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Biologically active peptides: from a laboratory bench curiosity to a functional skin care product

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Synopsis
Small, biologically active peptides (short sequences of amino acids) were first described about 40 years ago: TRH, angiotensin, vasopressin, oxytocin, bradykinin. Since then, many more peptides have been isolated from mammalian tissue and organs, and their activity investigated. Essentially, these molecules play a hormonal (messenger) role: released at one point in the body, they act at specific receptor sites at different locations in the organism. Mostly the peptides are transported from the site of release to the site of biological activity through the blood or lymphatic fluid.

The use of these molecules in cosmetics does not appear obvious, as the topical application of these highly soluble, fragile and extremely expensive molecules seems inappropriate, and systemic effects (blood transport) are not desired.

This paper shows that the obstacles to using highly specific, powerful peptides as `actives' in cosmetic products can be overcome. Cosmetically interesting activities such as stimulation of collagen synthesis, chemotaxis, antistinging effects and others, can be observed and substantiated with chemically modified peptide sequences. Long chain fatty acid conjugates improve skin penetration, specific activity and economic feasibility of these molecules.

Résumé
De petits peptides (séquences courtes d'acides aminés), biologiquement actifs, ont été décrits pour la première fois il y a environ 40 ans: TRH, angiotensine, vasopressine, oxytocine, bradykinine. Depuis, beaucoup d'autres peptides ont été isolés à partir de tissus et organes de mammifères, et leur activité a été étudiée. Ces molécules jouent principalement un rôle hormonal (messager): libérées en un point de l'organisme, elles agissent en des sites récepteurs spécifiques à différents endroits de l'organisme. La plupart du temps, les peptides sont transportés du site de libération au site d'activité biologique par le sang ou le liquide lymphatique.

L'utilisation de ces molécules en cosmétique ne semble pas évidente car l'application topique de ces molécules fortement solubles, fragiles et extrêmement coûteuses, semble inappropriée et les effets systémiques (transport par le sang) ne sont pas souhaités.

Cet article montre que les obstacles à l'utilisation de peptides puissants, fortement spécifiques comme ‘principes actifs’ dans des produits cosmétiques peuvent être surmontés. On peut observer (et démontrer) des activités intéressantes pour la Cosmétique (stimulation de la synthèse du collagène, guérison de lésions, chimiotactisme, effets apaisants et autres) avec des séquences de peptides chimiquement modifiés (produits conjugués lipophiles à chaîne longue) lorsque les paramètres de pénétration dans la peau, d'activité spécifique et de faisabilité économique s'associent pour donner de nouveaux principes actifs, tant qu'aucun effet systémique ni pharmacologique (thérapeutique) n'est généré. Des exemples spécifiques de peptides concernant la peau sont donnés.

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Introduction

In the early fifties, du Vigneau and Tuppy isolated the first peptide hormone from the hypophysis and identified it as a cyclic octapeptide of the chemical structure Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GIy-NH_2. This structure was confirmed by synthesis, the peptide was named oxytocin.

Since that pioneering work, research in peptide synthesis has made enormous progress; isolation, followed by chemical, biochemical and biological characterization of hundreds of other peptides of shorter or longer sequences have been published. Table I fists a few of the major peptide families, their sequence and main activity.

Most of these molecules tan be classified as hormones: released at one site of the body, they are transported through blood or lymph vessels and act at specific tells located at different sites. Their activity is usually short-lived; renewal of hormone supply is necessary to sustain cellular activity. Many studies of receptor binding have been carried out to determine the affinity and specificity of the peptides for the target cells; so-called QSAR (quantitative structure activity relationship) studies, often correlated with conformational analysis, were undertaken to understand better the mechanisms of recognition, binding and signal triggering when the peptide interacts with the cellular surface. From all these investigations it became clear that peptides might have tremendous potential for medical and pharmaceutical applications. The use of purified natural insulin to treat diabetes highlighted this possibility, but also underlined the difficulties involved: synthesis vs. extraction, stability, safety and economy.

It is disappointing that so few of the naturally occurring peptide sequences have found therapeutic use. In some ways, these peptides are mostly valuable tools of biomedical/ physiological research, and are thus confined to the laboratory bench.

