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Proteomic analysis of the combined effects of cannabigerol and 3-*O*-ethyl ascorbic acid on kinase-dependent signalling in UVB-irradiated human keratinocytes

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Oxidative stress induced by medium-wavelength ultraviolet radiation (UVB) is one of the most dangerous environmental stressors for the skin. Therefore, various medicinal remedies aim to prevent the harmful effects of UVB or support the recovery of the damaged cells. This study aimed to evaluate the impact of bioactive phytocannabinoid cannabigerol (CBG) together with 3-*O*-ethyl ascorbic acid (EAA), a stable, lipophilic derivative of the antioxidant vitamin C, on UVB-induced changes of proteome in cultured human keratinocytes 24 h after treatment. Surprisingly, proteomic analysis revealed very prominent CBG and EAA effects on kinases. These changes mainly influenced ERK1/2, IKK, MAP3K7, MAPK14, RIPK2, and NLK. Their expression was decreased by CBG and EAA, especially if used together after UVB-irradiation, so the effects of UVB were abolished restoring the profile of kinases to non-irradiated control. Moreover, CBG and EAA also reduced the UVB-induced modifications of proteins by the lipid peroxidation product 4-hydroxynonenal, especially in the case of AKT, Camkk1, cJun, ERK1, IKKα, MAPK11 and PERK. We conclude that, by maintaining proteome stability and kinase-dependent signalling, both CBG and EAA may support the recovery of human keratinocytes exposed to UVB radiation, especially if applied together, while the time-dependence of these effects should be further studied.

Keywords Keratinocyte proteome, Kinases, UVB radiation, Cannabigerol, 3-*O*-ethyl ascorbic acid, 4-Hydroxynonenal-protein adducts

UVB radiation is one of the most common and the most dangerous physicochemical factors for human skin cells. While both UVA and UVB affect the skin along with the sunlight, UVB is characterized by a lower wavelength and higher energy levels than UVA[1]. Because of that, UVB radiation penetrates even the epidermis and significantly affects the metabolism and functioning of its cells, especially keratinocytes[2]. The primary effect of UVB irradiation of keratinocytes is increased generation of reactive oxygen species (ROS), as well as disturbances in the functioning of the antioxidant system, including the activity of antioxidant enzymes, the level of non-enzymatic antioxidants, and the action of redox-sensitive transcription factors, such as Nrf2, Ap-1, and Ref-1, that are up-regulated[3]. Such changes are partly related to the direct effect of UVB on cellular structures but are also a consequence of the indirect effects of UVB on metabolism, including, among others, the functioning of mitochondria[4]. As a result, the mitochondrial electron transfer chain is interrupted, which leads to increased ROS production and the damage of mitochondrial membranes resulting in the release of pro-apoptotic molecules into the cytosol[5]. This is usually accompanied by an increased lipid oxidative metabolism, causing an increase in the level of lipid peroxidation products, including 4-hydroxynonenal (4-HNE), which is known as a signalling molecule involved in pro-inflammatory pathways[2]. Hence, in parallel to the lipid-based pro-inflammatory signalling, UVB radiation also induces inflammation by an increase of cytokine levels including

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TNF-α/β[6,7]. Moreover, UVB radiation stimulates the overproduction of proliferation factors by keratinocytes and promotes their differentiation thus enhancing the replication of damaged or mutated cells and accelerating their metabolic ageing[8]. Therefore, UVB induces disorders of intracellular signalling[9] including changes in the level and activities of protein kinases[10,11], and may even lead to skin cancer development[12]. Therefore, there is a demand for compounds/remedies that could be used to counteract the harmful effects of UVB radiation on the skin cells.

