CALLUNA VULGARIS EXTRACT MODULATES NF-κB/ERK SIGNALING PATHWAY AND MATRIX METALLOPROTEINASE EXPRESSION IN SKH-1 HAIRLESS MICE SKIN EXPOSED TO ULTRAVIOLET B IRRADIATION

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Photochemoprevention with natural products represents a new concept in the attempt to reduce the occurrence of skin cancer. However, the molecular mechanisms caused by ultraviolet light exposure remain still unclear. The aim of the study was to assess the mechanisms involved in the action of a Calluna vulgaris (Cv) extract, administered in single or multiple doses (10 consecutive days), on UVB-induced skin damage in SKH-1 hairless mice. The extract was topically applied 30 min before each UVB exposure in two doses (2.5 and 4 mg total polyphenolic content/40 μl/cm²). At 24 hours after the last treatment, total mitogen-activated protein kinase (p44/42MAPkinase, ERK 1/2), nuclear factor-κB (phospho-NF-κB p65), matrix metalloproteinases (MMP-2, MMP-9) and metalloproteinase inhibitor 1 (TIMP-1) levels were measured in skin using enzyme-linked immunosorbent assay (ELISA). MMP-2 and -9 activities were additionally evaluated by zymography. One topical application of Cv extract reduced the secretion (p<0.004) and inhibited MMP-9 activity UVB-mediated (54% inhibition). In multiple UVB exposures, both doses of Cv extract induced the increase of ERK 1/2 level in correlation with activation of NF-κB and reduced the secretion (p<0.04) and activation of MMP-9 (62% inhibition). Pretreatment with Cv diminished the MMP-2 protein secretion only in one dose UVB-irradiated group (p<0.0001) and decreased TIMP-1 level (p<0.001). These results demonstrated the dual behavior of Cv extract in skin protection against single versus multiple doses of UVB irradiation.

Key words: flavonoids, metalloproteinases, signal transduction, skin, ultraviolet radiation, metalloproteinase inhibitor 1

INTRODUCTION

Skin cancer is a significant health problem associated with mortality and morbidity; therefore concerted efforts were made aiming to develop novel strategies for the prevention of ultraviolet radiation (UV)-mediated damages. Chemoprevention, defined as “the use of agents capable of ameliorating the adverse effects of UVB on the skin” by natural compounds, represents a new concept in the attempt to control the carcinoogenesis process UVB-induced (1).

Calluna vulgaris L. Hull (known as common heather) is the sole species in the genus Calluna in the Ericaceae family. It was used in traditional folk medicine as an antiseptic, antibacterial (2), cholagogue, diuretic (3), expectorant, antirheumatic and antiinflammatory agent (4, 5).

Further, both in vitro (6) and in vivo studies (7-9) revealed antioxidant and anti-inflammatory effects of Cv extract. Recently, we demonstrated that pretreatment with Cv extract reduced lipid peroxides and nitric oxide generation and inhibited the UVB-induced apoptosis and inflammation in mice (8, 9) as well as the formation of DNA photolations in HaCaT keratinocytes (10). These effects were assigned to the high content of polyphenols identified in the extract composition (10).

It is known that phenolic compounds are plant secondary metabolites with antioxidative, antiinflammatory and photoprotective properties. In vitro and in vivo studies demonstrated that kaempferol and quercetin, the most important compounds identified in Cv extract, have important antioxidant, anti-inflammatory and antiproliferative activities (11, 12). Lee et al. (13) showed that kaempferol suppressed UVB-induced cyclooxygenase-2 (COX-2) expression and Src kinase activity in mouse skin epidermal JB6 P+ cells and attenuated the UVB-induced phosphorylation of mitogen-activated protein kinase (MAP kinase). Quercetin was found to suppress activation-UV induced of phosphorylated extracellular signal regulated kinase (ERK) and p38 MAP kinase in lipopolysaccharide-stimulated macrophage, and to inhibit nuclear factor-κB (NF-κB) activation, respectively proinflammatory cytokines and metalloproteinases (MMPs) expression (14, 15).