Peptides and the skin

Most of the peptides mentioned above are specifically cleaved fragments of larger, often much larger, precursor peptides. It has long been thought that these precursor peptides originate mainly from hypophysal or hypothalamic regions, and thus represent real hormones, acting at a distance. More recent investigations have shown that various cell types are often able to synthesize these

Table I. Major peptide families, sequence and main activity

Peptide	Sequence *	Function
ACTH (Corticotropin)	SYSMEHFRWGKPVGKKRR PVKVYPNGAEDESAEAFPLEF	Corticoid synthesis in kidney
Angiotensin II	DRVYIHPF	Blood pressure regulation
Bradykinin	RPPGFSPFR	Vasodilator
Enkephaline:	YGGFL	Analgesic
LHRH	< EHW SYGLRPG-NH_2	Ovulation
Saralasin	Sar-RVYVHPA	Angiotensin II inhibitor
Substance P	RPKPQQFFGLM-NH_2	Pain mediator
TRH	< EHP	Regulates thyrotropin release
Vasointestinal Peptide (VIP)	HSDAVFTDNYTRLRKQMAVKK YLNSILN-NH_2	Gastric secretion

*Sequences given in one-letter amino acid code.

these precursor molecules, to process them into active fragments which then act locally or very close nearby. Instead of hormones then, we should talk of mediators to describe this fact.

It seems that more and more of these biologically active peptide sequences are almost ubiquitous, i.e. found in many peripheral cell types and organs as opposed to only the brain. The skin is not an exception and therefore it made sense to investigate the possible use of peptides in connection with the skin, and thus within the framework of cosmetology.

The major obstacles to the use of synthetic peptides in cosmetic products are related to the question of penetration and diffusion, to long-term stability and to possible long-range effects.

The most important question, of course, is which peptides have potential interest for topical application? Blood pressure regulation (angiotensin, vasopressin), thyrotropin release (TRH), induction of lactation (oxytocin) or stimulation of gastric juice secretion (VIP) do not seem ideal candidates for cosmetic activity. We shah see, however, in the following that there are answers to all of these problems.

Recent investigations

ß-AIa-His (Carnosin)

To begin with, we chose a model peptide of the sequence ß-alanyl-histidine, also called carnosin. This peptide is present in the blood plasma of mammals and is implicated in various biologically activities: wound-healing, protection against oxidation, prevention of cataract, etc.

In view of the fact that a peptide of this small size is highly water soluble, we wanted to find out if significant amounts of the peptide were capable of penetrating the highly lipophilic stratum corneum. For this; it was necessary to determine the skin affinity and diffusion characteristics of this peptide and to test the hypothesis that a specifically modified peptide would prove advantageous in this respect.

We synthesized the molecule by classical peptide synthesis, and then synthesized a chemically modified peptide by attaching a fatty acid drain (preferentially palmitoyl) to the terminal NHZ group. Aliquots of these two molecules were then labelled with radioactive iodine, which were incorporated into solutions of the ‘cold’ peptides as tracer molecules.

Standard Franz diffusion cells were then used to study the diffusion and penetration kinetics of the labelled peptides. The protocol consisted of applying a known amount of peptide solution to the surface of the skin, and analysing the amount of radioactivity distributed into the various skin layers (stratum corneum, epidermis/dermis) and the amount of peptide recovered in the receptor fluid of the Franz cell (transcutaneous flow). Figures 1 and 2 show two important results of these experiments.

The unmodified peptide ß-Ala-His has very low affinity for the skin and does not penetrate beyond the first layer of the stratum corneum, despite its small size. The lipophilic peptide palmitoyl-ß-Ala-His, however, diffuses into the stratum corneum and the epidermal and dermal skin layers.

Neither peptide diffuses beyond the dermal layer, thus there is no significant transcutaneous penetration; therefore, no uptake in blood or lymphatic fluids is to be expected (Figs 3 and 4). For cosmetic purposes these results are important: the modified peptide diffuses quite rapidly to the sites of action, and then stays there. There is no systemic activity.