Among the compounds with potential skin protective activities are natural plant antioxidants, including phytocannabinoids[13]. One of the main non-psychotropic phytocannabinoids is cannabigerol (CBG), the biological action of which is based on the cannabinoid receptors (CB1/2) activation[14]. As a result, CBG modulates the expression of pro-inflammatory cytokines, including TNF-α, interleukins (IL-1β, IL-6, IL-10), interferon-γ, as well as prostaglandin E2 (PGE2)[15]. On the other hand, CBG through PPARγ receptor activation increases superoxidase dismutase (SOD) expression achieving antioxidant action under oxidative stress[16]. In the case of the skin cells, CBG exerts both antioxidant and anti-inflammatory properties[17] by silencing the pro-inflammatory pathways and reducing the proliferation of stress-stimulated keratinocytes[18]. Moreover, previous findings have shown that the positive effects of CBG can be enhanced by using it in combination with other natural antioxidants with different spectrums of physicochemical properties and biological activities[18]. An example of such a compound is 3-*O*-ethyl ascorbic acid (EAA). Namely, a comparison of the chemical structure of CBG and EAA suggests that EAA has a potentially greater possibility of proton donation, which should result in stronger antioxidant properties (Fig. 1). Moreover, EAA, as a derivative of the ascorbic acid with additional ethyl group is characterized by great stability and a capacity to penetrate skin[19]. So far, its antioxidant activity in the context of skin cells has been proven at the level of direct free radicals scavenging[20], ROS generation suppression[21], and activation of transcription of the cytoprotective factor Nrf2[22]. Accordingly, EAA can be considered an effective antioxidant factor, which may further support the antioxidant and anti-inflammatory actions of CBG and enhance its capacities for skin protection against UVB irradiation.

Therefore, the aim of this study was to perform proteomic analysis of the effects of CBG and EAA applied in vitro after the UVB-induced changes of human keratinocytes.

Results

Changes in the proteome

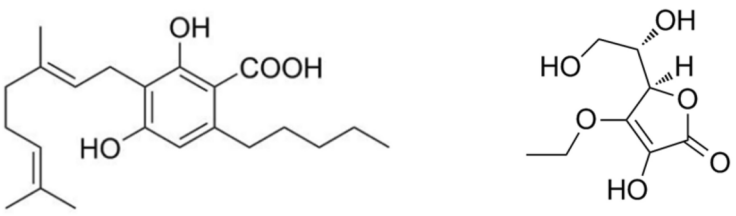
In total 890 proteins were label-free quantified and subjected to statistical analysis. The results obtained have shown significant differences in the proteome of keratinocytes treated with CBG and EAA, either if used separately or together, both in non-irradiated cells and in cells exposed to the UVB. Following non-informative variables filtering, including the omission of results with low repeatability, near-constant, and small values close to baseline or detection limit, the 599 proteins were detected as significantly changed using a significance threshold of $p < 0.05$ (Fig. 2A). These were mainly involved in the regulation of DNA expression (28% of all significantly changed proteins), antioxidant capacity (23%), or kinase activity (14%). When the significance threshold was narrowed to $p < 0.01$, the number of significantly changed proteins decreased to 357, while the function distribution ratio between these proteins also changed partially (Fig. 2B).

Namely, the proportion of proteins with kinase activities increased from 14 to 24%, which was surprising because the samples were analyses 24 h after treatment, while changes of the kinases occur usually much earlier after stress. Because 84 kinases were identified as significantly changed, the effects of CBG and EAA on kinases are presented in this paper.

Modification of the kinases

The analysis of biological functions of these kinases allowed us to divide them into 4 main clusters (Fig. 3). The biggest cluster 1 (in the figure marked with red) contained 27 proteins involved in the cellular process (GO:0009987), phosphorylation (GO:0016310), and signal transduction (GO:0007165). Cluster 2—marked in yellow, contained 11 proteins responsible for the nucleotide/carbohydrate derivative metabolic process (GO:0009117/GO:1901135) and phosphorylation (GO:0016310). Cluster 3 was marked in green and contained 10 proteins involved in the phosphate compound/lipid metabolic process (GO:0006796/GO:0044255), while in Cluster 4 (blue) were 10 proteins that positively regulate nitrogen compound metabolic process, macromolecule, and cellular metabolic process (GO:0051173, GO:0010604, GO:0031325).

