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ABSTRACTS OF THE INTERNATIONAL CONFERENCE ON OXIDATIVE STRESS IN SKIN BIOLOGY AND MEDICINE

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OXIDATIVE STRESS, ANTIOXIDANTS AND SKIN

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Healthy aging and disease prevention by antioxidant nutrition is increasingly a subject of academic and public health interest and research. Antioxidants play an important role in maintaining the physiological redox status of cellular constituents. Antioxidants may change their redox state, quench free radicals, be targeted for destruction, regulate oxidative processes involved in signal transduction, affect gene expression and pathways of cell proliferation, differentiation and death. This talk will provide an overview of the antioxidant defense system with special relevance to the skin. The action of antioxidants that interact with the redox antioxidant network will be highlighted, including the bioflavanoid rich pine bark extract, Pycnogenol®, Vitamin E and Carotenoids.

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REACTIVE OXYGEN AND NITROGEN SPECIES AND THE CONTROL OF ONCOGENESIS

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Nontransformed (effector) cells induce apoptosis selectively in transformed (target) cells. This process, originally described for the interaction of nontransformed and transformed fibroblasts, has been termed “intercellular induction of apoptosis”. Meanwhile, this concept of a potential control step during oncogenesis has been shown to be relevant for a variety of other effector and target cell systems. For example, macrophages and granulocytes cause selective elimination of transformed cells, using the same signaling chemistry as fibroblasts. Intercellular induction of apoptosis seems to act selectively on transformed cells, as induction of the transformed state causes induction of sensitivity, whereas abrogation of the transformed state causes insensitivity to intercellular induction of apoptosis. Intercellular induction of apoptosis is independent of direct contact between effector and target cells. Transforming growth factor type-beta triggers the onset of intercellular induction of apoptosis in the fibroblast system, but does not seem to be required by the other effector cell systems. The selectivity, as well as efficiency, of intercellular induction of apoptosis are based on reactive oxygen and nitrogen species (ROS and RNS). Extracellular superoxide anions generated specifically by transformed target cells represent the central element in this signaling system. In a first signaling pathway, target cell-derived superoxide anions spontaneously dismutate to hydrogen peroxide, which fosters HOCl generation by a target cell-derived peroxidase. HOCl then interacts with target cell-derived superoxide anions and generates apoptosis-inducing hydroxyl radicals. In a second signaling pathway, target cell-derived superoxide anions interact with effector cell-derived nitric oxide and form the apoptosis-inducer peroxynitrite. In a third signaling pathway, HOCl and nitrite form toxic nitryl chloride. Our data show that nontransformed effector cells cause selective apoptosis induction in transformed cells through specific ROS and RNS interactions. Thereby, the efficiency and the selectivity of the reaction are determined by target cell-derived superoxide anions. In addition to intercellular induction of apoptosis, transformed cells are eliminated by TGF-beta-triggered, ROS-mediated autocrine/paracrine self-destruction. In this process, the effector molecules, peroxidase and NO, are generated by the transformed target cells themselves and cooperate with their own extracellular ROS to induce apoptosis. Tumor formation seems to require interference with or resistance to intercellular induction of apoptosis and to apoptotic self-destruction. Understanding and subsequent abrogation of tumor cell resistance may have a therapeutic potential.

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THE ROLE OF IRON IN DAMAGE TO CULTURED SKIN CELLS – IMPLICATIONS FOR PROTECTION

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The ultraviolet A (320 – 380 nm) component of sunlight generates an oxidative stress in skin which contributes to both the acute (sunburn) and chronic (aging, skin cancer) effects of sunlight. The damaging effects occur *via* generation of active oxygen species and will be exacerbated by the presence of catalytically reactive iron, so that the observation that UVA radiation causes an immediate release of "free" iron in human skin fibroblasts and keratinocytes *via* the proteolysis of ferritin is likely to be biologically significant. UVA radiation also breaks down heme-containing proteins in the microsomal membrane to release free heme. The well-characterised activation of heme oxygenase 1 (HO-1) by UVA radiation will lead to breakdown of heme and further release of iron. Overall,

these interactions generate a strong oxidative stress on cells. Both the basal and UVA-induced levels of labile iron are 2-4 times higher in fibroblasts than keratinocytes and this is consistent with the higher resistance of keratinocytes to UVA-induced necrotic cell death. This resistance is also consistent with the presence of constitutively high levels of heme oxygenase (HO-2) and ferritin in keratinocytes in contrast to UVA-inducible HO-1 and ferritin in fibroblasts. Modulating cellular iron levels by hemin (to enhance the levels) or iron chelators (to reduce the levels) has the predicted effect on levels of necrotic cell death. Overall, these studies further illustrate the potent oxidising nature of UVA radiation and point to potential strategies for protection.

4

CHOLINERGIC REGULATION OF GENE EXPRESSION IN KERATINOCYTES AND FIBROBLASTS

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The continuous cycle of cell birth and death in the skin is a self-sustaining process controlled, in part, by locally produced acetylcholine (ACh) through the signaling pathways that couple each type of ACh receptors (AChRs) to a particular function of skin cells. Research evidence indicates that cholinergic pathways mediate paracrine signaling between epidermal keratinocytes (KCs) and dermal fibroblasts (DFs), regulating skin responses to environmental hazards. Downstream signaling from nicotinic AChRs (nAChRs) accelerates the biological clock of chronological aging which, in the most part, is counterbalanced by the effects of muscarinic AChRs (mAChRs). Both KCs and DFs respond to ACh *via* classical AChR types that use calcium as a second messenger. KCs and DFs can be simultaneously stimulated through two distinct types of cholinergic signaling pathways: 1) the ionic events, generated by opening of ACh-gated ion channels represented by nAChRs; and 2) the metabolic events, elicited by ACh binding to the G protein-coupled single-subunit transmembrane glycoproteins, or mAChRs. The repertoire of cholinergic enzymes and receptors changes with cell maturation so that, at each stage of their development, KCs and DFs respond to ACh *via* different combinations of AChRs. This diversity could allow the single cytotransmitter ACh to exert diverse effects on KCs and DFs at various stages of their development, which helps explain the plethora of biological effects of ACh in the skin. Nicotine (Nic), too, may use a cholinergic network to accelerate cell senescence by skewing this equilibrium. Nic up-regulates the expressions of p21, cyclin D1, PCNA, Ki-67, Bcl-2 and caspase 3, whereas a3 nAChR deletion is associated with a decrease of p21, cyclin D1, PCNA, Ki-67 and Bcl-2, and an increase of caspase 3, Bax and p53. Chronic Nic exposure alters AChR gene expression in KCs and DFs, and the altered receptor profile leads to aberrant ACh signaling, resulting in changes in cell cycle/state. Skin aging is also associated with changes in the repertoire of cholinergic enzymes and receptors in KCs and DFs.

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NEUROPEPTIDE INDUCTION OF NITRIC OXIDE IN HUMAN KERATINOCYTES *IN VITRO*

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Nitric Oxide (NO) has already been proven to be a critical biological mediator with many physiologic and patho-physiologic functions. Recently, it has been suggested that NO participates in UV-induced immune suppression by mediating Langerhan's cell depletion from the epidermis. It is not clear, however, by what mechanism NO might do this or how NO interfaces with other signaling mediators known to be involved in UV-induced immune suppression such as IL-10, CGRP and TNF-α. The purpose of this study was to investigate the possibility that CGRP, known to be up-regulated in response to UV exposure, stimulates NO production by epidermal keratinocytes. Furthermore, we intended to elucidate any relationship between eNOS, iNOS and nitric oxide production. Exposure of human telomerase immortalized keratinocytes (h TERT) to either CGRP or UVB-induced NO in both treatments, but the time-course was different. CGRP from 0.01 nM to 10 nM was as potent as 1 to 100 ng/ml LPS at inducing NO when measured at time-points earlier than 24 h. SP and VIP (0.1 to 100 nM) were less potent, as was TNF-α. In ELISA study, exposure of keratinocytes to either UVB or CGRP induced iNOS and eNOS, but CGRP up-regulation of NOS was much less marked than UVB. *In vitro*, CGRP-treated keratinocytes displayed a time-course of NO induction that contrasts with keratinocytes exposed to UVB. We conclude that CGRP can stimulate production of NO by two separate mechanisms: an early induction phase, which is independent of iNOS or eNOS protein synthesis, and a later induction phase, which results from iNOS and eNOS up-regulation. Although the exact mechanism is yet to be elucidated, CGRP stimulation of NO may contribute to UV-induced erythema and immune suppression following UV stimulation of neuronal CGRP release.

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NITRIC OXIDE (NO) AND ULTRA VIOLET (UV) ERYTHEMA

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We have demonstrated that UVB (290-320 nm) radiation of human keratinocytes, in the presence of extracellular Ca^{2+}, stimulated both cNOS and XO activities. Because XO generates O_2^- anion that reacts with NO to form the noxious oxidant $ONOO^-$, the chemilugenic substrate luminol was used to evaluate UVB-induced $ONOO^-$ production from human keratinocytes. The potent $ONOO^-$ produced by UVB-irradiated keratinocytes is dependent on the enhanced production of both NO and O_2^-. Inhibition of UVB-irradiated keratinocytes-mediated luminol chemiluminescence by desferrioxamine and L-cysteine further supports $ONOO^-$ as the major oxidant species. Additionally, the efficient inhibition of UVB-irradiated keratinocyte-mediated luminol chemiluminescence by urate is consistent with $ONOO^-$ being the oxidant species. We also demonstrated that $ONOO^-$ increased cGMP synthesis in cultured human keratinocytes and that $ONOO^-$, or its by-products released within keratinocytes, may interact with the various components of these cells.

Our findings demonstrating that, in a defined system consisting of purified cNOS and purified XO, the formation of NO and O_2^-, respectively, was increased after UVB radiation, are consistent with the widespread signal transduction system which involves Ca^{2+}/calmodulin-regulated NO and $ONOO^-$ formation and activation of sGC. These reconstitution experiments were also verified by placing purified NOS and purified XO in a special incubation chamber, with two compartments separated by a thin teflon membrane through which NO and O_2^- gas could pass. $ONOO^-$ increased when irradiated XO and cNOS were placed in the opposite compartment.

In summary, our studies are broadly viewed as refocusing the concept of preventing "erythema and photoaging" to preventing the increase of skin oxidative stress following UV exposure.

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SKIN: FIRST BARRIER AGAINST ENVIRONMENTAL POLLUTION. SHOULD OZONE CONCERN US?

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Ozone (O_3) is among the most reactive environmental oxidant pollutants to which cutaneous tissues are exposed. O_3 exposure has been shown to induce antioxidant depletion as well as the oxidation of lipids and proteins within the outermost skin layer, the stratum corneum. However, relatively little is known regarding the potential effects of O_3 on the cellular constituents of the underlying skin epidermis and dermis. In the present study, hairless mice exposed for 6 h to 0.8 ppm O_3 showed increases in lipid peroxidation, as quantitated by increases in 4-hydroxynonenal-protein adducts. O_3 exposure caused an induction of the stress proteins HSP27 and heme oxygenase-1 (HO-1), starting at 6 h and increasing up to 18 h after O_3 exposure. This was accompanied by an increase in matrix metalloproteinase-9 (MMP-9) mRNA and activity levels and induction of iNOS, indicative of possible injurous-reparative processes. Collectively, our data demonstrate that skin exposure to O_3 not only affects antioxidant levels and oxidation markers in the outermost stratum corneum layer, but also induces cellular stress responses in the deeper cellular layers of the skin.

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WHAT IS THE PRINCIPAL ROLE OF ELASTIC FIBERS IN ANIMAL SKIN?

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Initial studies with mice suggested that specialized epithelial cells lining the hair follicle were responsible for virtually all the non-vessel-associated elastin in the dermis of these animals. Studies were conducted to determine if this was true for all animals and perhaps to rationalize the reason for this distribution.

Avian species have a different architectural arrangement of elastin fibers from other animals, but here also the elastin is either derived from, or associated with, the feather follicle. All feather follicles are surrounded by a fine network of elastic fibers, which are attached at various sites along the follicle to discrete bundles of smooth muscle, thereby interconnecting each feather follicle. The smooth muscle bundles act like a fulcrum to raise and lower the feathers in response to a stimulus and take the place of the pilli muscle in other animals. In non-feathered areas, a cable-like structure, constructed of alternating stretches of elastic bundles and smooth muscle bundles, leads from the apex of one feather follicle through the

apteral area to the apex of the next closest feather follicle, where they are again interconnected through elastic fibers. The function of elastic fibers in the dermis of avian species is almost assuredly to assist in coordinating feather movement and maintaining the feathers in a precise position.