Recent studies indicated that the MAPkinase signal transduction pathways are involved in the cell function regulation, including MMPs expression and cell growth (16). Once activated, MAP kinases can translocate towards the nucleus to phosphorylate and activate the activator protein 1 (AP-1) and NF-κB factors. In the nucleus, NF-κB coordinates the transcriptional activation of inflammatory, anti-apoptotic genes and genes that positively regulate cell proliferation, metastasis and angiogenesis (17). Taking into account the potential therapeutic application of Cv extract on humans, this study intended to evaluate the mechanisms involved in Calluna.
vulgaris effects in single or multiple doses of UVB-induced damage to the skin of SKH-1 hairless mice. Specifically, we assessed comparatively the modulatory effects of Cv extract on UVB-mediated activation of ERK1/2 and NF-kB by using ELISA tests. Also, we assessed the MMP-2 and -9 activities by zymography and MMP-2, MMP-9 and TIMP-1 levels by ELISA.

MATERIALS AND METHODS

Reagents

Sodium dodecylsulfate (SDS), Triton X-100, 2,2-diphenyl-1-picryl-hydrazyl (DPPH free radical), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate (ABTS), Bradford and Folin Ciocalteu reagents were purchased from Sigma-Aldrich Chemicals GmbH (Germany). ELISA tests for measuring total p44/42MAPKinase (ERK1/2) and phospho-NF-κB p65 (ser536) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). ELISA tests for MMP-2, MMP-9 and TIMP-1 levels were obtained from R&D Systems (Minneapolis, MN, USA). Other chemicals were of reagent grade and purchased from Sigma-Aldrich Chemicals GmbH (Germany).

Plant material

The aerial parts of Calluna vulgaris L. Hull (known as Common Heather, fam. Ericaceae) were used. The aerial parts of Calluna vulgaris L. Hull were collected in August 2010 from Ciucea (altitude 650 m, 46.95 N 22.82 E), Cluj, Romania. The plant was identified by Prof. Dr. Mircea Tamas and a voucher specimen was deposited at the Herbarium of the Botany Department (index no. 825), Faculty of Pharmacy, University of Medicine and Pharmacy, Cluj-Napoca.

Sample preparation and total polyphenolic content

The Calluna vulgaris (Cv) fluid extract was obtained as described (9). Total polyphenolic content (TPC) was assessed by the Folin-Ciocalteu colorimetric reaction (18) and expressed as equivalents (Eq) gallic acid (GA) per unit of volume. The analysis was performed in triplicate.

High-performance liquid chromatography-mass spectrometry analysis of polyphenols from Calluna vulgaris extract

The composition of the Cv extract in polyphenols was determined by high-performance liquid chromatography (HPLC) analysis as previously described (10), using an Agilent 1100 HPLC Series system (Agilent, USA) coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL).

Determination in vitro of antioxidant capacity

2,2-diphenyl-1-picryl-hydrazyl (DPPH)-free radical assay (19) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate (ABTS) test were used to evaluate the antioxidant activity (8).

Animals and experimental protocol

Female SKH-1 hairless mice, 8 weeks old, from Charles River (Germany) were acclimatized for one week in a 12-h light/12-h dark cycle, 35% humidity, and free access to water and normocaloric standard diet (VRF 1). Two series of experiments were performed in order to evaluate the effects of Cv extract on mice exposed to a unique dose of UVB (experiment I) and respectively on mice treated with multiple doses of UVB irradiation, 10 days consecutively (experiment II).

In experiment I, 6 groups of 7 animals each, were treated as follows: Group 1 – control; Group 2 – vehicle (acetone 40%); Group 3 – UVB irradiation (240 mJ/cm²); Group 4 – vehicle + UVB irradiation; Group 5 – Cv 2.5 mg TPC/cm² + UVB irradiation; Group 6 – Cv 4 mg TPC/cm² + UVB irradiation.