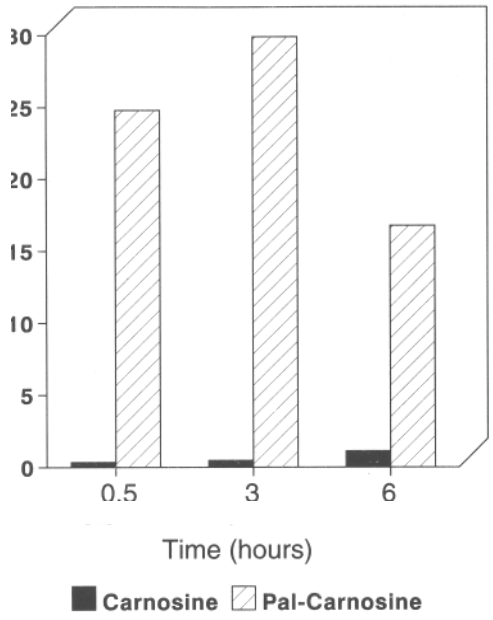

Fig. 1. Total amount of radiolabel found by gamma counter in the sum of the first 10 stratum corneum layers (obtained by stripping), expressed in percent of total label initially applied.

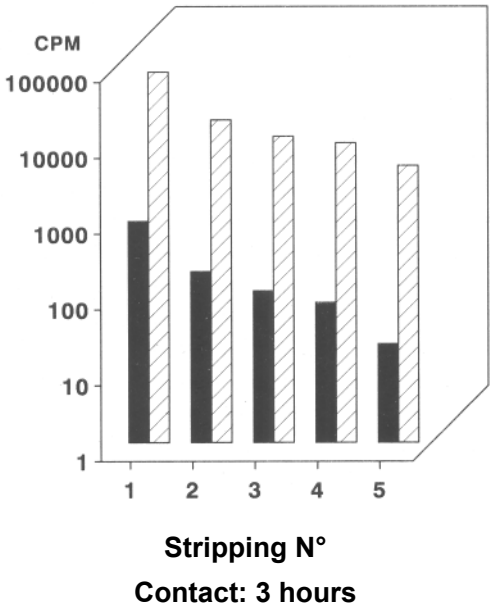

Fig. 2. Radiolabel (counts per minute) found in the successive strips of stratum corneum layers (after 3 h of contact time). Y-axis = logarithmic scale.

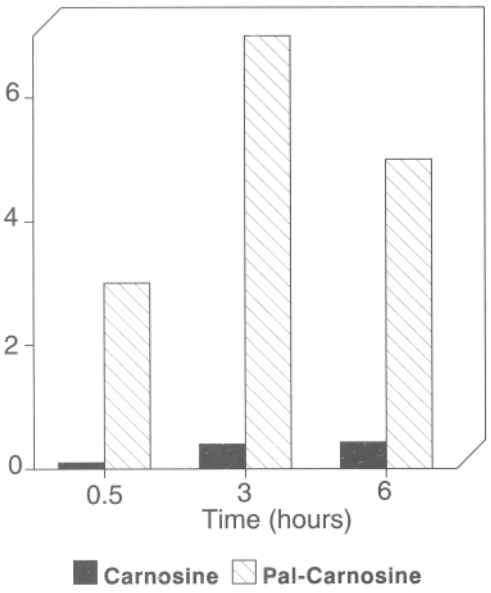

Fig. 3. Total amount of radiolabel found by gamma counter in the epidermis/dermis layers (after stripping of the stratum corneum), expressed in percent of total label initially applied.

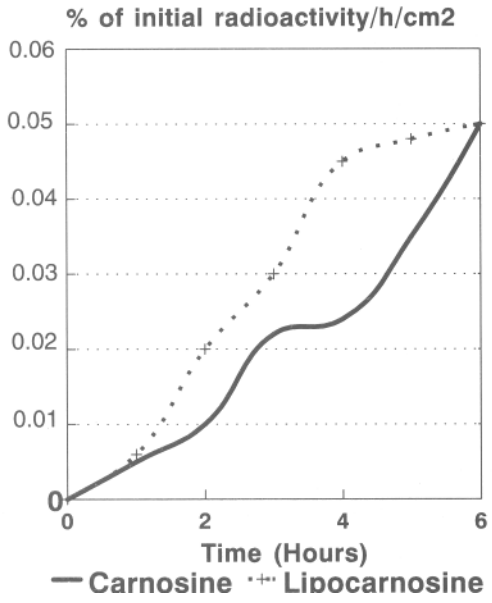

Fig. 4. Radiolabel flux detected in the receptor fluid, expressed as percentage of initial amount per hour per cm^2 of skin surface.