The dermis of over 20 different mammals was investigated and here, too, we suggest that the primary role for elastin in the skin of most mammals is directed toward hair movement. In species such as the horse, cow, mouse or deer with single, uniform hair follicles, the erector pilli muscle lies adjacent to each single hair follicle and is attached at the lower end of the follicle through elastic fibers. The other end of the pilli muscle transforms into elastic fibers just under the epithelium, where it is firmly embedded in the matrix. Flowing out from the hair follicle, and down virtually its whole length, are elastic fibers that appear to tie into fibers stretching out from adjacent follicles. When the pilli muscle is stimulated to contract, all follicles move in unison, due to the elastin network which ties them together. In wool-bearing animals with one major hair follicle adjacent to bundles of much smaller follicles, only the major follicle is directly attached to the pilli muscle. This would include sheep, rabbits, foxes, raccoons and some dogs. The small, woolly hairs have their own fine elastic network which interconnects these fine hair follicles as well as connecting the whole bundle to the main follicle – a very efficient means of interconnecting all hair follicles with a minimum number of pilli muscles. Each of the species studied had its own unique architectural arrangement, yet they all suggested that the primary function of elastic fibers in skin is either feather or hair movement. Any elasticity of the skin contributed by elastic fibers may simply be a residual of the intended function. We were unable to show this exclusive relationship with human and porcine skin.

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REDOX-RELATED RESPONSES IN MICE SKIN AFTER A WHOLE BODY HIGH DOSE X-RAY IRRADIATION

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The skin is at risk of photo-oxidative damage due to the generation of various ROS. Ionizing radiation also causes damage to the skin such as inflammation, loss of hair and cancer in the worst case. In these pathological processes caused by ionizing radiation, formation of free radicals is thought to be a primary event. Therefore, it is important to study the free radical generation in the skin caused by oxidative stress such as an ionizing radiation. Since the direct *in vivo* spin trapping method to monitor the free radical generation in the skin is not established yet, we try to detect products, the amount of which is regulated or affected by the oxidative stress. In the present study, GSH/GSSG ratio, iNOS protein and Bax protein expressions, and ascorbyl radical formation in mouse skin were measured after whole body X-ray irradiation.

Hairless mice (male, 6-10 weeks old) were used for the experiments. After the whole body 50 Gy irradiation, the skin at the back of the mouse was pealed off and immediately frozen in liquid nitrogen. The frozen sample was weighed and crushed to a powder. For measuring GSH and GSSG, the powder was mixed with a buffer containing 6.8% meta phosphoric acid and 1 mM bathophenanthroline disulfonic acid. The homogenate was derivatized with dinitro- fluorobenzene for HPLC detection of GSH and GSSG. For the Western blotting analysis, the sample was prepared by mixing the powder with a buffer containing protease inhibitors. For the measurement of ascorbyl radical, the skin peeled off was directly set in a tissue cell and the ESR spectrum was measured with an ESR spectrophtometer (FR30, JEOL).

The GSH/GSSG ratio in the skin at 2 h post irradiation was significantly decreased, indicating that severe oxidative stress was challenged on the skin by the high-dose X-ray irradiation. By the irradiation, iNOS protein expression was increased to 150% of the control level at 1.5 h post irradiation and then it was decreased to the original level. In contrast, Bax protein, an apoptosis indicator, was gradually increased and reached a maximum, about 300% of the control level, at 72 h post irradiation. The ascorbyl radical in the skin was also increased gradually by the X-ray irradiation. Since the increase of Bax protein reflects the progress of apoptosis, the parallel time-course of ascorbyl radical and Bax protein suggests the positive association between free radical formation and apoptosis. The change in the iNOS expression is an earlier event than the appearance of apoptosis, and it is not clear if it is related to the later apoptosis or not.

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THE EFFECTS OF UVB IRRADIATION ON THE PRODUCTION OF NITRIC OXIDE (NO) IN MALIGNANT MELANOMA

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This study investigates the production of nitric oxide (NO) in malignant melanoma before and after UVB 100 mJ/cm^2 exposure. It also reports the activities of nitric oxide synthase (NOS) and xanthine oxidase (XO) with relation to oxidative stress and total antioxidant capacity. *Materials and Methods*: Tissue samples of malignant melanoma were used and compared to normal tissue samples of corresponding weight. Trials were performed on all samples before and after UVB 100 mJ/cm^2 exposure for 10 minutes. NO was determined using luminol-enchanced chemiluminescence (Berthold LB953). NOS and XO activities were measured by a method based on the oxidation of scopoletin that can be detected fluorophotometrically. Oxidative stress was estimated using t-butylhydroperoxide (t-BHP) in a luminol-enhanced chemiluminescence. Finally, total antioxidant capacity was estimated by means of a chemiluminescence reaction using sodium perborate, $NaBO_2$-H_2O_2, in the presence of horseradish peroxidase. *Results*: The production of NO by the melanoma tissues was six times higher than that of the corresponding weight normal/control tissues. NOS and XO activities were approximately three times that of the normal/control. Oxidative stress was approximately five times that of the normal/control, whereas the total antioxidant capacity was 472 min as compared to the normal tissue value of 520 min, suggesting a decrease in total antioxidant capacity of melanoma. The effect of UVB 100 mJ/cm^2 irradiation for 10 minutes on both malignant melanoma and normal/control tissues showed a marginal increase in the production of NO, as well as an increase in the activities of both NOS and XO. *Conclusion*: NO is directly related to the homeostasis of the skin and is capable of modifying several skin reactions such as vasodilatation, melanogenesis and defense mechanisms against environmental challenges. Our results show that the increased activity of NO, NOS, XO and oxidative stress, as well as the decrease in antioxidant capacity after UVB irradiation, are the major factors with regards to skin damage. This was seen in both malignant melanoma tissues and the normal/control tissues. It appears that NO has a dual role. It can contribute to carcinogenesis *via* chronic inflammation, either by increasing the concentration of nitrosocompounds locally or directly by damaging the DNA with strand breaks and mutations. However, NO also possesses a protective role in the skin in which it removes damaged cells, thereby contributing to apoptosis. Knowledge of the dual role of NO promises to be a significant source in the understanding of the biochemistry of malignant melanoma.

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PURINE NUCLEOTIDES AS REGULATORS OF THE OXIDATIVE EQUILIBRIUM OF FINITE AND IMMORTAL ANIMAL CELL LINES: A MECHANISM FOR CONTROLLING CELL DIVISION?

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In all eukaryotic cells, free radical species are produced as by-products of normal metabolism, during the mitochondrial transport of electrons to molecular oxygen. Using two animal cell lines (LT and Vero), we demonstrated, for the first time, that the intracellular dilution of reactive oxygen species is one of the driving forces for cell division. Furthermore, we have identified a novel group of proteins that are induced in cells, tissues or organisms treated with purine nucleotides such as ADP or ATP. The addition of the proteins has been associated with a remarkable change in cell number (3- to 4-fold compared to control). Changes in certain mitochondrial functions may trigger the mitotic process when the accumulation of free radicals exceeds a threshold value. This finding justifies cell division in an evolutionary context and could offer an explanation for the (repeatedly documented *in vitro*) antimitotic properties of various antioxidants, since their ROS-scavenging effect would deprive cells of a major mitotic stimulus. On the other hand, low antioxidant dosages might exert an opposite, mitosis-promoting effect by adjusting free radicals at a non-toxic concentration. From a practical point of view, cell division could be controlled by regulating free radical concentration through the administration of oxidants, the partial degradation of biological membranes or the promotion of cellular metabolism.

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THE NF-Î B TRANSCRIPTION REGULATOR AS A THERAPEUTIC TARGET IN CHRONIC INFAMMATION

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NF-Î B is a collective name for dimeric transcription factors (TFs) comprising members of the Rel family of DNA-binding proteins that recognize a common sequence motif. In unstimulated cells, NF-Î B resides in the cytoplasm due to its interaction with inhibitory proteins, called IÎ Bs. NF-Î B is a key regulator of inducible gene expression in many different cell types. It is activated in response to a wide variety of agents including mitogens, inflammatory cytokines, bacterial/viral products and oxidative/physical stress. Upon receipt of an appropriate stimulus, the IÎ B protein is specifically phosphorylated by the IÎ B kinase(s) and subsequently poly-ubiquitinated and degraded by the 26S proteasome. Released, dimeric NF-Î B is translocated to the nucleus where it activates transcription of target genes. A large number of these genes play a role in several aspects of

cellular immune responses and inflammatory processes (*i.e.*, genes encoding adhesion molecules, immunoreceptors, cytokines, acute phase proteins). Additionally, several viruses such as HIV-1/2 and CMV utilize NF-Î B TFs to regulate their gene expression. The NF-Î B/IÎ B system has received considerable attention, because of its pivotal contribution to physiological cellular development and function, and its involvement in a wide gamut of pathogenic conditions. Accordingly, NF-Î B TFs have been discussed in the context of a variety of clinical situations, ranging from cancer and AIDS to rheumatoid arthritis, septic shock and various autoimmune diseases. In this vein, the activity of NF-Î B TFs has been shown to be influenced by well-known therapeutic drugs, such as glucocorticoids, nonsteroidal anti-inflammatory drugs, tacrolimus and the anti-psoriatic drug anthralin. Unfortunately, from the standpoint of individual disease treatment, NF-Î B TFs are not unique targets for these drugs, and the side-effects associated with their use comprise a well-documented issue in clinical praxis. The exploitation of accumulated information concerning NF-Î B structure and molecular details of the delicate biochemical machinery regulating NF-Î B activation, open novel routes for the development of highly specific and potent inhibitory drugs. For example, the mechanism by which IÎ B proteins interfere with NF-Î B function provides a selective target for the design of small-molecule ligands "grouping" steric/chemical features that are indispensable for its execution. IÎ B can retain NF-Î B protein complexes in the cytoplasm by masking the NLS on the NF-Î B subunits. NLS masking excludes recognition by nuclear import machinery, therefore interfering with the active transport of these proteins to the nucleus. Re-masking of the NLS in the released, after IÎ B destruction, NF-Î B subunits by direct targeting of the responsible region may prevent the TF from reaching the nuclear compartment and exercise its effect on transcription. Clearly, the continual insights into the molecular and biochemical basis of this rapid response system will pinpoint additional important "microloci" within the structural framework of its constituents and will guide our thinking about potential inhibitors to antagonize NF-Î B activity in different subcellular compartments or at defined stages of activation.

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MOLECULAR DISSECTION OF THE SIGNALING PATHWAYS IN PHOTODYNAMIC THERAPY-INDUCED APOPTOSIS OF CANCER CELLS

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Photodynamic therapy (PDT) is an attractive, emerging therapeutic procedure suitable for the management of a variety of tumors and non-malignant disorders. PDT involves the administration of a photosensitizing compound (photosensitizer) which specifically accumulates in the tumor or in hyperproliferating tissue, followed by irradiation with visible light of the appropriate wavelength, usually in the red or near-infrared region. Light irradiation of the photosensitizing drug leads, in the presence of molecular oxygen, to the photo-generation of reactive oxygen species (ROS), which ultimately kill the target cells. Hypericin, a naturally occurring photosensitizer isolated from St. John's wort (*Hypericum perforatum*) with promising activities in clinical PDT, is an efficient inducer of apoptosis in tumor cells, both *in vitro* as well as *in vivo*. Photosensitization of hypericin, which localizes in cytosolic membranes of the endoplasmic reticulum and golgi apparatus, results in rapid cellular calcium overload, disruption of the mitochondrial membrane potential along with the release of cytochrome c from mitochondria and activation of the apoptosome, leading to cell death. Concurrently to the mitochondria-mediated caspase activation cascade, photosensitized hypericin also induces the sustained activation of the p38 MAPK signaling pathway, which initiates a cellular rescue response against PDT-induced apoptosis. Cyclooxygenase-2 (COX-2) has been recently identified as a down-stream target of the p38 MAPK. COX-2 up-regulation and the release of prostaglandins in response to PDT with hypericin depends specifically on the activation of p38 MAPK, which extends the half-life of the COX-2 transcripts. The current knowledge on the signaling pathways regulating the cell death/survival balance following hypericin-mediated PDT will be discussed.