Heatmap creation allowed the identification of the top 15 modified kinases within significantly changed proteins (Fig. 4). UVB radiation strongly induced expression of ERK ½, IKKα, ribose-phosphate protein kinase,



A. Cannabigerol [CBG] **B. 3-O-ethyl ascorbic acid [EAA]**

Fig. 1. The chemical structure of (**A**) cannabigerol [CBG] and (**B**) 3-*O*-ethyl ascorbic acid [EAA].

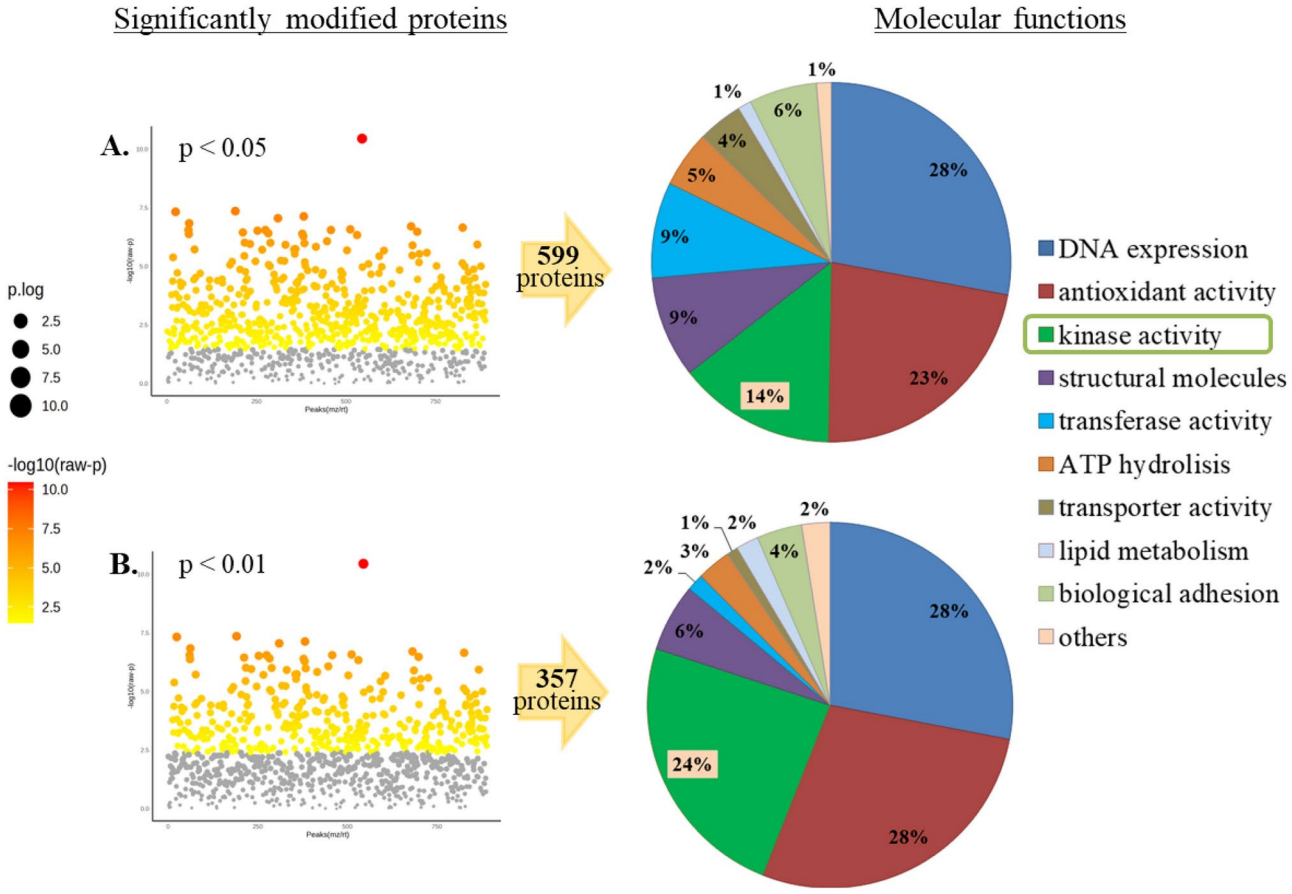


Fig. 2. The one-way univariate analysis (ANOVA), $p < 0.05$ (**A**) and $p < 0.01$ (**B**) for significantly changed proteins of keratinocytes exposed to UVB and treated with cannabigerol [CBG] or 3-*O*-ethyl ascorbic acid [EAA] or with both compounds used together. The molecular functions of proteins were determined using Protein ANalysis THrough Evolutionary Relationships Classification System (PANTHER 18.0).

serine/threonine kinase, MAP3K7, MAPK14, RIPK2, adenylate kinase 5, and NLK, while using CBG and EAA together strongly reduced the levels of these proteins both in non-irradiated keratinocytes and in the cells exposed to UVB. A different situation was observed in the case of another group of significantly changed kinases, notably PKC α/δ, IKKβ, cJun, and MEK2. The expression of these kinases was increased by EAA and decreased by CBG and by CBG + EAA given together to the non-irradiated cells. Following UVB irradiation, the expression of these kinases was increased, while CBG alone or CBG + EAA together significantly reduced their expression. These results were also confirmed by the western blot analysis. Additionally, the level of the phosphorylated form of chosen kinases (Akt, cJun, ERK1/2, PERK, and PKC) changed in a similar way indicating that their activity was changed in the same scope (Fig. 5).