Having found a solution to the first problem (penetration into the skin but not through it), we investigated the application of this concept to peptides that might have activity in cutaneous tissue. We shah describe two examples out of the many peptides studied.

Palmitoyl-Gly-His-Lys

In 1988, a study was published [1] showing that a copper complex of the tripeptide glycyl-L -histidyl-L-lysine activated the synthesis of collagen by fibroblasts in culture. As the tripeptide is found to be a fragment of collagen, released during hydrolysis of collagen by collagenases (in inflammation and wound healing processes), it appears to be a natural feedback signal to fibroblasts to stimulate neosynthesis of tissue matrix molecules.

For cosmetic purposes, therefore, we applied the concept of the palmitoylated peptide. We also dispensed with the copper ion as non-essential.

We synthesized palmitoyl-Gly-L-His-L-Lys by solid phase synthesis and obtained a peptide of high purity (> 97%). Its amphiphilic structure makes the peptide difficult, but not impossible, to solubilize in cosmetic excipients.

In vitro studies. We studied the stimulation of collagen synthesis in human fibroblast culture. Figure 5 shows that the attachment of the palmitoyl-drain did not modify the capacity of the peptide to act on the cells.

A strong signal of collagen synthesis (monitored by incorporation of tritiated proline) is observed at the concentration of 0.5 μm L^{-1}, not far from the active concentrations published previously on the unpalmitoylated peptide.

Ex vivo study. The described *in vitro* result led us to do further studies *ex vivo*[1] and *in vivo*. Histological examination of skin samples (human biopsies from plastic surgery, abdominal tissue) that had been irradiated with daily doses of UVA light for a week shows a strong degradation of dermal

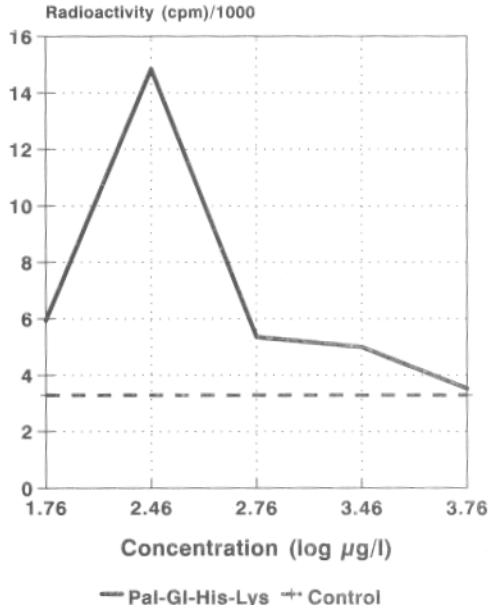

Fig. 5. Incubation of human fibroblasts in presence or absence of increasing concentrations of PalGIy-L-His-z-Lys and 3H-proline. Collagen was recovered and radiolabel was measured by scintillation counter.

[1] *Ex vivo* is the term we apply to studies carried out on living tissue but removed from a whole body organism, i.e. biopsies, explants, reconstituted skin models. It is an intermediate category between pure cell-free systems and/or monolayer cell cultures (*in vitro*) and studies on human volunteers (*in vivo*).

collagen [2]. Treatment of the skin samples after irradiation with retinoic acid (500 p.p.m.) or with palmitoyl-Gly-His-Lys (5 p.p.m.) during the same week, shows almost total preservation and/or renewal of the tissue collagen (Figs 6-9).

In vivo study. An *in vivo* clinical study on 23 healthy female volunteers was then carried out. The peptide was incorporated at 4 p.p.m. concentration into a vehicle, and the cosmetic efficacy of the peptide was tested against the vehicle alone. The parameter measured was skin layer thickness, as monitored by ultrasound echography. After 4 weeks of product application, the peptide-treated site showed a small but statistically significant increase in skin thickness of about 4% (Fig. 10). This value is not negligible, given that the thinning of ageing skin occurs at the rate of about 6% every 10 years [3]. We presently do not know if longer application of the product would lead to further increases in skin thickness or if a plateau has or will be reached.

From a cosmetic point of view, the validity of the use of peptides for topical application was therefore established. Non-toxicity (confirmed in numerous toxicological studies), efficacy (as described above), and stability were found to correspond to the demands of cosmetic use.