For experiment II we used the following groups: Group 7 – control; Group 8 – vehicle; Group 9 – UVB (multiple doses, 240 mJ/cm²/day, 10 days); Group 10 – vehicle + UVB; Group 11 – Cv 2.5 mg TPC/cm² + UVB irradiation; Group 12 - Cv 4 mg TPC/cm² + UVB irradiation.

Before treatment, the animals were anesthetized with an i.p. injection of ketamine xylazine cocktail (90 mgkg⁻¹ b.w. ketamine, 10 mgkg⁻¹ b.w. xylazine). The extract was applied topically on the skin in a dose of 2.5 (low dose) respectively 4 mg/cm² (high dose), in vehicle, 30 min before each UVB exposure. UVB irradiation was performed with Waldmann UV 181 broadband UV-B source as described previously (8, 9). The UVB emission was monitored with a radiometer Variocube radiometer (Waldmann GmbH, Germany), Animals were weighed and observed during the experiment. 24 hours after the last treatment, the skin was macroscopically examined, animals were weighed and under anesthesia, fragments of dorsal skin were excised from each mouse to be used for investigations. All experiments were performed according to the approved animal-care protocols of the Ethical Committee on Animal Welfare of the “Iuliu Hâțieganu” University of Medicine in accordance with the internationally accepted principles for laboratory animal use and care (European Community Guidelines, EEC Directive of 1986; 86/609/EEC).

Isolation of skin samples

After the removal of subcutaneous tissue, skin tissue fragments were homogenized with a Polytron homogenizer (Brinkman Kinematica, Switzerland) for 3 min, on ice, in phosphate buffered saline (pH 7.4) added at a ratio of 1:4 (w/v). The suspension was centrifuged for 5 min at 3000 g and 4°C in order to prepare the cytolsic fraction. The proteins content in homogenates was measured with the Bradford method (20).

Quantitative estimation of total p44/42 MAP Kinase (ERK 1/2) and phospho-nuclear factor-xB p65 by ELISA

Total p44/42 MAP (ERK1/2) protein and phospho-NF-KB p65 (ser536) level were evaluated by path scan sandwich ELISA tests according to the manufacturer’s protocol (Cell Signaling Technology Inc.). Results are expressed in terms of OD units/mg protein of different treatment samples.

Measurement of MMP-2, MMP-9 and TIMP-1 levels by ELISA

For the analysis of MMP-2, MMP-9 and TIMP-1 ELISA assays were used according to the producer instructions (R&D Systems). The two MMPs levels and their inhibitor were expressed as pg/mg protein.

Determination of MMP-2 and MMP-9 activities by zymography

The activity of gelatinases was determined by zymography (21). Skin samples were homogenized with an Ultra Turrax homogenizer in a medium with 2% glycerin and 2% SDS in 62.5 mM Tris-HCl buffer. After centrifugation at 12,000 g, the protein content in the supernatant was determined by a slight modification of Lowry’s method. Five-microgram protein samples were subjected to electrophoresis on 10% polyacrylamide gels copolymerized with 1 g/mL gelatin at 100 V for 2 hours. After
electrophoresis, the gels were washed with 2.5% Triton X-100 for 1 hour, then incubated for 18 hrs, at 37°C, in 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM CaCl₂, 200 mM NaCl and 0.02 % BRIJ-35. The gels were then stained with 1% Coomassie brilliant blue R-250, for 2 hours and discolored in 10 % acetic acid solution. Activity was evaluated with an automated Vilber-Lourmet (France) image analyzer using the Bio 1-D program. Proteolytic activity was determined against a weight marker, confirmed with an enzymatic marker and reported as arbitrary units/µg protein.

Statistical analysis

Experimental data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey’s multiple comparisons post-test using the GraphPad Prism 5.0 software (GraphPad, San Diego, Ca., SUA). The four groups treated with Cv extracts were compared with control group (untreated), groups treated with vehicle and UVB irradiated. The data were expressed as means ±standard deviation. The effects of Cv extracts and UVB and their interaction were considered statistically significant at p<0.05.