Proteome/kinase modifications by 4-HNE

Furthermore, CBG and EAA used separately or together influenced levels of the protein modifications by the lipid peroxidation product 4-HNE both in non-irradiated and in UVB-exposed keratinocytes. However, as shown in Fig. 6, CBG and EAA, as well as CBG + EAA increased only a few several percent the level of total 4-HNE-protein adducts in non-irradiated cells, while in the case of UVB-irradiated keratinocytes, CBG and EAA decreased the UVB-induced 4-HNE-modified proteins for about 50% and 30%, respectively and even by 60% when used together.

Modifications by 4-HNE were also revealed for the cellular kinases (Fig. 7), although CBG and EAA either used separately/ or together did not have any significant effect on the level of these modifications in non-irradiated cells. However, UVB irradiation strongly increased the 4-HNE-adducts with AKT, Camkk1, cJun, ERK1, IKKα, MAPK11, and PERK.

The application of CBG and EAA used separately/together significantly decreased UVB-induced modification of kinases by 4-HNE, however in various degrees in a kinase-dependent manner. The 4-HNE-Camkk1 level was reduced most strongly by CBG and EAA, restoring the levels of 4-HNE adducts to the level of the non-irradiated control cells. Similar effects of CBG and EAA were observed also for 4-HNE-ERK1 and 4-HNE-cJun adducts, which were reduced by half. In the case of 4-HNE-AKT/IKKα/MAPK11/PERK, the UVB-induced increases of 4-HNE adducts were only slightly reduced by EAA but much more in the case of treatments with CBG or CBG + EAA.