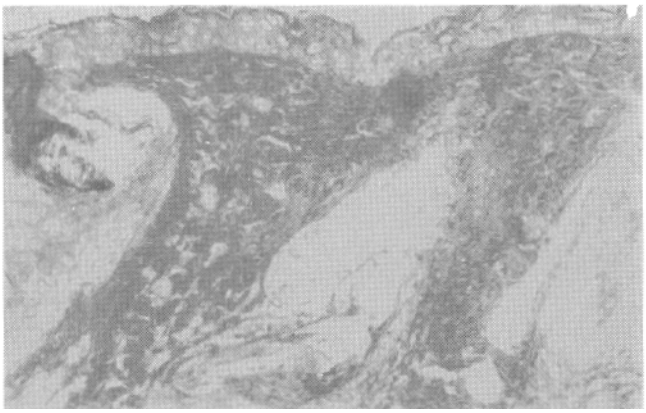

Fig. 6. Histological coloration of skin tissue before UVA irradiation. The dark colour indicates the high density of collagen present.

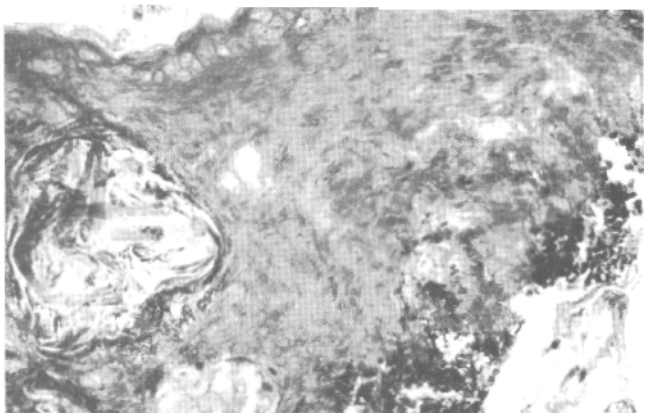

Fig. 7. Histological coloration of skin tissue after UVA irradiation (2.7 Jcm^{-2}, 5 min, 7 days). The significant degradation of collagen is clearly visible.

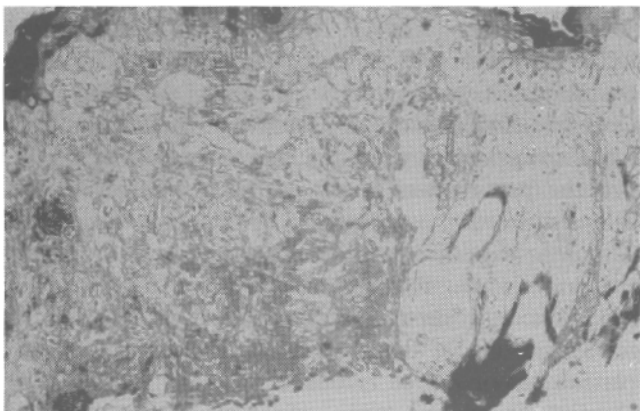

Fig. 8. Identical UVA irradiation as in Fig. 7, but skin treated with 5 p.p.m. of Pal-Gly-His-Lys after each irradiation period. The skin tissue contains a high density of collagen.

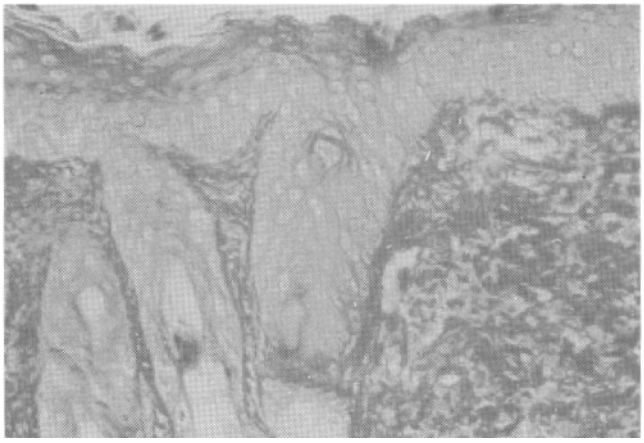

Fig. 9. Identical UVA irradiation as in Fig. 7, but skin treated with 500 p.p.m. of retinoic acid after each irradiation period. The skin tissue contains a high density of collagen.