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OXIDATIVE DNA DAMAGE, FACTORS DETERMINING THE RATE OF FORMATION, REPAIR OF OXIDATIVE DNA DAMAGE AND THE RELATION TO CANCER DEVELOPMENT

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Skin ageing and skin cancer are epidemiologically closely linked to ultraviolet light (UV) exposure from the sun. Epidemiology shows that repeated sunburn in childhood, ambient sun exposure (latitude) and genetic pigmentation of the skin are the most important factors for cancer development, particularly malignant melanoma and basal cell carcinoma. Molecular biology tells us that genetic

alterations (mutations) are necessary events in carcinogenesis. Genomic stability denotes a condition where protective and repair mechanisms are able to maintain the genome at a low level of changes, whereas genomic instability denotes a situation where these mechanisms are insufficient and believed to be able to lead to cancer development. The most evident type of damage to the skin is believed to be oxidative stress from UV light; particularly UV-B light clearly leads to cyclobutane dimers and at least 6 modifications that are potentially mutagenic. It is recognised that changes from oxidative stress occur at a rate that would render the genome oxidized within a few years, and that DNA repair is an extremely important process operated by a variety of different mechanisms including back-up systems: direct reversal, base-excision repair, nucleotide excision-repair, mismatch-repair, repair of double-strand breaks. Each of these systems seems to have several sub-types, and different responses of the repair systems may explain why the result of UV is sometimes cancer and sometimes ageing. Methodologies to measure oxidative damage, DNA repair and the mutations that are consequences of UV exposure are challenging from a methodological approach, particularly in the *in vivo* situation. Understanding the mechanisms of damage and repair are of great importance as the number of individuals with skin cancer is steadily growing, and because preventive and protective measures are difficult.

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LIPIDS AND BARRIER FUNCTION IN HUMAN SKIN

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Approximately 10% of the dry weight of human stratum corneum from most regions of the skin consists mainly of a mixture of ceramides, cholesterol and fatty acids. These lipids are in the intercellular spaces of the stratum corneum and determine the permeability barrier properties of the tissue. Because of their location at the skin periphery, these lipids must resist oxidative damage on exposure to the atmosphere. Accordingly, the free fatty acids and most of the acyl chains in the ceramides are saturated. The trans double-bonds present in some of the long-chain base components of the ceramides and the isolated double-bond in cholesterol, although potentially oxidizable, are not particularly reactive. There are, however, three structurally unusual acylceramides in human stratum corneum that contain ester-linked linoleic acid (C18:2Δ9,12). When isolated, these acylceramides are rapidly oxidized on exposure to air. However, there is no evidence for acylceramide oxidation in the stratum corneum, presumably because they are protected by vitamin E. All three acylceramides contain 30- through 34-carbon ω-hydroxyacids with linoleate ester-linked to the ω-hydroxyl group. They differ in the nature of the base component. The most studied acylceramide (CER EOS) contains sphingosine. One acylceramide (CER EOH) contains 6-hydroxy-sphingosine and the third acylceramide (CER EOP) contains phytosphingosine. The main unit of organization of the intercellular lipid in the stratum corneum is a trilaminar 13 nm repeat unit that appears to include broad-narrow-broad lucent bands on transmission electron micrographs. CER EOS has been shown to be essential for formation of this trilaminar unit. Little is known of the roles of CER EOH and CER EOP. A new molecular model to account for the role of CER EOS in stratum corneum lipid organization and barrier function will be presented.

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MECHANISMS FOR IMPROVING DERMAL DRUG ABSORPTION

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The main barrier properties of the skin are in the outermost layer, the stratum corneum. This is a unique and remarkable membrane that has evolved to prevent excessive water loss from the body and ingress of xenobiotics into the body. The main reasons for the excellent barrier properties are: (a) a tortuous pathway for permeation; (b) structured lipids within the tortuous pathway (Repeated partitioning across the lipid bilayers or Slow diffusion through the polar headgroup regions of the lipids).

There are passive and active mechanisms for improving dermal absorption. The passive processes include increasing the thermodynamic activity state of the permeant in the formulation, modifying the solubility properties of the skin lipids (with simple solvents) and disrupting the structured skin lipids, thereby facilitating diffusion. Often it is difficult to demonstrate exactly which mechanism dominates and biophysical techniques can be used to try and separate the different effects. In addition, synergy can be seen if more than one enhancement process occurs. For example, a combination of a 'solvent' and 'lipid disrupting' effects will cause a multiplicative increase in the permeant flux. A novel infrared technique coupled to chemometric analysis can be used to follow the various events that take place.

It is perhaps surprising that more effort is not given to the rational design of topical agents. The physicochemical parameters that optimise dermal delivery have been examined and simple rules are available. Small molecules that have a log (octanol water partition coefficient) around 2.00, have low melting point and are soluble in both oils and water

permeate well. There are very few dermal and transdermal (with the exception of nitroglycerin and nicotine) drugs with these characteristics. Future developments should take more account of the impermeability of the skin and how delivery can be optimised with judicious choice of both the active and the formulation. Both should take due consideration of the important physicochemical properties of the active.

Active mechanisms include the use of iontophoresis, electroporation, ultrasound, radio frequency, and physical damage such as microneedles and laser ablation. It may be possible, in the future, to couple these types of delivery system to small analytical sensors that will sample the blood (such as the GlucoWatch) and administer the active in response to the biological need.

Over recent years, there has been a marked increase in the sensitivity and sophistication of biophysical techniques. This has allowed a much better understanding of the process of skin permeation, such that it is possible to understand it at a molecular level. These advances will continue and allow us to generate topical and transdermal formulations that are far more effective.

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LOW MOLECULAR WEIGHT ANTIOXIDANTS RELEASED FROM THE SKIN'S UPPER LAYER: AN AGE-DEPENDENT PHENOMENON

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Skin is one of the tissues most exposed to oxidative stress both from endogenous and exogenous sources. Therefore, it can be speculated that skin should possess an extremely efficient antioxidant defense mechanism, particularly in its upper layers. The present study shows that human and rat skin surface possess a unique antioxidant activity, which can be washed out into the surrounding environment. Non-invasive measurements, based on a well containing extraction solution placed on the surface of the rat back skin and human frontal wrist, were conducted. The results indicated that skin releases low molecular weight antioxidants from its outer layers into the solution. Cyclic voltammetry measurements have shown that rat skin releases three major groups of reducing antioxidants at peak potentials of 476 and 889 and 1044 mV, while human skin releases two major groups at peak potentials of 779 and 1068 mV. The lack of a first anodic wave in human skin and the difference in peak potential of the second wave suggests that the type of scavengers released from rat skin are different from those released from human skin. The overall concentration of the low molecular weight antioxidants (LMWA) secreted was found to decrease significantly with age. The major components of the LMWA, composing the first anodic wave in rats, were identified as uric acid and ascorbic acid. Human skin was found to release uric acid in relatively low concentrations that could be detected only in HPLC equipped with an electrochemical detector. Ascorbic acid was not released in human skin. Differences in the ability to release high levels of LMWA among species were well correlated with their metabolic rates. It is suggested that the released LMWA may serve as a possible marker for aging of the skin.

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CELLULAR SENESCENCE AND ADAPTIVE ANTIOXIDANT DEFENSE IN PHOTOAGING OF THE SKIN

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As the skin is always in contact with oxygen and is increasingly exposed to environmental and artificial ultraviolet (UV) irradiation, the risk of photooxidative damage induced by reactive oxygen species – finally leading to phototoxicity, photoaging and skin cancer – has increased substantially.

The term reactive oxygen species (ROS) includes oxygen-centered radicals like the superoxide anion radical and the hydroxyl radical, but also nonradical species such as hydrogen peroxide and singlet oxygen – all being produced in skin upon UV irradiation. In response to the attack of reactive oxygen species, the skin has developed a complex antioxidant defense system including enzymatic and non-enzymatic antioxidants. As a first line of the enzymatic antioxidative defense, superoxide dismutases reduce superoxide anion radicals to hydrogen peroxide, which subsequently is detoxified to water by catalase and glutathione peroxidases.

We were interested whether the antioxidant enzymes manganese superoxide dismutase (SOD2) and glutathione peroxidase (GPx1) are inducible upon UV irradiation and whether repetitive UV exposure, as practiced for the light-hardening during phototherapy of photodermatoses, can even enhance the adaptive antioxidant response. To address this question, skin fibroblasts and keratinocytes were exposed *in vitro* to single and repetitive UV low-dose irradiation in different time-intervals and afterwards challenged by high-dose irradiation. The antioxidant response was measured in terms of steady state mRNA levels and activity changes of SOD2 or GPx1, as well as of the viability after challenge with high-dose UV irradiation.

Interestingly, only UVA but not UVB irradiation was able to induce the mRNA steady state levels and the activity of SOD2 in fibroblasts. However, fibroblasts incubated with the supernatants from UVB-irradiated epidermal cells responded with an increase in SOD2. This increase in

mRNA and activity levels was mediated by paracrine-acting secreted factors produced by the keratinocytes. If fibroblasts were exposed repetitively to sublethal UVA doses, the further up-regulation of SOD2 correlated with the protection against high UV doses. Importantly, SOD2 basal levels of protein content and activity substantially differed within cultivated cells and skin biopsies from different individuals. These results provide evidence of an adaptive antioxidative UV response of the skin. Interindividual differences might account for differences in the susceptibility of developing photodermatologic disorders related to photosensitivity, photoaging and skin cancer.

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THE EFFECT OF OXYGEN-TRANSIENTS AND ANTIOXIDANT VITAMINS IN BIOLOGICAL SYSTEMS. A SURVEY.

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The oxidative stress caused by a number of oxygen species plays a crucial role in the organism. It mediates an important step in the induction of various diseases. The reason for the enhanced formation of oxygen radicals is still not completely understood.

In the present overview, the generation and action of strong active oxygen transients, as well as the role of antioxidant vitamins, will be discussed. The combined action of oxygen and vitamins on the enhancement of cytostatic efficiency will also be briefly reported.

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METALS AND SKIN ABSORPTION, INTERACTION AND FREE RADICAL FORMATION

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Metals or metal compounds are present in the environment as particles and play a crucial role in life as essential elements, as well as hazards. They can enter the body through the semi-permeable membrane of the skin, through food, drinks and inhalation. Skin acts as a barrier membrane, but could absorb the metal compounds, which in high concentrations may produce toxic effects. The positively-charged metal ions interact directly or indirectly with sites characterized by high electron density or negatively-charged residues, such as nucleophilic amino acid residues of proteins, imidazole ring and phosphate groups of membrane or epidermal DNA. Metal ions exist as "free" or "bound" in the body. They are "free" hexahydrated, when they can move around in the body liquids, and they are "bound" when they form complexes with the biological molecules and form covalent bonds.

Transition or d-block metals have the d-orbitals partially filled and, thus, they can qualify as free radicals. They can generate reactive oxygen species (ROS), which lead to DNA single- or double-strand breaks, DNA-protein cross-links and a broad spectrum of oxidative DNA base modifications.

Magnesium, the second most abundant intracellular metal, activates and regulates a large number of enzymes. In the skin, magnesium maintains structural integrity between the dermis and epidermis. Magnesium deficiency, or the displacement of the Mg^{2+} ion by other divalent toxic metal ions, leads to increase of oxidative stress, aging effects and carcinogenicity, while inhibiting DNA repair by enhancing genomic instability. Recently, it was found that superoxide anions *in vivo* decrease the intracellular concentration of free magnesium cations in amnion cells and this is possibly a reason for the appearance of stress and diseases, which are associated with magnesium deficiency.

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DESIGNING NOVEL SUBSTITUTED MORPHOLINES WITH HYPOLIPIDEMIC, ANTIOXIDANT AND NITRIC OXIDE-DONATING PROPERTIES

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Oxygen free radicals and oxidative stress are involved in numerous pathologic conditions, *e.g.* cancer, mutagenesis, autoimmune diseases, ageing and cardiovascular disorders. The main cause of cardiovascular diseases is associated with the increased concentration of plasma lipids, oxidative damage of low density lipoproteins (LDL) and dysregulation of endogenous nitric oxide (NO) activity. High levels of LDL are the initiating event in atherogenesis. LDL can undergo extensive lipid peroxidation, resulting in modified LDL, formation of foam cells, fatty streaks, development of inflammation processes on the vascular tissue and, finally, formation of atheromatic plaques and arteriosclerosis. Furthermore, the amount of NO at the site of endothelial injury and NO-mediated vasodilatation are dramatically decreased. The reduced levels of NO are

attributed to the formation of the most toxic peroxynitrite *via* the reaction of NO with superoxide anion radical. It has also been proposed that NO possesses hypocholesterolemic activity, due to its ability to interfere with the production of apoprotein B (apoB), the main constituent of LDL.

Based on the above, in the present study we report the design of substituted morpholine derivatives that combine structural requirements for hypocholesterolemic activity, while they are effective antioxidants and, in some cases, have NO-donating properties as well as lipoxygenase inhibitory action.

Several of the compounds were found to be potent hypolipidemics and antioxidants. The NO-donating ability improved their hypolipidemic and antioxidant profile.

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POLYPHENOLS AND SKIN PROTECTION: THE CASE HISTORY OF PROCYANIDINS

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UV exposure is possibly the most typical example of oxidative stress leading to tissue damage. Besides by quenching or filtering radiation, protection can be achieved by increasing the antioxidant capacity of the skin. This restores the oxidative equilibrium and prevents the biological responses to injury, which, cumulatively, account for inflammation, photoaging and, ultimately, also carcinigenesis.