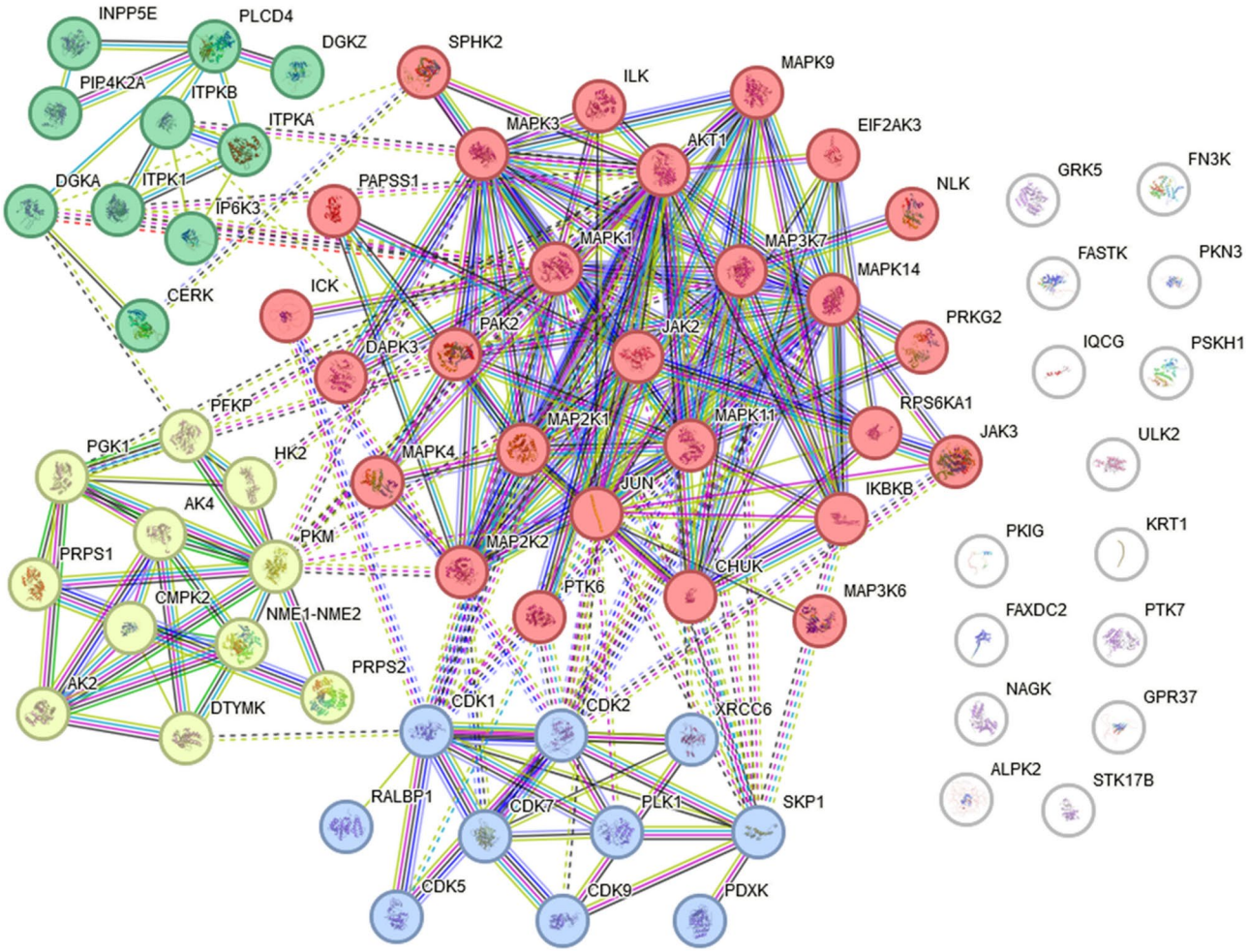


Fig. 3. The clustering of kinases the expression of which was significantly changed in keratinocytes exposed to UVB [60 mJ/cm^2] and treated with cannabigerol [CBG] or 3-*O*-ethyl ascorbic acid [EAA] or with both compounds used together. Analysis was done using STRING.11.5 based on the Gene Ontology (GO) database. Full lines represent known interactions (experimentally determined from curated databases) protein–protein associations—blue, gene co-occurrence—brown, gene fusions—grey, protein homology—pink, co-expression—violet, gene neighbourhood—yellow. Dashed lines represent predicted interactions.

Discussion

UVB irradiation is known as an environmental stressor, which induces oxidative stress in the skin and has a strong impact on DNA expression, including the biosynthesis of proteins involved in the antioxidant system of keratinocytes[23]. On the other hand, UVB influences the signal transduction pathways by the modulation of kinase-dependent signalling cascade activities[24].