N-acetyl-tyrosyl-arginyl-hexadecylester

The dipeptide Tyr-Arg was discovered by Japanese scientists at the University of Kyoto, and was named kyotorphin because of its morphine- (or endorphin-) like analgesic activity [4]. When injected into rat brain, this peptide shows strong pain-inhibiting power. It acts through the release of endogenous opiate peptides such as enkephalins that bind to the morphine receptor.

Today, a particular field of cosmetic research is devoted to the phenomenon of 'sensitive skin'. Studies show that sensitivity to climate (heat, cold) or to chemical substances (pollutants, irritants) is a real, consumer-perceived problem. A cosmetic response to this issue might consist of diminishing the sensitivity of the skin to some of these forms of aggression, all the while making sure that no durable effect of analgesia or anaesthesia is obtained.

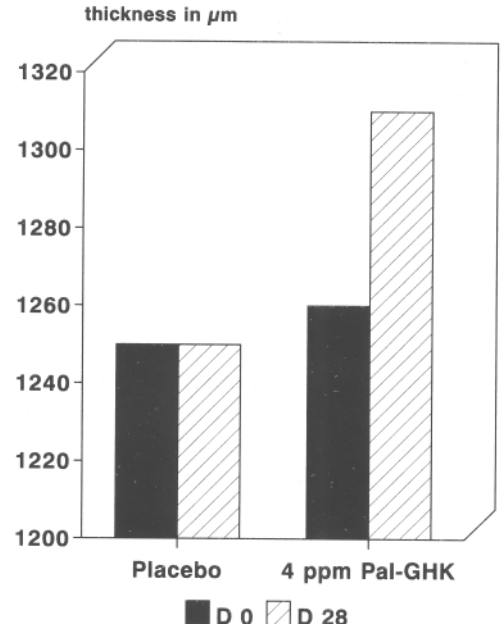

Fig. 10. Variation of skin thickness. Average value *(n* = 23) after 4 weeks of application of a cream containing 4 p.p.m. of Pal-Gly-L-His-L-Lys in a vehicle, compared to vehicle (placebo). Measurement by ultrasound echography (DERMASCAN A) on volar forearm *(n* = 4 measures). The difference between D_0 and D_{28} is significant ($P < 0.05$) for the peptide-treated site and with respect to placebo ($P < 0.05$).

The following points led us to study the dipeptide Tyr-Arg with the aim of cosmetic applications:

- certain skin cells (keratinocytes) are able to synthesize and secrete pro-opio melanocortin, a precursor molecule of ß-endorphin and enkephalins [5]
- immunological studies have revealed the presence of enkephalines at the epidermal/dermal junction, close to the Merkel cells [6]
- Merkel cells are presently thought to transmit mechanical and other nerve signals (pain, heat sensation).

It is therefore worth investigating if the application of a molecule known to stimulate the release of enkephalines in the brain might not do so also at the periphery and thus lead to local decrease in the intensity of signal transmission of heat or irritation. In order to test this hypothesis, we again modified the peptide by attaching lipophilic groups to the ends, thus synthesizing N-acetyl-Tyr-Arg-hexadecylester.

Again, stability tests in cosmetic excipients and toxicological evaluation were carried out before *in vivo* clinical studies on human volunteers.

Heat sensitivity. Twenty-one human volunteers were trained to evaluate their skin sensitivity to heat. A heat generating probe was attached to the skin on the back of the panellist at the height of the shoulder blades; the temperature of the probe was increased by 0.1 °C increments, and the panellists were asked to indicate four levels of heat sensation: warm, hot, very hot, painful. The temperatures at which the

panellists orally signalled these levels were recorded. After cooling, the peptide (300 p.p.m. in a vehicle) was then applied to the skin, and after a waiting period of 2 resp. 4 h, the measurement of the level temperatures was repeated.

The results show a numerically small but statistically significant reduction in sensitivity of the skin to heat, compared to vehicle alone, perfectly in line with cosmetic requirements. For clarity, Fig. 11 shows only the 2 h results. Four hours after product application, the sensitivity-decreasing activity diminishes slightly, but remains significant for the levels hot ($P < 0.05$), very hot ($P < 0.01$) and painful ($P < 0.05$).