Grape seed extract (GSE) is a standardized mixture of polyphenols particularly rich in procyanidins (PC: non-hydrolysable polymers of catechin and epi-catechin and the corresponding gallates). It has been observed that GSE protects the irradiated skin in terms of an increase of the MED. Moreover, PC have been reported to play an anti-tumor promoting activity and to facilitate dermal wound healing.

In this study, we report the kinetic and thermodynamic features of the antioxidant effect of PC, as compared to other polyphenols bearing the redox active catechol moiety.

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***IN VITRO* OVEREXPRESSION OF THE RATE-LIMITING ENZYME OF GLUTATHIONE SYNTHESIS, γ-GLUTAMYLCYSTEIN LIGASE, AS A PROTECTION AGAINST CHEMICAL TOXICITY. POTENTIAL THERAPEUTIC ALTERNATIVE TO OXIDATIVE STRESS-RELATED DISEASES**

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Oxidative stress is related to a wide number of diseases where the prooxidant/antioxidant balance has been disturbed towards the former. A decrease in glutathione (GSH), the most important water-phase antioxidant and essential cofactor for antioxidant enzymes, has been linked to most of these medical conditions. In this study we evaluated the use of γ-glutamylcystein ligase (γ-GCL), the key enzyme of glutathione synthesis, as a potential therapy agent against arsenic and cadmium, two known oxidative stress and carcinogenic agents. *Materials and Methods:* Ten micrograms of an expression plasmid carrying the catalytic domain of the γ-GCL gene was used to transfect HeLa cells 48 h before their exposure to a sub-lethal concentration, 25 μM, of As^{3+} or Cd^{2}. Cell health status and expression of the transgene was evaluated 24 h after the toxic treatment through the analysis of metabolic activity by the MTT assay, GSH, lipid peroxidation (LPO) and γ-GCL activity. *Results:* Exposure to arsenic and cadmium decreased the GSH content, 1.6 and 1.8 times, and increased the LPO levels by 34% and 42%, respectively, compared to controls. When cells were pretreated with plasmid γ-GCL, γ-GCL activity was up-regulated about 1.5-1.7 times in each group. GSH concentration was not significately afected after As^{3+} or Cd^{2+} exposure [18±3 (Ctrl) *vs* 16±4 μmol/mg prot, (γ-GCL+As^{3+}), and 15±3 μmol/mg prot (γ-GCL+Cd^{2+})], and metabolic activity presented a similar pattern of response (92% and 89%, respectively). LPO levels were reduced after the γ-GCL treatment [86±5 (Ctrl) *vs* 76±7 nmol MDA/mg prot (γ-GCL+As^{3+}), and 80±4 nmol MDA/mg prot (γ-GCL+Cd^{2+})]. *Conclusion: In vitro* transduction of the γ-GCL gene results in an effective increase in the γ-GCL enzymatic activity and a concomitant rise in the GSH intracellular concentration, making cells more resistant to the oxidative stress caused by the toxic substances used. The protection conferred by the extra load of GSH driven by the increased synthesis of γ-GCL might be a potential gene therapy alternative against metal- and free radical-related diseases.

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CAROTENOIDS IN COMBINATION WITH VITAMINS E AND C PROTECT *IN VITRO* CULTURED HUMAN MELANOCYTES AND INFLUENCE PIGMENTATION

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Carotenoids and vitamins are used in dietary supplements and skin care products to protect the skin against photodamage. Carotenoids have been described to contribute to skin colour and, in combination with vitamins E and C, stimulate pigmentation. However, detailed investigation about the effects of carotenoids and their mixture with other vitamins on melanocytes and melanogenesis is scarce. We have tested the effects of a carotenoid mixture extracted from natural sources in combination with vitamins E and C on growth and pigmentation of melanocytes *in vitro*.

Melanocyte cultures of Caucasian origin were grown in standard Ham's F-10 medium supplemented with 1% Ultroser-G, 12-O-tetradecanoyl-phorbol-13-acetatete, isobutylmethylxanthine and cholera toxin. In order to induce melanogenesis, the melanocytes were also grown with 5 or 10x tyrosine concentrations (0.05 or 0.1 mM). We used extract from palm fruit (PE) as a rich source of carotenes (30%) and tomato extract (TE) naturally rich in lycopene (6%). Mixtures of both extracts were tested at various concentrations and in combination with 35 μM vitamin E and 100 μM vitamin C (COMB) during one and repeated passages (short- and long-term incubations). Effects on morphology and cell growth of the melanocytes were studied. Influences on pigmentation were measured spectrophotometrically using synthetic melanin as the standard, or by detection of the specific eumelanin degradation products by HPLC and fluorimetry. Induction of DNA damage by UVA irradiation of the cells was measured with the comet assay. Light- and darkly-pigmented melanocytes were preincubated with COMB for 24 h before irradiation of the cells and measurement of the comet tail moment.

We discovered that TE and PE extracts could be added to the culture medium with no signs of toxicity up to 200 μg/ml and 100 μg/ml, respectively. Short- and long-term incubations were performed with 20 μg/ml TE and 4 μg/ml PE. In the medium containing high tyrosine, both melanocyte cultures showed more than a 10-fold increase in pigmentation, which was accompanied by a reduction of growth. This reduction was much less when TE was used in the medium. Especially in the long-term cultures, optimal growth advantage was found for the cells maintained in high tyrosine medium containing the combination of TE/PE with vitamin E and C (COMB). Treatment with higher concentration of TE/PE (200/40 μg/ml) resulted in higher dendricity of the melanocytes and a reduction in cell numbers after one passage. Under these conditions, the presence of vitamin E and C (COMB) resulted in a higher degree of pigmentation of the cells compared to those grown with TE/PE alone. Tyrosine-induced pigmentation resulted in a higher degree of DNA damage, as observed by an increased tail moment measured with the comet assay. This was especially the case for the UVA-irradiated cells. Preincubation of the cells with COMB resulted in a considerable reduction of the comet tail.

In conclusion, the results demonstrate that melanocytes grown in high tyrosine medium show very active melanogenesis and are inhibited in their growth. The presence of the carotenoids TE/PE prevents this growth inhibition, especially in the presence of vitamins E and C, and can also stimulate melanin production. As shown earlier by others in melanocyte monocultures, tyrosine-induced pigmentation can result in a stronger induction of DNA damage by UVA. Pretreatment of the cells with COMB may offer considerable protection against this UVA-induced damage.

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THE EFFECT OF A COMMON SUNSCREEN INGREDIENT ON CELL VIABLITY IN CULTURED HUMAN KERATINOCYTES AFTER UVA EXPOSURE

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As an extension of our previous investigations on sunscreen ingredients, the present work was aimed at assessing the effects of a common UVA-absorbing agent, Parsol 1789 (4-*tert*-butyl-4'-methoxydibenzoylmethane), on human keratinocytes under UVA illumination. Cytotoxicity was evaluated by determining LDH release, uptake of ethidium bromide and fluorescein diacetate, total protein content and percentage of cell detachment. Apoptosis was determined by Annexin-V-FITC uptake, while the TOSC-assay was used to measure the total antioxidant capacity level in these cells. The results obtained show that, with respect to the illuminated control, 10 μM Parsol 1789 induces: i) a slightly higher mortality rate and loss of cells from the monolayer after 4 h from exposure, ii) decreased protein content after 4 h from exposure, and iii) a more consistent loss in all three parameters after 24 h from exposure (about 70%). However, no significant LDH release with respect to the control was observed after UVA illumination, suggesting that cell death might occur *via* apoptosis. Indeed, an additional increase in apoptosis was observed in cells illuminated and treated with Parsol 1789 compared to the control. These results confirm previous findings on the undesirable effects of this sunscreen, where both plasmid DNA and bovine serum albumin are damaged under UVA illumination in the presence of Parsol 1789. The damage is most probably due to the formation of the free radicals it generates upon illumination. However, the biological role and significance of these results to the consequences of sunscreen use in humans are not known, hence extrapolation from laboratory experiments must be done with caution.

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IN SITU COLLAGEN IRRADIATION. AN INFRARED SPECTROSCOPIC STUDY

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Skin is influenced by several factors, such as pollutants, xenobiotis, mechanical stress, UV (ultraviolet) and X-ray irradiation, *etc.* The influence of irradiation is of considerable importance for skin changes. On the other hand, in many protocols the treatment of bone tumors includes radiotherapy, while radiotherapy is the only treatment for metastatic bone pain. The aim of this work was the study of the changes of human collagen, which are produced when it is irradiated *in situ* with γ-rays (^{60}Co). It was found from infrared spectra that, after irradiation with a dose of 60 Gy, the band at 1661 cm^{-1}, which corresponds to the C=O group of the peptide bond, decreases significantly, suggesting damage of the peptide bond. Furthermore, the appearance of a new band at 1635 cm^{-1} leads to the result that the collagen helix changes its conformation from an alpha helix to a random coil one. Furthermore, from the new band at 1672 cm^{-1}, which was observed after irradiation, it becomes clear that the collagen chain is reduced and acquires a size of about ten carbon atoms.

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TUMORIGENESIS AND DERMAL CONNECTIVE TISSUE CHANGES IN RESPONSE TO UVB IRRADIATION IN HAIRLESS SPARC-NULL MICE

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In this study, we investigated the role of the extracellular matrix protein SPARC in skin tumorigenesis and dermal connective tissue changes in response to UVB. Hairless SPARC-null mice were bred and subjected to UV radiation for 22 weeks (cumulative 13.2 J/cm^2 UVB). Tumorigenesis was dramatically reduced in SPARC-null mice compared to SKH-1 controls. There was a total of 390 papillomas and 64 squamous cell carcinomas in the 20 control mice, while only 16 papillomas and 0 squamous cell carcinomas in the SPARC-null mice. SPARC was actively produced in the skin tumor mass of the SKH-1 mice, but was not observed in the cancerous keratinocytes of the epidermis. Normal histological and biochemical changes in the skin matrix in response to UV were similar between both groups. The striking difference in the onset, progression and severity of tumor formation between SPARC-null and wild-type mice suggests that SPARC has a significant role in the process of tumorigenesis in UVB-induced skin cancer.

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NOVEL STEPS IN SIGNAL TRANSDUCTION

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Radiation and prooxidants target numerous cellular molecules. A particularly sensitive class of macromolecules are the protein tyrosine phosphatases (PTPs). PTPs exert numerous regulatory roles in the intracellular signal transduction network. All PTPs yet tested seem similarly sensitive to UV irradiation of cells. As result of radiation by UVA, UVB or UVC, two immediate processes are induced: oxidative inactivation and calpain-mediated degradation.

A major consequence of impaired PTP activity is elevated signal flow. We have identified some of the PTPs negatively regulating receptor tyrosine kinases (RTKs). Their inactivation causes ligand-independent RTK activation, obviously indicating relatively high spontaneous dimerization of RTK subunits.

Following receptor-dependent signaling from the PDGF receptor, from Met and from the EGF receptor, we detected an unexpected critical step in signal transduction. The signal transfer from the receptors to the small G proteins, *e.g.* Ras, requires an intact link of the F-actin cytoskeleton to the plasma membrane. The link is accomplished by the ERM proteins. Dominant-negative mutants of ERM proteins, *e.g.* those unable to bind F-actin, sequestering ERM proteins away from the plasma membrane, or various types of interference with F-actin polymerization abolish signal transfer to Ras and to downstream signaling components. This novel step of signal transduction is subject to physiological regulation: a tumor suppressor protein, NF2, mimics the action of the dominant-negative ERMs, in that it binds to the same plasma membrane proteins but carries no F-actin-binding motif.