RESULTS

Composition of Calluna vulgaris extract and the antioxidant capacity

Chemical analysis of the polyphenols content indicated a value of TCP in Cv extract of 67.50±3.25 mg GAE/ml (in 3 repeated extractions). The antioxidant activity of Cv extract evaluated by ABTS test was 38.92±0.01 eq. mM Trolox, in agreement with the results obtained previously (8). The antioxidant activity can be explained by the amount of phenolic compounds found in Cv extract as shown in Fig. 1 (10). The most representative compounds identified by HPLC-MS analysis in non-hydrolyzed sample were: hyperoside (855.7 µg/ml), quercetin (388.32 µg/ml) quercitrin (273.88 µg/ml) and kaempferol (99.912 µg/ml). The hydrolyzed sample revealed elevated quantities of quercetol (1290.636 µg/ml) and kaempferol (307.528 µg/ml).

Macrosopic changes

It is known from literature that UVB radiation induces erythema, edema and hyperplasia of epidermis (22). Thus, we first compared the visual aspect of the skin of non-irradiated and irradiated animals. No visible macroscopic changes (redness, swelling) in the skin of unirradiated mice and of animals treated with vehicle were observed. The groups exposed to a single dose of UVB (240 mJ/cm²), unprotected or protected with vehicle and Cv extracts, had the same macroscopic aspect as the unirradiated skin (Fig. 2A-E). In the 10 days experiment, multiple doses of UVB induced hyperplasia of the skin, scaling and erythema (Fig. 2F, 2G). Topical application of Cv extracts on 2 cm² skin area protected the skin, the epidermis was normal as color, thickness, without scaling or pigmentation (Fig. 2H). No toxic effects of Cv extract on treated animals (weight loss, morbidity and mortality) and on the organs (liver, brain, spleen) were not observed.
Effects of Calluna vulgaris extract on UVB-induced activation of p44/42 MAPK in SKH-1 hairless skin

The p44/42 MAPK (ERK1/2) signaling pathway can be activated as response to a diverse range of extracellular stimuli including mitogens, growth factors and cytokines (23) and is an important target in cancer diagnosis and treatment. One single dose of UVB did not change the protein level of total form of enzyme (1.64±0.02 vs 1.71±0.04 U OD/mg protein). Both doses of Cv extract administered before UVB exposure maintained the total ERK1/2 concentration close to control or irradiated groups (1.62±0.06 respectively 1.62±0.08 U OD/mg protein; p>0.05) (Fig. 3A).

In the 10 days experiment the total p44/p42 ERK 1/2 level, evaluated after the last treatment, revealed in UVB irradiated groups (0.16±0.04 vs 0.33±0.15 U OD/mg protein; 50% inhibition; p<0.05) lower but insignificantly amounts of protein compared to control or vehicle treated groups (0.16±0.04 vs. 0.33±0.15 U OD/mg protein; 50% inhibition; p>0.05) (Fig. 3B).

Topical applications of Cv extracts increased significantly phosphorylation of ERK (p<0.0001), suggesting that this pathway was activated in skin by treatment with natural products. The activation was not dose dependent, the highest value of ERK 1/2 being found in group treated with low dose of polyphenols (0.96±0.07 U OD/mg protein; 6.0 times; p<0.0001). High dose of TPC (4 mg/cm²) maintained a high concentration of ERK 1/2 in skin (0.73±0.21 U OD/mg protein).

