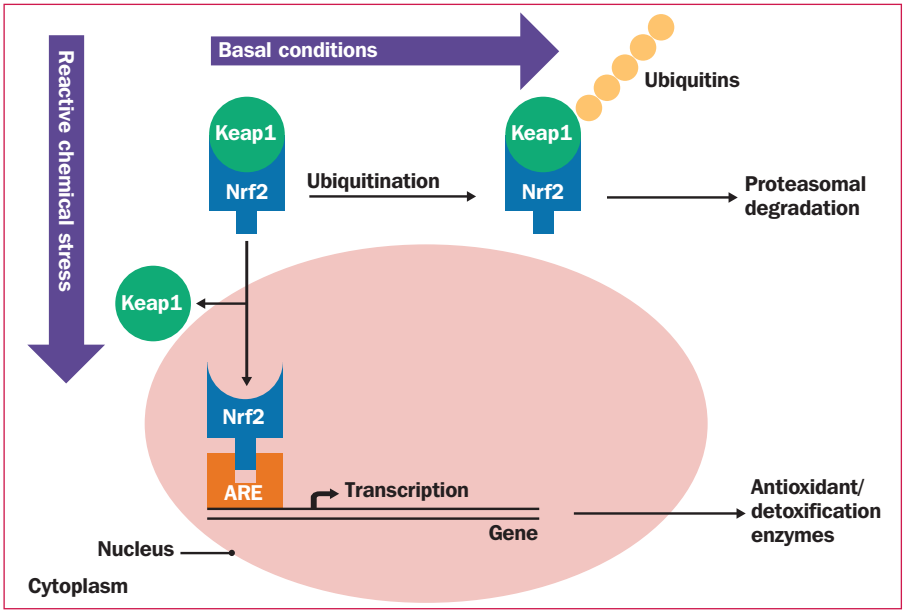

Figure 1: *Transcription factor Nrf2 as central player in the expression of antioxidant/detoxification enzymes.*

the Kelch-repeat domain of the Keap1 protein.[5] Responsible for binding are salt bridges between glutamic and aspartic acid residues in the Neh2 domain and arginine residues in the Kelch-repeat domain.
The peptide acetyl-DEETGEF corresponding to a Neh2 sequence containing the 'ETGE' motif was developed to compete with Nrf2 for binding to Keap1. The heptapeptide was incorporated into solid lipid nanoparticles for stabilisation and for better penetration into the skin and increased cellular uptake. The size of the peptide (868 Dalton) and its hydrophilic nature would otherwise not allow significant penetration into the skin. Solid lipid nanoparticles were originally developed as drug delivery systems for intravenous and parenteral applications. They make possible a controlled release, protection of unstable ingredients and intracellular traffic. The acetyl-DEETGEF peptide in solid lipid nanoparticles (PerfectionPeptide P7; INCI: Acetyl sh-Heptapeptide-1, Glycerin, Butyrospermum Parkii (Shea) Butter, Lecithin, Phenethyl Alcohol, Ethylhexylglycerin, Tocopherol and Water) was tested in cell culture assays and clinical studies for stimulation of the skin's own defence systems against toxic reactive chemicals.