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PROTEIN OXIDATIVE DAMAGE IN ATOPIC SKIN AND REMARKABLE EFFECTIVENESS OF TREATED NATURAL ANTIOXIDANT OINTMENT IN ATOPIC PATIENTS

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The rise in prevalence and severity of atopic dermatitis (AD) has recently been seen, especially in urban and industrial areas of developed countries including Japan. We have ascribed this to increased reactive oxygen species (ROS) generated from environmental pollution and solar radiation. Direct oxidative protein damage of atopic skin was investigated. Carbonyl moieties in skin biopsies from 75 AD patients were assessed using both spectrophotometric and immunohistochemical detection of the formation of dinitrophenylhydrazone (DNP) from dinitrophenylhydrazine (DNPH). Lipid peroxidation was also assessed by staining with antibody to 4-hydroxy-2-nonenal (4-HNE), an aldehyde product of oxidized omega-6 fatty acids. The activity of superoxide dismutase (SOD), an effective scavenger of ROS, was also assessed. The level of protein carbonyl moieties in patients' skin was elevated and correlated with the severity of the disease. SOD activity was increased, except for those with extra-severe disease. Positive staining with anti-DNP antibody and anti-4-HNE-antibody were found in the most superficial layers of the stratum corneum. Atmospheric nitrogen oxide concentration for the top five cities of origin of the AD patients directly paralleled the number of patients from those cities who had severe AD requiring hospitalization. This supports the hypothesis that environmentally-generated ROS induce oxidative protein damage in the stratum corneum, leading to the disruption of barrier function with resultant loss of moisturization and enhancement of antigen presentation, hallmarks of AD (Br J Dermatol, 149, 2003). In this communication, we have developed an ointment, named "AOA (antioxidant analogs) ointment", which consists of an extract of natural plants and seeds, treated by the following procedures: irradiation with far infrared-ray heating, brewing with *Aspergillus oryzae*, and lipophilization with sesame oil. This treatment procedure contributes to activate antioxidant activity contained in natural plant products by liberating the repeated subunits of low molecular weight antioxidant polymers (Inflammation *10*: 79, 1986; Planta Med *57*: 299, 1991; Int J Tiss Reac *20*: 63, 1998). After being added with 0.0218% beta-methasone valerate (glucocorticoid, GC) and hydrophilic petrolatum as vehicle, this preparation was studied for its clinical effect in 750 patients with AD. In patients with mild or moderate AD, the AOA ointment showed remarkable effects in reducing the extent and severity of the disease. It was less effective in patients with severe AD, although the same effect was observed. AOA was significantly more effective in mild to moderate AD than control ointments, including an equivalent concentration of GC with or without the natural untreated extract, and the untreated extract without low-dose GC. In individual patients, the AOA ointment was far more effective than the control GC ointment. During the test of the GC ointments, 50 cases of severe AD patients were given high concentrations of 0.12% beta-methasone valerate ointments. Out of 25 severe AD cases which were treated by the lower GC ointments alone without AOA extract, 23 cases relapsed when 18.2% of commonly used 0.12% beta-methasone (*i.e.* 0.0218% beta-methasone) was applied. Among 25 severe AD patients, 19 cases improved by 0.0218% beta-methasone valerate combined with AOA extract. Thus, the AOA ointment appears to be an effective agent for the treatment of AD, which may support our hypothesis of protein oxidative damage in AD patients.

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UV-PROTECTIVE EFFECTS OF CAROTENOIDS

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Carotenoids are natural colorants present in various fruits and vegetables, such as carrots, tomatoes, spinach, oranges, or peaches. Dietary carotenoids are absorbed and distributed into blood and tissues. The major carotenoids in human skin are similar to those found in serum and other tissues and include α- and β-carotene, lycopene, lutein, zexanthin, phytoene, phytofluene and β-cryptoxanthin. In biological systems, UV-light exposure leads to the formation of reactive oxygen species which are damaging to biomolecules and affect the integrity and stability of subcellular structures, cells and tissues. Irradiation with UVB-light induces lipid peroxidation, which can be inhibited with lycopene, β-carotene, or lutein (1). In the presence of carotenoids, UVB-induced formation of thiobarbituric acid-reactive substances was lowered in human skin fibroblasts. The amounts needed for optimal protection were divergent for lycopene, β-carotene and lutein. Beyond optimum levels, further increases in carotenoid levels in cells led to prooxidant effects. Photooxidative processes play a role in the pathobiochemistry of several skin disorders. When the skin is exposed to UV-light, erythema is observed as an initial reaction. There is evidence from *in vitro* and *in vivo* studies that carotenoids prevent photooxidative damage and protect against sunburn (2-4). When β-carotene was applied alone or in combination with α-tocopherol for 12 weeks, erythema formation induced with a solar light simulator was significantly diminished from week 8 on (2). Protective effects can also be achieved with dietary intervention. Ingestion of tomato paste, corresponding to a dose of 16 mg lycopene/day over 10 weeks, led to increases in serum levels of lycopene and total carotenoids in skin (3). Erythema formation was significantly lower in the group that ingested tomato paste as compared to the control.

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OMEGA-3 FATTY ACIDS IN INFLAMMATION AND AUTO-IMMUNE DISEASES

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Among the fatty acids, it is the ω3 polyunsaturated fatty acids (PUFA) which possess the most potent immunomodulatory activities and, amongst the ω3 PUFA, those from fish oil [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] are more biologically potent than α-linolenic acid (LNA). Some of the effects of ω3 PUFA are brought about by modulation of the amount and types of eicosanoids made and other effects are elicited by eicosanoid-independent mechanisms, including actions upon intracellular signaling pathways, transcription factor activity and gene expression. Animal experiments and clinical intervention studies indicate that ω3 fatty acids have anti-inflammatory properties and, therefore, they might be useful in the management of inflammatory and auto-immune diseases. Coronary heart disease, major depression, aging and cancer are characterized by an increased level of interleukin 1 (IL-1), a pro-inflammatory cytokine. In other words, these chronic diseases have a pro-inflammatory component. Similarly, arthritis, Crohn's disease, ulcerative colitis and lupus erythematosis are auto-immune diseases characterized by a high level of IL-1 and the pro-inflammatory leukotriene LTB_4 produced by ω6 fatty acids.

The anti-inflammatory properties of ω3 fatty acids, especially EPA, are due to competition with arachidonic acid (AA) as a substrate for cyclooxygenases and 5-lipoxygenase. The eicosanoids from the ω6 and ω3 fatty acids have opposing properties. The eicosanoids are considered a link between PUFA, inflammation and immunity. In addition to their effects on prostaglandins, thromboxanes and leukotrienes, ω3 fatty acids suppress the production of interleukin 1 (IL-1α) by suppressing the IL-1β mRNA, as well as the expression of Cox2 (cytooxygenase) mRNA that is induced by IL-1β. Cox2 is overexpressed in colon cancer cells. Both LNA, and EPA and DHA are involved in immune function. The precise effect of LNA depends on the level of linoleic acid (LA) and total PUFA content of the diet. A high dose of LNA (about 15 g/day) will suppress human IL-1 and TNF (tumor necrosis factor). It is unclear whether LNA itself exerts these effects, or whether they are the result of its conversion to EPA. Excessive intake of ω6 fatty acids, characteristic of Western diets, produces an imbalance of ω6 to ω3 PUFAs, which leads to an overproduction of the pro-inflammatory prostaglandins of the ω6 series and cytokines. Supplements of LA-rich vegetable oils increase IL-1 and TNFα. Humans given ω3-rich flax seed oil or fish oil supplements have sharply reduced stimulated production of IL-1, IL-2 and TNFα, as well as suppressed mononuclear cell proliferation and expression of IL-2 receptors. Thus, in humans, LA increases pro-inflammatory cytokine secretion, whereas fish oil reduces pro-inflammatory cytokine secretion. There have been a number of clinical trials assessing the benefits of dietary supplementation with fish oils in several inflammatory and auto-immune diseases in humans, including rheumatoid arthritis, Crohn's disease, ulcerative colitis, psoriasis, lupus erythematosus, multiple sclerosis and migraine headaches. Many of the placebo-controlled trials of fish oil in chronic inflammatory diseases reveal significant benefit, including decreased disease activity and a lowered use of anti-inflammatory drugs.

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PROTECTION AND REVERSAL OF PHOTODAMAGE OF THE SKIN WITH TOPICAL ANTIOXIDANTS

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Controversy exists as to whether topical antioxidants can be effective in protecting and reversing photodamage to the skin. Indeed, topical vitamins C and E, as well as selenium, can protect from sunburn, suntan and skin cancer and can dramatically reverse the mottled pigmentation and wrinkles of photoaging. Recent research confirming these effects will be presented with a description of the stringent criteria regarding the formulations necessary for efficacy. The concentrations, molecular forms and requirements for stability and topical delivery of each antioxidant are described. Data showing the impressive protection against acute and chronic photodamage by topical genistein (a soy isoflavone) and topical silymarin will be reviewed.

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ANTIOXIDANT ENZYMES IN KERATINOCYTES AND THEIR PROTECTION AGAINST PHOTO-INACTIVATION: IMPLICATIONS FOR SKIN CANCER THERAPY

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Cellular antioxidant enzymes protect against damage caused by exposure to endogenous or exogenous pro-oxidants. Singlet oxygen ($^1O_2$) is a reactive form of oxygen that can be produced *in vivo* either by UV light, as during sunlight exposure, or by photosensitizing chemicals, as during photodynamic therapy. We hypothesized that $^1O_2$ would decrease the activities of cellular antioxidant enzymes and render them susceptible to reactive oxygen-mediated damage. To test this hypothesis, we treated cultured mouse keratinocytes with the photosensitizer Photofrin®, illuminated them for varying times with visible light to produce $^1O_2$, and then measured copper- and zinc-containing superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD) and catalase activities with both in-gel and spectrophotometric enzyme activity assays. Our results demonstrated that the enzymatic activities of cellular CuZnSOD, MnSOD and catalase were significantly decreased by about 80% after keratinocytes had been treated with Photofrin® plus visible light. In contrast, the enzymatic activities of cellular CuZnSOD, MnSOD and catalase were unaffected in control cells treated with Photofrin® only or visible light only. In spite of the decreased enzymatic activities, the protein levels of all three antioxidant enzymes remained constant after photodynamic treatment, as determined by Western blotting. L-histidine, a $^1O_2$ quencher, protected against the inactivation of cellular CuZnSOD, MnSOD and catalase enzymes induced by photodynamically generated $^1O_2$ in a dose-dependent manner. The conclusion from these experiments is that the primary cellular antioxidant enzymes CuZnSOD, MnSOD and catalase can be inactivated by photodynamically generated $^1O_2$. These findings may be useful in the development of antineoplastic adjuvant therapies that employ the photodynamic generation of $^1O_2$ to inactivate antioxidant defenses, followed by treatment with ionizing radiation or drugs that produce reactive oxygen species to produce synergistic cytotoxicity.

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OXIDIZED ELICOSANOIDS IN UV-IRRADIATED HUMAN SKIN AND CULTURED KERATINOCYTES AFTER ADMINISTRATION OF ANTI-INFLAMMATORY DRUGS USING MICRODIALYSIS TECHNIQUE

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UV-irradiation of the human skin leads to the induction of oxidative stress and inflammation mediated by reactive oxygen radicals, lipid peroxidation, liberation of arachidonic acid from membrane phospholipids and formation of prostaglandins and leucotrienes. The precise role of free radical-induced lipid peroxidation as well as enzymatic arachidonic acid oxygenation and the possible connections to inflammatory diseases are not yet thoroughly evaluated, partly owing to the unavailability of reliable analytical methods. It was the aim of our studies to analyze the levels of oxidized eicosanoids, such as 8-iso-$PGF_{2\alpha}$,9α, 11α-$PGF_{2\alpha}$ PGE_2, monohydroxyeicosatetraenoic acids (HETEs) and LTB_4 in the dermal interstitial fluid obtained by cutaneous microdialysis technique and for comparison in cultured keratinocytes (HaCaTs) after UV-irradiation and application of diclofenac, a nonsteroidal anti-inflammatory drug. Defined areas on the volar forearm of 10 healthy volunteers were exposed to UVB irradiation (20-60 mJ/cm^2). After selected time-intervals (starting with 3 and 24 hours), microdialysis membranes were cutaneously inserted beneath the irradiated area. The membranes were perfused with isotonic saline solution and microdialysate samples were collected at 20-minute intervals for up to 4 hours. They were immediately frozen in liquid nitrogen and deep-frozen until quantification.

Analyses of oxidized arachidonic acid derivatives were performed using highly sensitive gas chromatography-mass spectrometry in the negative ion chemical ionisation mode (NICI-GC-MS). Our data provide evidence for the identification of HETEs (5-, 8-12- and 15-HETE), LTB_4, 8-iso-$PGF_{2\alpha}$, $PGF_{2\alpha}$ and PGE_2 in microdialysate samples of normal skin, their quantification and increase of their levels (except $PGF_{2\alpha}$ and LTB_4) following UVB-treatment. Diclofenac, topically applied on the UVB-irradiated areas, was able to reduce the enhanced levels of 8-iso-$PGF_{2\alpha}$ and HETEs in microdialysates. In HaCaTs, diclofenac doses between 0.1 and 10 μM suppressed the UVB-induced 8-iso-$PGF_{2\alpha}$ release, but had no effect on HETE levels.

The sensitive GC-MS assay for the quantification of oxidized eicosanoids, in combination with the cutaneous microdialysis technique, opens new opportunities to study the role of these lipid mediators in inflammatory skin disorders and may also be relevant to validate therapeutical strategies. Furthermore, microdialysis allows the study of skin penetration of various topically applied substances, such as diclofenac and others, in skin inflammation and may contribute to the improved treatment of various skin diseases.