Effects of Calluna vulgaris extracts on UVB-induced secretion and activity of MMP-2 and MMP-9 in SKH-1 hairless skin

Exposure to UVB radiation is known to upregulate the synthesis of matrix degrading enzymes. MMPs are a family of at least 24 zinc-dependent endopeptidases involved in photoaging, angiogenesis and metastasis (24). Among the family members, MMP-2 and MMP-9 have mainly been characterized as important factors involved in skin ageing and tumor invasion. Therefore, we evaluated the effect of Cv extract on UVB-induced MMPs protein secretion and activity in skin after UVB exposure. A single dose of UVB increased 3.7 fold MMP-2 (24.07±7.26 vs 6.28±3.10 ng/mg protein; p<0.0001) and 2.0 fold MMP-9 protein levels (33.04±7.73 vs 13.73±2.23 ng/mg protein) (Figs. 4A and 4B). Pretreatment of skin with Cv extracts given 30 minutes prior
to single UVB exposure reduced the MMP-9 level (11.10±9.26 respectively 17.05±2.44) and significant differences were obtained at low dose of TCP (p<0.004). ELISA data indicated that Cv extracts decreased the secretion of MMP-2 in skin (5.84±3.89 ng/mg protein; respectively 5.78±1.36; 41% inhibition p<0.0001). Analysis of zymographic images revealed the presence of MMP-9 (active form) and MMP-2 (active and inactive forms) in all groups studied (Figs. 4C-E). The inactive form of MMP-9 in skin was not detected. The activity of total MMP-9 was significantly increased (3.0 fold) in UVB irradiated group (523.8±2.05 U/µg protein) and in group pretreated with vehicle (410.0±120.1 U/µg protein; 2.0 fold; p<0.005). Low dose of Cv extract (2.5 mg TPC/cm²) significantly inhibited the total MMP-9 activity (54% inhibition; 283.6±34.22 U/µg protein; p<0.05). The activity of total MMP-2 evaluated by zymography did not change significantly after UVB irradiation (227.3±72.79 vs. 177.1±18.51 U/µg protein) or after treatment with Cv extracts (174.0±5.3 vs. 200.7±4.2 U/µg protein; p>0.05).

In the 10 days experiment, multiple UVB exposures did not increase the secretion of MMP-2 protein (6.85±1.16 U/µg protein vs. 6.28±3.10 respectively 7.05±2.83 pg/mg protein in control and vehicle treated groups; p>0.05) (Fig. 5A). Similar values were obtained in groups pretreated with both doses of Cv extract (6.22±1.79 and 4.74±1.08 pg/mg protein; p>0.05). Total MMP-2 activity was maintained at the same level in UVB irradiated groups and in groups treated with Cv extracts (p>0.05) as in the control group (Figs. 5C and 5E).

MMP-9 protein level quantified by ELISA (pg/mg protein) showed that the multiple UVB exposures insignificantly increased MMP-9 protein synthesis in skin cells (17.79±7.11) compared to control or vehicle treated groups (13.73±2.23 respectively 14.68±5.32 pg/mg protein) (Fig. 5B). MMP-9 protein secretion decreased 4.0 fold in group treated with 2.5 TPC/cm² (4.33±0.14; p<0.04). High concentration in TPC (4 mg/cm²) significantly maintained the reduced MMP-9 level (3.99±0.40 pg/mg protein) compared to UVB irradiated group (78% inhibition; p<0.04). Also, the activity of total MMP-9 decreased significantly in the group treated with 2.5 mg TPC/cm² (158.5±44.68 U/µg protein; 62% inhibition; p<0.01) compared to irradiated groups (381.3±417.4±6.78 U/µg protein) (Figs. 5D and 5E).