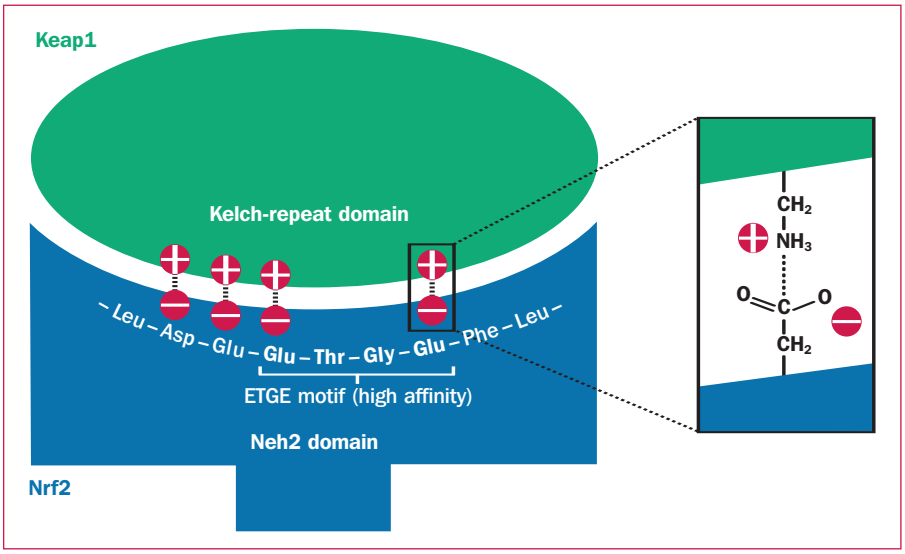

Figure 2: *High-affinity interaction region between Nrf2 and Keap1: responsible for binding are salt bridges between glutamic and aspartic acid residues of Nrf2 and arginine residues of Keap1.*

Methods

Analysis of the expression of Nrf2-regulated genes

A basic O/W formulation with 2% PerfectionPeptide P7 (now referred to as 'acetyl-DEETGEF peptide') was topically applied at 5 mg/cm^2 for 6 hours to human skin explants from an abdominal biopsy. The placebo formulation was applied to control skin explants. For gene expression analysis, 3 punches were performed on each skin explant (n=3) at the end of the incubation. The expression of the Nrf2-regulated genes NAD(P)H dehydrogenase quinone 1, heme oxygenase 1, thioredoxin, thioredoxin reductase 1, peroxiredoxin 1, ferritin light polypeptide and ferritin heavy polypeptide 1 was analysed by RT-qPCR. The PCRs were performed using the LightCycler system (Roche Molecular System Inc).

Analysis of protection against UV-induced formation of sunburn cells and depletion of Langerhans cells

Acetyl-DEETGEF peptide was applied at 0.037% and 0.11% to human skin explants (3 explants per test condition) received from an abdominal biopsy. The explants were pre-treated or not (control) for 24 hours with the test compound (systemically and topically applied) or the reference compound, sun cream SPF 30 (topically applied at 5 mg/cm^2). UV-irradiation was done with 1.25 J/cm^2 UVB and 18.7 J/cm^2 UVA. After irradiation, the treatments with the test and reference compounds were renewed and the explants were incubated again for 24 hours. Formation of sunburn cells was analysed by Hematoxylin-Eosin-Saffron staining and Langerhans cells were detected by immunohistolabelling.

Clinical trial to demonstrate protection against UVA-induced DNA damage

In a clinical study with 10 subjects aged between 42 and 64, a basic o/w formulation with 2% acetyl-DEETGEF peptide was applied twice daily for two weeks on the inner side of the forearm. The placebo formulation was applied to the other forearm. Two hours after the last product application, the test sites were irradiated with 20 J/cm^2 UVA. Immediately after irradiation, suction blister biopsies were generated for immunohistochemical analysis of the DNA damage marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the epidermis.

Results and discussion

Skin explants were used as models to analyse the expression of the typical Nrf2-regulated genes NAD(P)H dehydrogenase quinone 1 (NQO1), heme oxygenase 1 (HMOX1), thioredoxin (TXN), thioredoxin reductase 1 (TXNRD1), peroxiredoxin 1 (PRDX1), ferritin light polypeptide (FTL) and ferritin heavy polypeptide 1 (FTH1). In order to enhance penetration into deeper skin layers, acetyl-DEETGEF peptide was formulated into a basic O/W cream and applied topically. Compared to skin explants treated with the placebo formulation, all of these genes were up-regulated by the cream with acetyl-DEETGEF peptide, up to 105% for NAD(P)H dehydrogenase quinone 1 (Fig. 3). These results demonstrate an efficient competition of the heptapeptide with Nrf2 for binding to Keap1 and the resulting activation of Nrf2. A classical stressor used to induce production of