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IRON LEVEL AND GLUTATHIONE REDOX RATIO IN THE SKIN OF PATIENTS WITH CONTACT DERMATITIS

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In many pathological circumstances, including all inflammatory diseases, the level of reactive oxygen species (ROS) overcomes the endogenous antioxidant defense of the host. In contact dermatitis, including both allergic and irritant type, the main source of ROS is inflammatory cellular infiltrate. As a consequence of inflammation, cellular antioxidant defense is insufficient to keep the amount of ROS permanently below the toxic levels and, therefore, the skin has to sustain additional damage. *Materials and Methods*: To evaluate oxidative damage in contact dermatitis, we compared the glutathione redox ratio (GSSG/GSH), diene conjugate (DC) level, iron content and unbound iron-binding capacity (UIBC) in inflammatory and normal skin. Positive patch test reactions served as a model of acute contact dermatitis and were biopsied in 15 patients. In 14 patients, skin biopsies were obtained from chronic hand dermatitis lesions. Uninvolved back skin of all participants was also biopsied. *Results*: Acute dermatitis in a positive patch test area was characterized by a significant increase in iron level ($p<0.001$), UIBC ($p<0.05$) and DC ($p<0.05$) in comparison to apparently healthy skin. There was no substantial elevation in GSSG/GSH ratio when comparing lesional to apparently healthy skin. In patients with chronic contact dermatitis, a significant difference between clinically normal and affected skin was observed for DC ($p<0.001$) and the GSSG/GSH ratio ($p<0.001$), but not for the iron level. Treatment of chronic contact dermatitis with topical corticosteroids and emollients for up to 10 weeks resulted in a decrease in DC ($p<0.02$) and, especially, in the GSSG/GSH ratio ($p<0.00005$). The comparison of the iron status and GSSG/GSH ratio in the normal skin of patients with acute and chronic contact dermatitis gave surprising results. In the normal skin of patients with contact dermatitis restricted only to the patch test area, the GSSG/GSH ratio significantly ($p<0.05$) exceeded the ratio in normal skins of patients with chronic hand dermatitis. Conversely, in patients with chronic hand dermatitis, the iron level of normal skin was significantly higher when compared to normal skin of patch-tested patients ($p<0.02$). The percentage saturation of iron-binding capacity was also higher in the chronic contact dermatitis group ($p<0.02$). *Conclusion*: The GSSG/GSH ratio is both an indicative and sensitive marker of cellular oxidative stress. The significantly higher GSSG/GSH ratio in the normal skin of patients belonging to the acute dermatitis group might be explained by the predominance of nickel-allergic subjects. According to several investigators, exposure to nickel produces measurable levels of free radicals in the cells. Nickel also has a direct negative effect on glutathione, depleting it. This raises the question of whether the patch testing is truly a harmless procedure. In the case of chronic hand dermatitis, the iron store of the entire skin might increase proportionally to the duration of the disease. As a consequence, the increase of "free" iron in the skin of our patients was accompanied by more intensive lipid peroxidation, which might damage skin barrier lipids, too. Therefore, the function of the entire skin might be disturbed.

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MAST CELLS IN THE SKIN OF AGED RAT: RELATIONSHIP TO ANTIOXIDATIVE DEFENCE

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Mast cells are normally present in the skin dermis between collagen fibers and surrounding blood vessels and are usually involved in cell proliferation and growth. They are implicated in a variety of pathophysiological skin functions. The cellular mechanisms of their activation are still unknown. However, the reactive oxygen species may be involved in the degranulation of mast cells.

In this work, changes of mast cell phenotype, antioxidative defense (CuZn superoxide dismutase, EC 1.15.1.1; catalase, EC 1.11.1.6; glutathione peroxidase, EC 1.11.1.9; glutathione reductase, EC 1.6.4.2; thioredoxin reductase, EC 1.6.4.5 and glutathione S-transferase, EC 2.5.1.18) and blood vessels in the rat skin (Dark August rat strain) during the lifespan (3 days to 21 months), were studied.

From our results, it may be concluded that the higher number of mast cells and level of antioxidative defence in young rats may contribute to the skin cell development and differentiation. The altered phenotype of mast cells in old animals, accompanied with increased levels of antioxidative defence and lipid peroxidation, could be the consequence of disturbed redox equilibrium in the skin. The results also suggest the possible role of reactive oxygen species in these processes related to skin aging.

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ENZYMATIC PREPARATION OF LIPOPHILIC DERIVATIVES OF GLYCOSYLATED FLAVONOIDS WITH ANTIOXIDANT ACTIVITY

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Flavonoids have multiple biological properties including anti-carcinogenic, anti-inflammatory, anti-bacterial and anti-viral activity. However, the relative hydrophilic nature of these antioxidants reduces their effectiveness in stabilizing oil-based formulae. Therefore, the preparation of lipophilic derivatives of flavonoids *via* esterification of the hydroxyl functions with aliphatic molecules can be used as a tool to alter their solubility in oil-based formulae and emulsions (1). Moreover, the selective modification of these natural antioxidants is expected to produce analogs that may be useful models to study the structure-activity relationships, in particular as regards their amphiphilic and lipophilic properties and, therefore, their ability to penetrate the cell membrane (2). In addition, esters of flavonoid glucosides with phenolic acids (such as ferulic and caffeic acid) may serve an important antioxidant function in preserving the physiologic integrity of cells exposed to both air and impinging UV radiation (3).

The use of conventional chemical acylation methods for the modification of the hydroxyl function of flavonoids leads to a mixture of products with various degrees of esterification, since these methods show almost complete lack of regio-selectivity. To overcome the disadvantages of the conventional processes, the use of enzymes in non-aqueous media has opened new avenues for producing many valuable products, such as lipophilic derivatives of natural antioxidants (3, 4).

In this work, we investigated the ability of various lipolytic enzymes to catalyze the regio-selective acylation of glucosylated flavonoids such as rutin and naringin, as well as chrysoeriol-7-O-β-D-(3''-E-p-coumaroyl)-glucopyranoside isolated from the aerial parts of a greek endemic plant, *Stachys swainsonii* ssp. *argolica* (Boiss.) Phitos and Damboldt. Various acyl donors, including fatty and phenolic acid vinyl esters, were used as substrates in anhydrous organic media. The effect of the lipophilic derivatives of flavonoids on Cu^{2+}-induced oxidation of low-density lipoprotein (LDL) and total serum was investigated.

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ENZYMIC RELEASE OF PHENOLIC ANTIOXIDANT FERULIC ACID FROM PLANT CELL WALL MATERIAL

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Gramineous plant cell walls are known to contain phenolic constituents, such as ferulic acid (FA), covalently bound to polysaccharides. FA is esterified to the O-2 or O-3 position of arabinose in dicotyledons, such as spinach and sugar beet and to the O-6 position of galactose in pectin (1, 2). There has recently been considerable interest in feruloyl esterases (FAE) and their potential application in obtaining FA from agro-industrial waste materials such as those produced by the milling, brewing and sugar industries. FA can be used as an antioxidant as it has been shown to possess some activity toward peroxynitrite (3) and oxidized low-density lipoprotein (oxLDL) *in vitro* (4). FA has also been evaluated in synaptosomes and neuronal cultures exposed to peroxyl, and hydroxyl radical insult *via* several oxidative stress indices and may be a promising candidate as an antioxidant in neurodegenerative disorders such as Alzheimer's disease (5). In this study, we report the ability of two purified feruloyl esterases from *Fusarium oxysporum* (FoFAE-I & FoFAE-II) and one from *Sporotrichum thermophile* (StFAE-A) to release FA from a complex substrate, destarched wheat bran (DSWB), both in the presence and absence of a xylanase from *Sporotrichum thermophile*.

FoFAE-I (45 mU g^{-1} DSWB) was able to release FA from the wheat bran preparation without the aid of other xylan-degrading enzymes (1.4% of total FA in 4 hours incubation). In the presence of xylanase (100 U g^{-1} DSWB), FoFAE-I (400 mU g^{-1} DSWB) removed 92% of the total FA after 4 hours incubation.

Similarly to FoFAE-I, StFAE-I, StFAE-A (400 mU g^{-1} DSWB) was able to release FA (0.7% of total FA after 1-hour incubation). When the esterase (800 mU g^{-1} DSWB) was incubated together with xylanase (300 U g^{-1} DSWB), 34% of total FA was released after 1-hour incubation.

As opposed to the above FAEs, FoFAE-II was unable to release FA in the absense of xylanase. Adding 150 mU g^{-1} DSWB esterase, a maximum of 23% of the total FA was

released in the presence of xylanase (300 U g^{-1} DSWB) in 1-hour incubation. The xylanase alone did not release FA.

In summary, all the feruloyl esterases were able to release significant FA from DSWB in the presence of *Sporotrichum thermophile* xylanase, indicating a synergistic interaction between feruloyl esterases and xylanases. These results demonstrate the potential use of feruloyl esterases and xylanases for the release of ferulic acid from cell wall materials.

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IMPROVEMENT OF ENCAPSULATION EFFICIENCY OF DICLOFENAC SODIUM INTO UNCOATED AND CHITOSAN–COATED LIPOSOMES

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Liposomes, prepared from dipalmitoylphosphatidylcholine (DPPC) phospholipid with or without cholesterol, were used to study the encapsulation efficiency of diclofenac sodium (DS) as well as its retention properties into liposomes. In order to improve the percentage (%) encapsulation of DS into liposomes, natural phospholipids were isolated from *Triticum* sp. (wheat germ) and were used for preparing the liposomes, while chitosan was used to coat the liposomes. DS was encapsulated into uncoated and coated liposomes using the thin film hydration method, with a trapping efficiency of more than 90%. Improvement of the percentage (%) encapsulation of DS into liposomes was achieved by employing phospahatidylethanolamine, dicetyl phosphate and chitosan. DS was encapsulated into liposomes, which were prepared from *Triticum* sp. phospholipids, with a high efficiency of 99%, compared to 59% when DPPC was used. Chitosan-coated liposomes (DPPC, DPPC/cholesterol and *Triticum* sp. lipids) were prepared and their encapsulation efficiency was studied. The results showed that the presence of cholesterol in DPPC bilayers produces a significant decrease in the encapsulation efficiency of DS, while its encapsulation was also affected by chitosan due to the presence of diacetyl phosphate rather than the presence of chitosan. The retention of DS in uncoated and in coated liposomes (pH 7.4 normal saline) at 37°C was studied. The Differential Scanning Calorimetry (DSC) technique was applied in order to get information on the interaction of DS with lipid bilayers and the role of cholesterol on the retention of the drug into liposomes

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INFLUENCE OF COMPLEXATION OF A COMMON SUNSCREEN AGENT WITH CYCLODEXTRIN ON BIOLOGICAL MOLECULES UNDER UVA ILLUMINATION

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Several studies have shown that the common UVA sunscreen, Parsol 1789 (4-tert-butyl-4'-methoxydibenzoyl-methane), designed to absorb UVA light, is not photostable and decomposes to produce an array of breakdown products deriving from the initial formation of free radicals. This not only leads to a reduction in photoprotective power, but it also leads to DNA strand breaks and oxidation of proteins. Recently, however, the complexation of the sunscreen agent with hydroxypropyl-β-cyclodextrin (HP-β-CD) reduced the photo-induced formation of free radicals, as measured by spin-trapping experiments. Therefore, the purpose of the present work was to investigate whether this diminished formation of free radicals, due to complexation, would lead to a concomitant decrease in damage to biologically relevant biomolecules. Exposure of deoxy-D-ribose, PC liposomes and bovine serum albumin (BSA) to artificial UVA light led to an increase in oxidative damage, as measured by

TBARS-reactive substances in the first two cases and carbonyl residues in the latter. The presence of Parsol 1789 enhanced the damage to deoxy-D-ribose and BSA, but this effect was neither increased nor reduced by complexation of the sunscreen agent with HP-β-CD. Interestingly, in liposomes, Parsol 1789 had opposite effects (protective or oxidative) according to the mode of exposure to the UVA source. Again, its complexation had no significant effects compared to the free form. The overall results thus demonstrate that complexation of this sunscreen agent is ineffective in reducing its damaging potential to biological molecules.

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INTERACTION OF NO SYNTHASE-II OXYGENASE DOMAIN WITH t-BUTYL ISOCYANIDE. THE ROLE OF TETRAHYDROBIOPTERIN

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Nitric oxide synthases (NOS I-III) constitute a family of homodimeric heme-containing enzymes that catalyse the stepwise formation of nitric oxide and L-citrulline from the oxidation of L-arginine by oxygen and NADPH. Nitric oxide, a diatomic radical, is an important signalling molecule implicated in processes such as vascular tone, neurotransmission and immune response.