**Effects of Calluna vulgaris extract on UVB-induced secretion of TIMP-1 in SKH-1 hairless skin**

Extracellular matrix metabolism is controlled by MMPs and their tissue inhibitors, TIMPs. The balance between MMPs and TIMPs plays an important role in the homeostasis of tissue. Therefore, we studied the effect of Cv extract on UVB-mediated altered TIMP-1 level. One single UVB irradiation (240 mJ/cm²) did not change the TIMP-1 level in skin compared to non UVB exposed skin.
Cv extracts applied before one UVB exposure insignificantly reduced the secretion of protein in skin (498.0±129.4 respectively 409.3±77.35 pg/mg protein; p>0.05). Multiple doses of UVB maintained the same levels of TIMP-1 in skin as non UVB irradiated skin (658.1±80.98 vs 611.1±62.44 pg/mg protein) (Fig 6B). Pretreatment with 2.5 mg TPC/cm² Cv extract resulted in significant inhibition of TIMP-1 level (311.2±82.11 pg/mg protein; p<0.001) compared to UVB alone (48% inhibition). Also, 4.0 mg TPC/cm² significantly decreased the TIMP-1 generation (2.6 fold; 253.4±45.81 pg/mg protein; p<0.0001).

**DISCUSSIONS**

The present study examined some parameters assumed to be involved in of putative photochemopreventive effects of a natural extract obtained from *Calluna vulgaris* against the photodamaging action of a single dose and multiple doses of UVB. To define the efficacy of Cv extract at the molecular level we assessed the secretion and activation of MAPK/ERK 1/2, NF-κB and MMPs in SKH-1 hairless mice skin following UVB irradiation and the modulatory effect of Cv extract on these events. Perde et al. reported the isolation of quercetin, isoquercitrin, quercitrin, hyperozid and kaempferol from the plant of Romanian origin (10). It seems that the accumulation of high concentration of phenolic compounds depends on the collection area and period, as proved by phytochemical studies on *Calluna vulgaris* (25, 26). The protective effect of Cv extract was primarily confirmed by the macroscopic aspect of the skin, the epidemics of mice being normal in color, thickness, without scaling or pigmentation after UVB exposure. The macroscopic changes in UVB radiation-induced skin of SKH-1 hairless mice are suggestively presented in Fig. 2 as supplementary material.

It is well known that MAPKs, which belong to a family of serine/threonine protein kinases, have the role to mediate signal transduction.
transduction from the cell surface to nucleus. Among the three distinct MAPK pathways identified in mammalian cell, ERK1/2 is activated through UVB-induced oxidative stress (27), growth factors and protein kinase C. It has been also documented that NF-κB is a downstream target of the MAPK signal transduction pathway and that its activation is important in inflammation and cellular proliferation (27). Further, UV-induced MAPK kinase signaling is also involved in the activation of MMPs, enzymes involved in the degradation of skin tissues matrix. Reactive oxygen (ROS) and nitrogen (RNS) species and mediated activation of MAPK and NF-κB signaling pathways in UVB exposure has been shown to be inhibited by using plant origin antioxidants (28, 29). Therefore, we considered the activation of these pathways as being molecular targets for potential photochemopreventive agents.

In our study, exposure to a single dose of UVB with or without treatment with Cv extract, did not change the protein level of total ERK1/2 after 24 hours. These findings are in agreement with those of Vicentini et al. (30) which demonstrated that quercetin, an important component of Cv extract, did not have effect on activation-UV induced of the three MAP kinases, ERK, JNK, or p38, in human keratinocytes. It seems that a single dose of UVB induces the activation of ERK 1/2 with a maximum after 12-24 hours, without any change in total ERK1/2 protein level in first hours (31). Our results could be explained either by a transient effect of UVB, in the first hours after irradiation, or by activation of enzyme without increasing of total protein level. Moon et al. (32) confirmed that ERK and JNK kinases were activated within 30 min of UVB irradiation followed by a decline of enzymes to a baseline level.