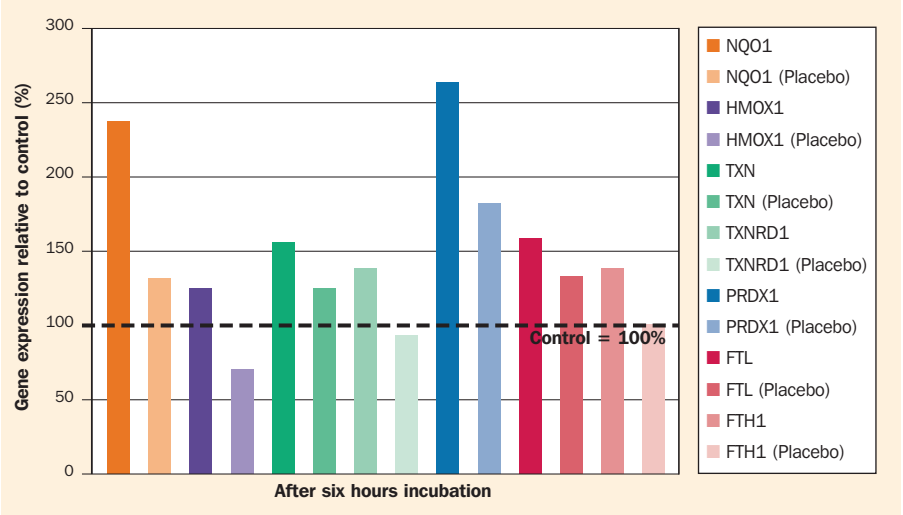

Figure 3: *PerfectionPeptide P7 stimulates expression of Nrf2-regulated genes.*

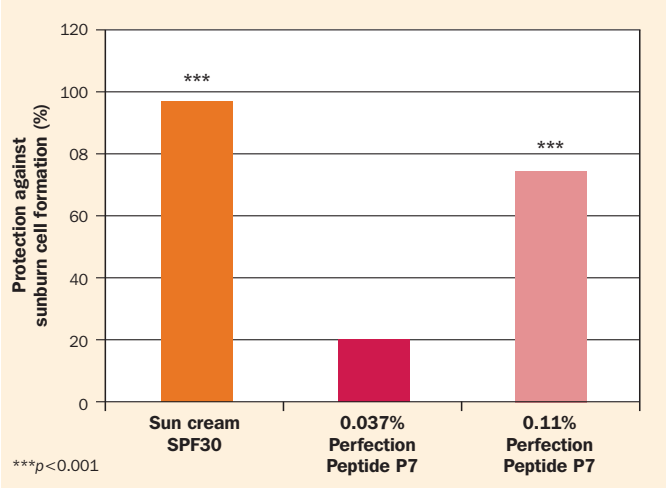

Figure 4: *PerfectionPeptide P7 protects skin against UV-induced sunburn cell formation.*

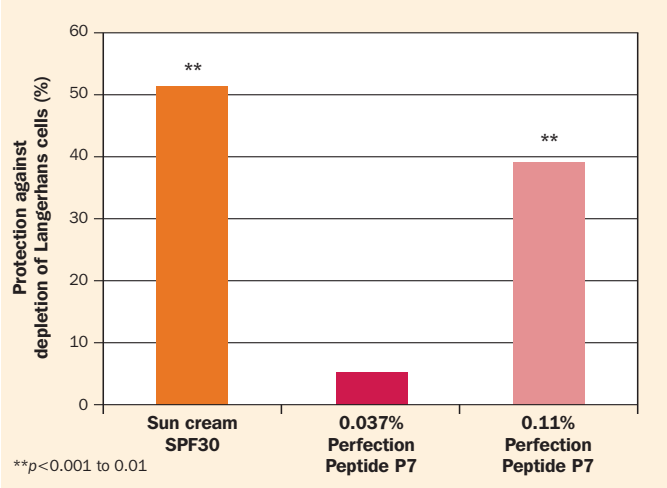

Figure 5: *PerfectionPeptide P7 protects skin against UV-induced depletion of Langerhans cells.*