All NOS isoforms include a reductase domain with FAD, FMN and NADPH binding sites, and an oxygenase domain containing tetrahydrobiopterin (H_4B), heme and the substrate binding sites. Although H_4B is crucial for catalytic activity, its exact role is not completely clarified.

The two domains can be expressed separately with preservation of their individual structural and catalytic properties, thus the oxygenase domain can be used for the investigation of the spectroscopic properties of this enzyme.

Considering that isocyanides are known to be potential ligands of iron in hemoproteins, and since NOSs are the only known heme-containing enzymes that require H_4B, we found it interesting to study the spectral binding properties of t-butyl isocyanide on recombinant NOS II, as a function of time, the oxidation state of iron and the presence of H_4B.

It was found that the ratio of the double Soret bands (at 430 nm and 450 nm) in the ferrous isocyanide complex varies with time, and that the presence of H_4B considerably reduces this effect, indicating an enzyme stabilising and protective effect of tetrahydrobiopterin.

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EFFECTS OF ENDOGENOUS COMBINATION OF ANTIOXIDANTS ON UV-INDUCED OXIDATIVE DAMAGE IN HUMAN SKIN *IN VIVO*

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Several vitamins, as antioxidants, are radical scavengers in a hydrophilic as well as in a lipophilic environment. It is well known that a deficit of these antioxidants considerably increases lipid peroxidation. Interestingly, such a deficit also appears in the stratum corneum of the skin after irradiation. ROS can modify macromelcules such as proteins in tissues to activate tissue degrading enzymes or to form carbonyl derivatives (oxidised proteins). These carbonyls accumulate, for example, in the papillary dermis of photodamaged skin. It was also shown that matrix-metalloproteases (MMPs), responsible for breakdown of connective tissue components, were induced dose-dependently *in vitro* and *in vivo* by UVA and UVB irradiation. The induction of MMPs is considered to be one of the important mechanisms that mediates cutaneous photoaging.

The present study was performed with 24 female, healthy volunteers receiving verum or placebo (double-blinded) twice a day for 10 weeks. The verum included a combination of antioxidants: 150 mg ascorbic acid, 100 mg alpha-tocopherylacetate and 6 mg beta-carotene in capsules. For placebo the capsules were identical, however without antioxidants. UVB/A irradiation was performed at weeks 9 and 10 using a Waldmann UV-light cabin for the entire body. The initial UV irradiation-dose was set depending on the skin type and on the individual MED. The measured mean MED in the UVB-range was 0.04 J/cm^2. The mean cumulative UV-dose at the end of the irradiation period was 0.94 J/cm^2. Blood samples were taken at baseline, weeks 2, 8 (before UV), 10 (after UV) and 12 (washout). Skin punch biospies (Ø 6 mm) were taken at baseline, before and after UV treatment. HPLC techniques were used for quantitation of ascorbic acid, alpha-tocopherol and beta-carotene in the plasma samples. ELISA was used to detect MMP-1 protein expression in the skin biopsies. Cryo sections of the skin were prepared for immunohistochemistry using

2,4-dinitrophenylhydrazine for derivation and a polyclonal anti-dinitrophenyl antiserum followed by peroxidase staining.

The supplied components accumulated within 2 weeks significantly in the plasma. Plateau levels were reached up to week 8 (before UV). In contrast to other studies, the plasma levels of ascorbic acid, alpha-tocopherol and beta-carotene were not altered during the UV irradiation period in both groups, indicating that the supply of the food supplement was sufficient to maintain the plasma levels.

MMP-1 levels of the verum group showed an insignificant reduction of protein expression even after UV irradiation, whereas the placebo group showed elevated, but insignificant, MMP-1 levels.

With respect to the oxidised proteins, the verum group showed heterogeneous results after UV treatment. Oxidised proteins were elevated in the majority of cases in the placebo group.

To a certain extent, this combination of antioxidants was able to protect skin from UV-induced oxidative damage, as well as from the overexpression of MMP-1. The data show a variety of individual reactions.

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CAN ENDOGENOUS ANTIOXIDANTS PROTECT HUMAN SKIN EQUIVALENTS FROM UV-INDUCED OXIDATIVE/NITROSATIVE DAMAGE?

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Reactive oxygen species (ROS) are an inherent part of the anabolism and catabolism of the skin. To test the effect of ROS *in vivo*, an organotypic skin equivalent was used as a skin model. ROS are important mediators in the etiology of UV-induced photoaging. The presence of 3-nitrotyrosine (3-NT) is a direct consequence of oxidative stress and an indirect chemical indicator of $NO^{\bullet}$ and toxic peroxynitrite-induced damage.

Three-dimensional (3D) skin equivalents were established to study the effects of α-tocopherol and ascorbic acid on UVA-induced 3-NT formation. Air lift cultures were maintained for 4 weeks. Antioxidants were supplied for 7 days prior to UVA (20 J/cm^2) treatment and the medium was renewed every second day. 3-NT was detected by immunohistochemistry of 4-μm paraffin sections of 3D skin equivalents using a polyclonal rabbit-3-NT antibody in combination with a FITC-conjugated secondary antibody (goat-anti-rabbit IgG). All sections were counterstained for cell nuclei with DAPI. For morphological observation, sections were routinely stained using Hematoxylin/Eosin and analysed by subjective rating of the blinded stained sections.

Histological sections of skin equivalents were in good coincidence with *in vivo* human skin, *i.e.* the normal layers of the epidermis were well-developed and fibroblasts were embedded in the collagen matrix. While in 3D skin equivalents incubated with 25 μM α-tocopherol a weak 3-NT reactivity after UVA treatment could be observed, the addition of 50 μM ascorbic acid did not show any further protection from 3-NT formation. Three control 3D skin equivalents (non-supplemented) showed the strongest reactivity for 3-NT in epidermal sections.

Results from the present study suggest that accumulation of 3-NT may be a sign of detoxification of peroxynitrite. Therefore, systemically supplied antioxidants are able to protect from UV-induced ROS, likewise, and both subsequently decrease cytotoxic peroxynitrite formation.

Signal transduction by tyrosine nitration might be possible *via* competition or cooperation with tyrosine phosphorylation-dependent signaling events, depending on the local peroxynitrite concentration. $NO^{\bullet}$ metabolism *via* formation of peroxynitrite, and as reflected by the subsequently formed 3-NT, could be more involved in signal transduction pathways than expected.

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QUANTITATIVE ASSESSMENT OF AGING

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A wide spectrum of mechanisms has been proposed to date in order to explain the complex and multi-factorial process of aging. Oxidative stress (exogenous and endogenous), that occurs *in vivo* during aging, is considered to be one of the principal contributors of molecular damage to cellular and tissue structures, along with the widely accepted synergistic contribution of mitochondrial dysfunctions and tmDNA mutations, deficiency of antioxidant systems and reduced degradation of modified proteins. *Aim*: The purpose of the present study was to investigate, based on the carbonyl content of plasma proteins, the intensity of free radical reactions and the extent of the resulting oxidative injury in adults of different ages. *Materials and Methods*: The levels of carbonyl groups were examined in the serum of 75 healthy, annually-checked, non-smoking subjects, 50-75 years of age, separated into three study groups (50-55, 55-65 and 65-75 years of age, respectively). Carbonyl proteins were measured by an enzyme-linked immunosorbent assay, using an anti-

DNP antibody, which is highly sensitive (Zentech PC test). *Results*: A distinct positive correlation was found between the fluctuations of the levels of carbonyl proteins and the age of the individuals included in this study, whereas no significant difference was observed among men and women of the same group. *Conclusion*: 1. The age-related increase in protein carbonyl content provides evidence that there is a substantial correlation between chronological aging and the accumulation of oxidatively-damaged proteins. 2. The quantitative assessment of aging based on the measurement of carbonyl protein levels presents considerable advantages as compared to that of other oxidation products, because of their relative early formation and stability. Moreover, the above method requires only microgram quantities of protein, it allows a large batch of samples to be run simultaneously (and therefore can prove useful in clinical practice) and, finally, it avoids the high and sometimes variable blanks due to unbound DNP that are limitations of the colorimetric method.

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HYDROGEN PEROXIDE-MEDIATED MELANIN SYNTHESIS: A CELLULAR DEFENCE MECHANISM AGAINST OXIDATIVE STRESS?

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Several mammalian cells are able to synthesise melanin. If one biological function of melanin in melanocytes is obvious (it absorbs sun light and protects the skin from the sun), why and how do other cells such as leucocytes, neurones or thyroid cells produce melanin? Since oxidative stress is often associated with increased melanin synthesis, and hydrogen peroxide is the predominant oxidative intermediate formed during oxidative stress, we wondered whether hydrogen peroxide was involved in the key step of melanin synthesis, *i.e.* tyrosine hydroxylation to DOPA. In this study, using an *in vitro* cell-free system, we analysed the conditions required for the production of melanin from tyrosine. The rate-limiting step of melanin synthesis is the hydroxylation of tyrosine to DOPA, a reaction catalysed in melanocytes by the enzyme tyrosinase, whereas the further oxidation of DOPA and its polymerisation to melanin is catalysed either by tyrosinase or a peroxidase. By HPLC analysis, we demonstrated that although hydrogen peroxide, alone or in combination with peroxidase, cannot mediate tyrosine hydroxylation *in vitro*, the presence of biologic concentrations of ascorbic acid with hydrogen peroxide results in the non-enzymatic hydroxylation of tyrosine to DOPA at a very high yield, similar to that achieved by tyrosinase. If peroxidase is initially present in the reaction mixture, the non-enzymatic DOPA formation is followed by the production of dopachrome and the subsequent formation of melanin pigments within 2 hours. On the other hand, the treatment of cultured mouse melanocytes B16 with H_2O_2-generating systems results in increased hydroxylation of tyrosine to DOPA and increased melanin formation. Thus, this H_2O_2-dependent pathway can readily explain the mechanism of melanin synthesis by extracutaneous cells devoid of tyrosinase, as well as increased melanin production by cutaneous melanocytes in conditions of oxidative stress such as UV irradiation and inflammation, in which high concentrations of intracellular hydrogen peroxide are produced locally. Based on these data, we propose that melanin synthesis is a cellular defence mechanism against oxidative stress by scavenging H_2O_2. In particular, we suggest that the two seemingly divergent concepts of hydrogen peroxide-mediated *versus* neuromelanin-associated cell degeneration in Parkinson's disease are, in fact, two relevant phenomena with cause effect nature, in which H_2O_2 is simultaneously responsible for melanin formation and the oxidative stress which can lead to cell degeneration.

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PHOTOPROTECTIVE EFFECT OF SILYBIN AND ITS OXIDATION DERIVATIVES ON KERATINOCYTES

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UV radiation is a potent inducer of reactive oxygen species (ROS), which have been implicated in cutaneous aging as well as in skin cancer and various cutaneous inflammatory disorders (1, 2). Interaction of ROS with cell biopolymers, fatty acids and saccharides can result in many harmful effects: disturbed cell metabolism, morphological and ultrastructural changes and attacks on the regulation pathways, alterations in the differentiation, proliferation and apoptosis of skin cells. One approach to protect human skin against the harmful effects of UV radiation is using antioxidants as photoprotectants. In recent years, naturally occurring herbal compounds have gained considerable attention as protective agents (3, 4). Silymarin, an extract from the seeds of *Silybum marianum*, is known to protect skin against UV. Flavonolignans, silybin and dehydrosilybin, constituents of silymarin, exhibit a wide range of biological activities based mainly on their antioxidant properties. Although dehydrosilybin is a

stronger antioxidant than silybin, its low solubility may limit its bioavailability and/or efficacy.

Selective oxidation of the primary hydroxyl group of silybin and dehydrosilybin to the carboxyl has the potential for increasing solubility without loss of antioxidant activity. For this reason, we prepared new C-23 modified derivatives of both flavonolignan, silybinic acid, dehydrosilybinic acid and silybin acetal, and evaluated their radical scavenging and antilipoperoxidant properties in comparison with the parent substances. In order to assess the photoprotective potency of these compounds, we tested their effect on human keratinocytes exposed either to hydrogen peroxide (the most frequent form of ROS produced in dermal cells after UV exposure) or UVA radiation (320-400 nm).

The radical-scavenging effect of the compounds was evaluated in the reaction with a stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH); inhibition of lipid peroxidation was measured using ADP/FeCl3/NADPH-treated rat liver microsomes.

Human normal keratinocytes (HaCaT, $1x10^5$ cells/cm^2) were incubated in Dulbecco's modified Eagles's medium supplemented with 7% fetal calf serum (FCS) for 48 h prior to protection experiments. To assess the protection against H_2O_2-induced damage, HaCaT were pretreated with the test compounds (10, 25, 50 μM) in the medium without FCS for 30 min and then exposed to H_2O_2 (0.5 mM) for 4 h. To determine the effect on UVA-induced cell injury, HaCaT were transferred to PBS, irradiated by UVA (9.25 J/cm^2) and then treated by test compounds (10, 25 μM, in the medium without FCS) for 4 or 24 h. The cytoprotective effect of the test compounds against H_2O_2/UVA was evaluated using the following: LDH assay, neutral red retention assay, MTT assay, intracellular ATP and GSH levels determination.