Stinging: Capsaicin is a well known molecule, extracted from hot pepper, that is able to elicit a stinging sensation when applied to skin in an appropriate amount and vehicle. We selected nine panellists responding to capsaicin in reasonable manner (some people do not react, others are extremely sensitive to it). As for the previous test, the volunteers established their base line of skin sensitivity to capsaicin. Then one side of the face was treated with the product containing 300 p.p.m. of peptide; the other side was treated with vehicle. After 30 min the capsaicin solution was applied to both sides of the face and the intensities of stinging and burning sensations were recorded over time by the panellists. Figure 12 shows the significant difference in chemical reactivity of the skin that had been pre-exposed to the Tyr-Arg peptide.

Thus, the initial hypothesis has received some support from the clinical, placebo-controlled *in vivo* studies: the Tyr-Arg peptide, appropriately modified for topical application, appears to reduce skin sensitivity to external factors such as heat or chemical irritants.

Further studies are planned to investigate on a more fundamental level the interaction of the peptide with skin cells. Does the peptide release enkephalins locally? What other mechanism might explain the observed effects?

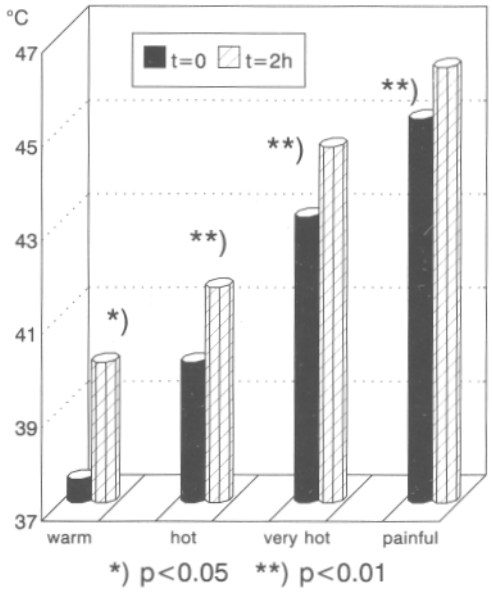

Fig. 11. Mean values of the temperatures indicated by the panellists ($n = 21$) for the four levels of heat sensation at the beginning of the experiment (t = 0 h) and 2 h after product application (t = 2h). Differences of up to 2°C can be observed in the temperatures at which panellist signals a change from one level to the next.

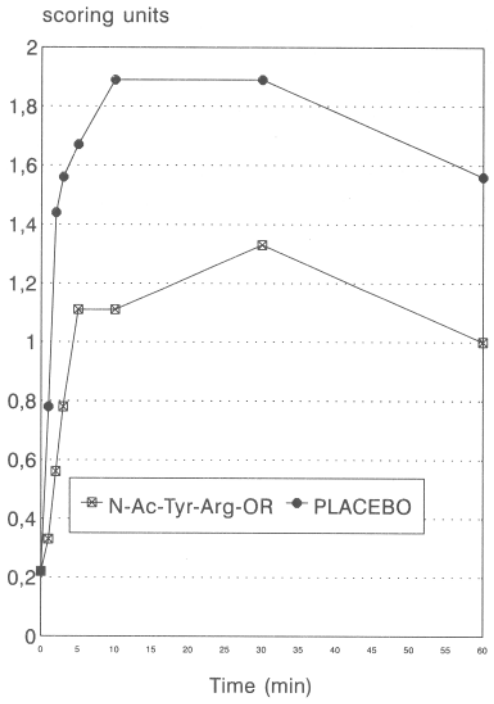

Fig. 12. Mean values of stinging scores (n = 9) vs. time. The capsaicin solution was applied 30 min after application of the products (300 p.p.m. of the peptide in vehicle or vehicle alone). Statistical difference ($P \leq 0.05$, student t-test) was observed for time points 2 min, 3 min and 30 min.

Conclusion

Ten years ago, no peptide chemist would have seriously considered topical delivery of synthetic peptides for cosmetic purposes as a viable strategy. Since then, research has shown that proper modification of peptide sequences allows the peptides to be targeted specifically for the cosmetically important skin layers. Peptide sequences with potential cosmetic activity, wisely chosen, have commercial potential. Non-toxicity and peptide stability can be guaranteed - if not wholesale, then for specifically selected peptides.

The experiments described in the present paper and many other observations clearly show that biologically active peptides can be used in topical cosmetic applications for improving the skin and its properties.

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