The treatment with Cv extracts before each UVB exposure, 10 consecutive days, resulted in an increase of total ERK 1/2 level, especially at low dose (2.5 mg TPC/cm²). The activation of MAP kinase by Cv extract is indeed surprising as well as intriguing. Generally, the chemopreventive effect of polyphenols is due on the one hand to action free radical scavengers and on

Fig. 5. Effects of Cv extract on UVB-induced secretion and activity of MMP-2 and MMP-9 in SKH-1 hairless skin, in the 10 days experiment: (A) MMP-2 level, (B) MMP-9 level, (C) MMP-2 and MMP-9 (D) activities in skin 24 hrs after the last treatment. The animals were treated as under Fig. 3. Pretreatment with Cv extract inhibited the protein secretion and activity of MMP-9. Data are means ±standard deviation. Statistical analysis was done by a one-way ANOVA, with Tukey’s multiple comparisons posttest (*p<0.01 compared to UVB group); (E) Zymographic patterns of MMP-2 and MMP-9 activities in UVB and Cv treated groups in SKH-1 hairless skin.
the other hand to the induction of phase II detoxifying enzymes via antioxidant response element (ARE). Recent studies showed the involvement of MAP kinase in the regulation of phase II enzymes gene expression and in induction of apoptosis (33). However, the active components from Cv extract which would activate the MAP kinase pathway remain unclear. Other investigators have found both prooxidant and antioxidant effects of quercetin depending on concentration used (34) and experimental design (35). There is evidence that quercetin, used in different doses, increased 1.3- to 2.0-fold the phospho-ERK1/2 levels in human epidermoid carcinoma A431 cells (36). These results suggested that multiple doses of quercetin could induce phosphorylation of ERK 1/2. The effect was probably due to quercetin degradation products which became unstable in any aqueous-based topical formulation. They may have prooxidant effects and initiate the apoptosis and also potenetate the UVB-induced c-fos gene expression and increase p38 and cyclic AMP response element binding (CREB) phosphorylation (37). Addition of ascorbic acid to quercetin stabilizes it and inhibits formation of reactive intermediate species (ortho-semiquinone radical and ortho-quinone) (38) and consequently reduces its proapoptotic effect on UVB-irradiated human keratinocytes (35).

An activating effect on MAPkinase expression was observed in treatments with other natural compounds. Thus, Chen et al. (33) have shown that (-)-epigallocatechin gallate (EGCG) induces the MAP kinase activation, with maximum effect at 2 hours after treatment. Other in vitro studies revealed that silybinin amplified the phosphorylation of ERK 1/2 induced by 15 and 30 mJ/cm² of UVB, without noticeable changes in total ERK 1/2 protein levels (39). It is well known that ROS play a key role in enhancing inflammation through the activation of stress kinases and redox sensitive transcription factors, such as NF-κB. In keratinocytes, NF-κB activates p21 which in turn is involved in growth arrest (40). Our results indicated that one exposure to skin to UVB activated NF-κB p65 compared to non UVB-exposed. Pretreatment with 2.5 mg TPC/cm² inhibited the single dose UVB-induced NF-κB p65 activation, while in the 10 days experiment Cv extract increased the phospho-NF-κB p65 protein level. The results obtained in acute experiment are consistent with those published by Vicentini et al. (30). The authors showed that quercetin decreased one single UV irradiation-induced NF-κB DNA-binding by 80%. In the 10 days experiment the data obtained suggested a possible role of ERK 1/2 in NF-κB activation in Cv treated groups. It was shown that NF-κB DNA binding activity was one of the essential events for the protection of cells under various kinds of stress (39). Probably by an up-regulation of phospho-NF-κB p65, Cv extract had protective effect against UVB-induced damage. More detailed studies, however, are needed to support this idea. Moreover, some authors demonstrated that natural extracts acted differently: in lower doses or if the damage was moderate, they protected the skin cells, but in higher doses or if the UVB damage was severe, they induced apoptotic cells death. This could be assumed as protective mechanism of natural compounds in prolonged exposure to ultraviolet radiation

UVB radiation has been reported to cause induction of different MMPs, enzymes able to cleave components of cell-cell and cell-matrix junctions within the epithelium to promote re-epithelialization. MMPs activities are modulated on several levels including transcription, pre-enzyme activation or by inhibitors, endogenous (tissue inhibitors of metalloproteinases – TIMPs) (14) or exogenous (doxycycline) (41). Gelatinases belong to MMPs group and include two members, MMP-2 (gelatinase A, 72kDa) and MMP-9 (gelatinase B, 92kDa). Substrates of both enzymes are: collagen denatured by MMP-1 UVB-induced (42), vitronectin, fibronectin and gelatin (43). MAPkinases also regulate the expression of MMP-9. Moreover, some MMP genes contain NF-κB binding sites and are shown to be involved in their active synthesis (44).