All test compounds exhibited the ability to reduce H_2O_2/UVA-induced keratinocyte damage in the following order (related to their radical-scavenging efficacy): silybin acetal ≥ dehydrosilybinic acid ≥ dehydrosilybin > silybin ≥ silybinic acid. Dehydrosilybinic acid, being more soluble than dehydrosilybin, proved more effective at higher concentrations. Silybin acetal showed better efficiency than the parent substance.

Our data provide evidence that oxidation of the primary hydroxyl at C-23 of silybin or dehydrosilybin may be a promising approach to improve cytoprotective activity. The studied flavonolignans and their derivatives have the potential to reduce UVA-induced oxidative stress-mediated cellular responses and may be beneficial as supplements in photoprotective dermatological preparations and skin care cosmetics.

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PROTECTIVE EFFECT OF FLAVONOIDS AGAINST H_2O_2-INDUCED OXIDATIVE STRESS IN HUMAN KERATINOCYTES

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UV skin exposure induces extensive generation of reactive oxygen species (ROS). These can react with DNA, proteins, fatty acids and saccharides causing oxidative damage. Such injuries result in a number of harmful effects: disturbed cell metabolism, morphological and ultrastructural changes and attacks on the regulation pathways, alterations in the differentiation, proliferation and apoptosis of skin cells. These processes can lead to photoageing and skin cancer development (1, 2). Hydrogen peroxide is one of the most frequent forms of ROS produced in dermal cells after UV exposure, mainly as a result of UVA action (320-400 nm). For this reason, H_2O_2 is often used as an experimental agent to simulate cell UV damage (3).

One approach to protecting human skin against the harmful effects of UV irradiation is to use antioxidants as photoprotectives and, in recent years, naturally occurring herbal compounds such as flavonoids, known antioxidants, have gained considerable attention as protective agents (4, 5). In this study, we tested the effects of the selected flavonoids – silybin, dehydrosilybin, quercetin, taxifolin – on H_2O_2-induced keratinocyte damage.

The normal human keratinocyte cell line (HaCaT, $1x10^5$ cells/cm^2) were incubated in Dulbecco's modified Eagles's medium supplemented with 7% fetal calf serum (FCS) for 48 h. HaCaT were pretreated with test compounds (1-50 μM) in a medium without FCS for 30 min and H_2O_2 (0.5 mM; 4 h) was then applied. The cytoprotective effect of the test compounds against H_2O_2 was evaluated using the following methods: LDH assay, neutral red retention assay, MTT assay, intracellular ATP and GSH levels.

All tested compounds exhibited the ability to reduce H_2O_2-induced keratinocyte damage in the following order: dehydrosilybin > quercetin > silybin = taxifolin. The double-bond in ring B (C_2=C_3) of dehydrosilybin and quercetin is

responsible for their better cytoprotectivity. At the tested concentration range (1-50 μM), silybin, quercetin and taxifolin had no cytotoxic effects. Although dehydrosilybin showed low cytotoxicity (20%) at 50 μM concentration, we found that at lower concentrations (1-25 μM) it proved much more active than the other tested compounds.

These results show that the flavonoids studied could be used as supplements in skin care products for prevention of oxidative stress-induced human skin disorders.

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PHOTODYNAMIC THERAPY *IN VITRO* ON MELANOMA CELLS

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Photodynamic therapy (PDT) is a new treatment modality for tumours. The photochemical interactions of photosensitizer, light and molecular oxygen produce singlet oxygen and other forms of active oxygen, such as peroxide, hydroxyl radical and superoxide ion. The tumour is destroyed either by reactive singlet oxygen species (type II mechanism) and radical products (type I mechanism) generated in an energy transfer reaction. The resulting damage to organelles within malignant cells leads to tumor ablation. The cellular effects include membrane damage, mitochondrial damage and DNA damage. This process is non-selective in principle, and tumour specificity depends on a relatively high accumulation of the sensitizer in tumour tissue after systemic administration, combined with directed illumination. The mechanism of PDT cytotoxicity (phototoxicity) is not fully understood. We studied cellular uptake and the phototoxicity of *meso*-tetrakis(4-sulphonatophenyl)porphine (TPPS4) and ZnTPPS4, PdTPPS4 sensitizers in the presence or absence of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) on G361 human melanoma cells. Twice-washed trypsinized cells (ATTC, USA) were divided in the amount of 10^4 to each well (Dynatech plates 8 x 12, flat bottom) and filled in DMEM with 10% FCS in a total volume of 80 μL. After 24 hours of cultivation at 37˚C in 5% CO_2, the sensitizer (20 μL) was added. Cells were cultivated with sensitizers at concentrations ranging from 0.1 to 125 mg/ml. The total volume of 100 μL (cells with additives) were cultivated for 24 hours. The controls contained cells in the cultivation medium only. After 24 hours of cultivation, the cells were subsequently irradiated by a halogen lamp (24V/250W) at a dose of 0.5 to 150 J/cm^2. The halogen lamp has a continuous irradiance spectrum (from 360 to 2,700 nm), with maximum in visible and near infrared region. The absorption maximum of sensitizers is in the visible region (peak at 420, 550 and 630 nm). Irradiance was measured by Radiometer RK 2500 (Meopta Prerov, Czech Republic). Morphological changes in cells were evaluated using inversion fluorescent microscope and image analysis. The quantitative changes of cell viability in relation to sensitizers concentrations and irradiation doses were proved by fluorimetric measurement with fluoroscan Ascent (Labsystems). The viability of cells was determined by means of molecular probes for fluorescence microscopy (LIVE/DEAD kit). Sensitizer uptake into tumour cells may vary depending on the metabolic state of individual cells. The results, using fluorometric assay of cell lysates, showed that the maximum distribution of sensitizers occurs before that of sensitizers in the supramolecular complex with cyclodextrin. The distribution of the sensitizers fixed in cyclodextrin carriers is slower and probably more effective. According to our results, ZnTPPS4 seems to be more phototoxic than TPPS4 and PdTPPS4. G361 cells are sensitive to photodynamic damage by all of the tested sensitizers. In conclusion, ZnTPPS4, PdTPPS4 and TPPS4 in the supramolecular complex with cyclodextrin represent efficient sensitizers with high phototoxicity to G361 human melanoma cells.

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PYCNOGENOL®, FRENCH MARITIME PINE BARK EXTRACT – MORE THAN JUST A SUPERANTIOXIDANT

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Several large clinical studies failed to show any benefit of supplementation with anti-oxidative vitamins to protect against cardiovascular disease. Recently, the Heart Protection Study in the U.K., involving more than 20,000 patients at cardiovascular risk in 69 clinics, showed no benefit at all of a combination of 600 mg vitamin E, 250 mg vitamin C and 20 mg betacarotene. No effects on mortality, cardiovascular events, stroke, heart infarction, cancer or lung diseases could be observed.

In contrast, in the 7 countries study, observing 12,763 middle-aged men over a period of 25 years, the intake of flavonoids was significantly inversely related to the 25-year coronary heart disease mortality, demonstrating a preventive effect of flavonoid intake. Observations made with Pycnogenol®, containing a cocktail of flavonoids and phenolic acids, may contribute to explain why flavonoids are more effective than antioxidative vitamins for cardiovascular protection.

The possibility that flavonoids are so effective because of their higher antioxidative capacity can be taken into consideration, however, oxidative stress is only one of many risk factors for the cardiovascular system. The important risk factors are hypertension, high cholesterol or high LDL and low HDL, diabetes and smoking, besides the untreatable risk factor age.

Supplements with Pycnogenol® can influence all these risk factors positively, in a mild way.

Pycnogenol®, by reducing endothelin-1 and thromboxane levels and increasing concentrations of endothelial nitric oxide and prostacyclin, reduces high blood pressure. Several studies showed a cholesterol and LDL lowering effect together with higher HDL values. These observations could also be made with diabetic patients. Especially for diabetics, the prevention of platelet aggregation by Pycnogenol® is of outstanding importance because of the high risk of thrombosis in this group of patients.

The reduction of all these risk factors points to protection of the cardiovascular system by flavonoids on a broad basis. Anti-inflammatory effects, coupled with vasodilatation and cholesterol lowering, reinforced by prevention of platelet aggregation, should have a great impact on cardiovascular health. Instead of having a pill combining low doses of multiple therapeutic agents, Pycnogenol®, with its many flavonoids, offers the possibility of modest reduction in multiple risk factors.

Even smokers can benefit from Pycnogenol® in a direct way through improvement of the quality of cigarette filters with Pycnogenol®. It could be demonstrated that cigarette filters, containing Pycnogenol®, reduced the acute and chronic toxicity of tobacco smoke as well as its mutagenic effect. Of course, cessation of smoking would be the best and easiest protection.

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INHIBITION OF MATRIX METALLOPROTEINASES BY MARITIME PINE BARK EXTRACT AND ITS TWO MAJOR METABOLITES

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Matrix metalloproteinases (MMPs) constitute a family of endopeptidases that degrade extracellular matrix components such as elastin and collagen. Controlled and concerted activity of these enzymes facilitate physiological processes during development, cell migration or wound healing. In contrast, dysregulation of MMP expression or activation contributes to pathophysiological states, such as chronic obstructive pulmonary disease (COPD), asthma, atherosclerosis or cancer invasion and metastasis.

We have investigated the effects of maritime pine bark extract (Pycnogenol®) and two metabolites that are formed *in vivo* after oral intake of the extract on activities of collagenase 1 (MMP-1), gelatinase A (MMP-2) and gelatinase B (MMP-9). Inhibition of proteolytic degradation of collagen, elastin and gelatine was determined in a rapid and sensitive colorimetric microassay. A significant decrease of substrate hydrolysis was observed in the presence of Pycnogenol® and its metabolites. Both metabolites exhibited more potent inhibition of the matrix degrading enzymes compared to their parent pine bark extract. Of all tested enzymes, gelatinase B (MMP-9) was most effectively inhibited by one of the Pycnogenol® metabolites. Notably, (+)-catechin, the metabolic precursor compound, displayed no MMP inhibition activity. These experiments, employing purified enzymes, were confirmed by cell culture experiments with freshly isolated human monocytes. Both metabolites as well as their parent pine bark extract effectively reduced gelatinase B (MMP-9) secretion of lipopolysaccharide (LPS)-stimulated monocytes.

These results indicate that the maritime pine bark extract Pycnogenol® and its two major metabolites inhibit activities of matrix degrading enymes and secretion of gelatinase B (MMP-9) from LPS-stimulated human monocytes. Thus, we provide evidence that intake of Pycnogenol® could be beneficial in various inflammatory diseases or cancer.

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PYCNOGENOL® IN DERMATOLOGY

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French maritime pine bark extract, Pycnogenol®, was shown in biochemical, pharmacological and clinical studies to be a

very versatile substance with anti-oxidant, anti-inflammatory and circulatory enhancing benefits.

In a study on fair-skinned human volunteers, orally administered Pycnogenol® dose-dependently and statistically significantly increased the minimal UV erythema dosage. It took almost twice as high a UV exposure to cause the first signs of sunburn when subjects were supplemented with Pycnogenol®. Pycnogenol® protects the skin from UV damage by neutralising free radicals developing as a result of UV exposure. Thus, Pycnogenol® prevents the development of oxidative stress in the skin, which would initiate an inflammatory condition, the mechanism causing sunburn. Oral supplementation with Pycnogenol® represents a considerable anti-photoageing effect.

In clinical study, Pycnogenol® was shown to be effective for reducing over-pigmentation of the skin, as occurs in melasma (chloasma). Pycnogenol® supplementation for 1 month statistically significantly reduced both the size of over-pigmented skin areas, as well as the pigmentation intensity. Pycnogenol® supposedly exerts its beneficial activity of over-pigmentation by virtue of its pronounced antioxidant activity.

In a pilot study conducted with patients suffering from the auto-immune disease Lupus erythematosus, Pycnogenol® administration was shown to improve various typical inflammatory parameters of this disease. Patients took either Pycnogenol® or placebo for 2 months as second-line therapy, in addition to their prescribed medication consisting of prednisone and/or hydroxychloroquine. As compared to placebo, Pycnogenol® reduced typical blood parameters such as auto-antibody titre, granulocyte oxidative burst and premature T cell apoptosis.

In conclusion, oral supplementation with Pycnogenol® exerts profound antioxidant activity to the skin with objectively measurable benefits. Pycnogenol® protects the skin from age-related degradative processes, UV light-induced damage and inflammatory conditions.