reactive chemical species is exposure to UV irradiation. Again skin explants were used as models and the formation of sunburn cells and the death of Langerhans cells were analysed as endpoints after UV exposure. Acetyl-DEETGEF peptide was applied topically and systemically at 0.037% and 0.11%, diluted in the culture medium. A sun cream with SPF30 applied topically served as a positive control. Compared to irradiated but untreated skin explants, treatment with 0.11% acetyl-DEETGEF peptide protected skin explants against sunburn cell formation and depletion of Langerhans cells almost as efficiently as the sun cream (Figs. 4 & 5). In contrast to UVB that is damaging also by direct interaction with cellular components, UVA-induced stress is mainly a consequence of formation of reactive oxygen species. These can cause oxidation of DNA bases. The oxidative DNA damage marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) was used to analyse the effect of an o/w cream containing 2% acetyl-DEETGEF peptide in a clinical study. The cream was applied by 10 volunteers to the forearm twice daily for two weeks. After the last product application, the skin was irradiated with UVA. Immediately after exposure, suction blister biopsies were generated. The blister roofs were used for immunohistochemical analysis of 8-OHdG in the epidermis. Compared to the placebo-treated forearm, treatment with acetyl-DEETGEF peptide significantly reduced UVA-induced formation of 8-OHdG (Fig. 6). This result clearly demonstrates the *in vivo* efficacy of the heptapeptide to protect against reactive oxygen species. The effect is based on stimulation of the Nrf2-regulated cellular defence system.

Conclusion

Antioxidants like vitamins C and E are not stable in cosmetic formulations and once applied to the skin, are quickly used. The authors therefore looked for an approach to stimulate the skin's own defence and protection systems. The transcription factor Nrf2 is known as a master switch in cellular defense, protection and repair. Nrf2 is normally repressed by binding to Keap1. Competition with a peptide for binding to Keap1 could increase active Nrf2. In assays with skin explants and clinical studies, the peptide acetyl-DEETGEF was found to stimulate Nrf2-regulated enzymes and to protect the skin against UVA-induced damage which is known to be a consequence of UVA-induced formation of reactive oxygen species. Thus, disruption of the Nrf2-Keap1 complex seems to be a successful strategy to stimulate cellular defence reactions against toxic free radicals.

Formation of reactive chemical species that are damaging our skin is induced by exposure to the sun's UV light and by certain unhealthy lifestyle factors. Beside, this formation is always ongoing as a side effect of the cellular metabolism. Thus, it makes sense to apply daily PerfectionPeptide P7 to protect the skin against premature appearance of ageing signs. PerfectionPeptide P7 fits perfectly with the emerging new trend of DD creams which stands for daily defence. PC

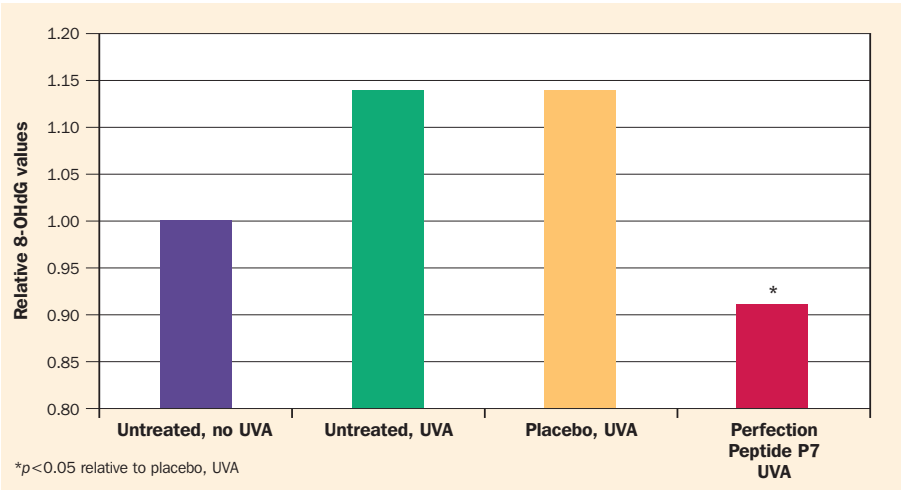

Figure 6: *PerfectionPeptide P7 protects skin against UVA-induced DNA damage.*

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