In our study, one single UVB exposure increased significantly the secretion of MMP-2 and MMP-9 in skin cells. Also, UVB increased MMP-9 activity, both in acute and in the 10 days experiment. It was known that the recruitment of inflammatory cells, including neutrophils, macrophages and mast cells in skin exposed to UVB, could lead to release of MMP-9 protein. It was also demonstrated, that UVB irradiation stimulated activation of MMP-9 in keratinocytes in a dose-dependent manner via hydroxyl radical and lipid peroxides generation (45). In our study, pretreatment with Cv extract inhibited induction and activation of MMP-9. This effect was

Fig. 6. Effects of Cv extract on UVB-induced secretion of TIMP-1 in SKH-1 hairless skin in a single dose of UVB (A) and 10 days experiment (B). The animals were treated with Cv extract in a dose of 2.5 and respectively 4 mg TPC/cm², in vehicle, 30 min before each UVB exposure (240 mJ/cm²). Both doses of Cv extract resulted in significant inhibition of TIMP-1 secretion compared to UVB in the 10 days experiment. Data are means± standard deviation. Statistical analysis was done by one-way ANOVA, with Tukey’s multiple comparisons posttest (*p<0.01; **p<0.001 compared to UVB group).
noticed at low concentration of polyphenols and was explained by antiinflammatory and antioxidant effects of \(CV\) extract, as demonstrated in our previous in vivo studies (8). These results are in agreement with those of Fisher et al. which reported that the inflammatory cytokines induced MMP-9 expression through NF-\(\kappa\)B in keratinocytes exposed to UVB (46).

Analysis of the gelatinase MMP-2 in skin after one single UVB irradiation showed increased levels of MMP-2 secretion, this effect being inhibited by \(CV\) extracts. The MMP-2 activity in skin was not significantly changed in these groups, neither in experiment I, nor in experiment II. A possible explanation is the absence of critical elements in the promoter region of this gene, critical elements present in other MMP promoters in response to UVB exposure (47).

TIMP-1 is important for extracellular matrix metabolism because it can inhibit the activity of all MMPs, except membrane-type MMP. Generally, UV irradiation induces a significant increase in TIMP-1 level (47). However, UV induction of MMPs exceeds that of TIMP-1, resulting in an imbalance in MMP and TIMP levels (48). Therefore, we evaluated the TIMP-1 secretion in skin in correlation with the secretion and activity of MMPs. In our experimental model, UVB irradiation did not influence the level of TIMP-1 in skin. Pretreatment with \(CV\) extracts reduced the secretion of protein, especially after multiple doses of UVB irradiation. These results suggested that the inhibitory effect of \(CV\) extract on MMPs induction was due mainly to inactivation of MMPs activators (ROS and cytokines) and not to the stimulation of inhibitors secretion. In summary, \(CV\) extract attenuated UVB-induced photodamage by modulating the activation of MMPs, MAPkinases and NF-\(\kappa\)B. The results demonstrated the dual efficacy of \(CV\) extract in skin protection against different doses of UVB irradiation and suggested the role of \(CV\) as a UVB damage sensor. In lower doses or if the damage was moderate, \(CV\) protected the skin cells, but when the UVB damage was severe or in multiple UVB exposures, activated ERK 1/2 kinase and NF-\(\kappa\)B, and possibly induced apoptotic cells death. The effect might be important in human skin protection that is exposed to sunlight in their daily life. Further studies must be carried out on other biomarkers and signaling pathways in order to elucidate the molecular mechanisms involved in \(CV\) effect in chronic UV irradiation.

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