Because metabolic responses of skin cells to UVB irradiation and various bioactive substances are dynamic, the first changes caused by irradiation appear already 30 min after treatment, manifesting structural modifications of signalling molecules including kinases phosphorylation[25]. Our previous study[25], has shown that the response of the cells (mainly on the level of the cellular membrane) to UV radiation is at the highest and afterward unchanged level in about two to six hours after treatment. The results of these UV-induced changes of intracellular signalling are visible with a certain delay in the functioning of cells—e.g., in their metabolism, proliferation, and differentiation[27], which require time to synthesize new proteins. The UVB radiation in the presented study was used as a stress factor, and the aim of the study focused on effects of the used protective substances, including CBG, whose long-term effects are based on the activation of CB1 and CB2 receptors by this phytocannabidiol.

In addition, there is also time needed for the tested substances to be transported into the cell. These parameters are important also while taking into consideration physical modifications of membrane phospholipids or protein structure/activity. For proteome analysis, we were also interested in protein expression changes, therefore, we extended the incubation to include activation of transcription factors, transcription/translation, and maturation of proteins with necessary post-translational modifications. Therefore, we have chosen the 24 h as a single time point, resembling several studies that also implemented the 24-h incubation, also showing that CBG induces expression of a wide panel of kinases in keratinocytes after 24 h[17].

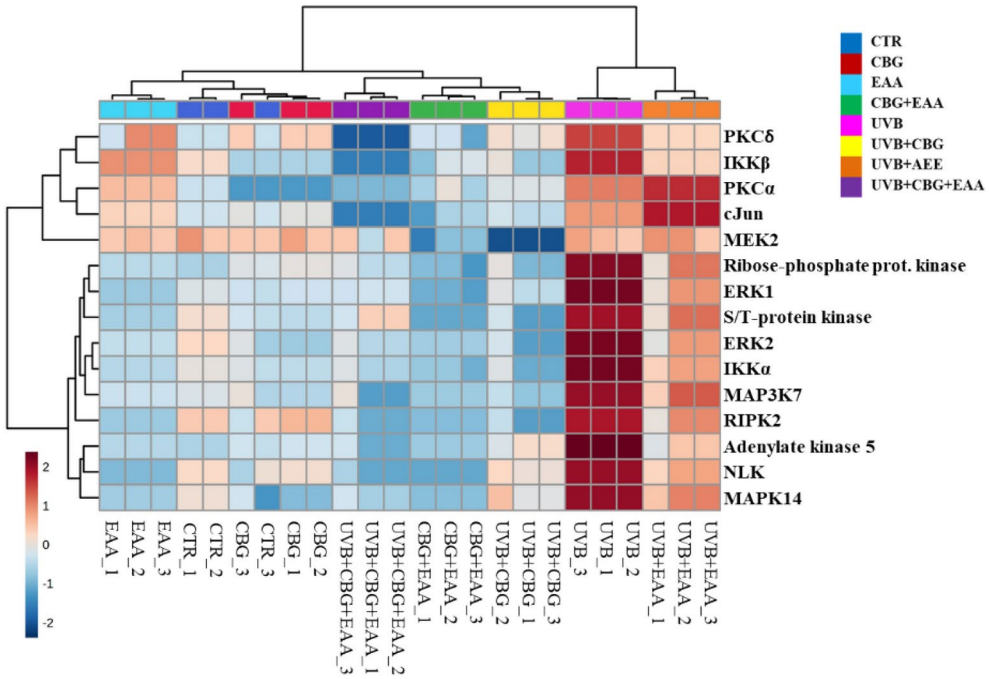


Fig. 4. Heatmap and clustering of top 15 modified kinases in keratinocytes exposed to UVB and treated with cannabigerol [CBG] or 3-*O*-ethyl ascorbic acid [EAA] and with both compounds used together. Analysis was done using MetaboAnalyst 5.0 software.

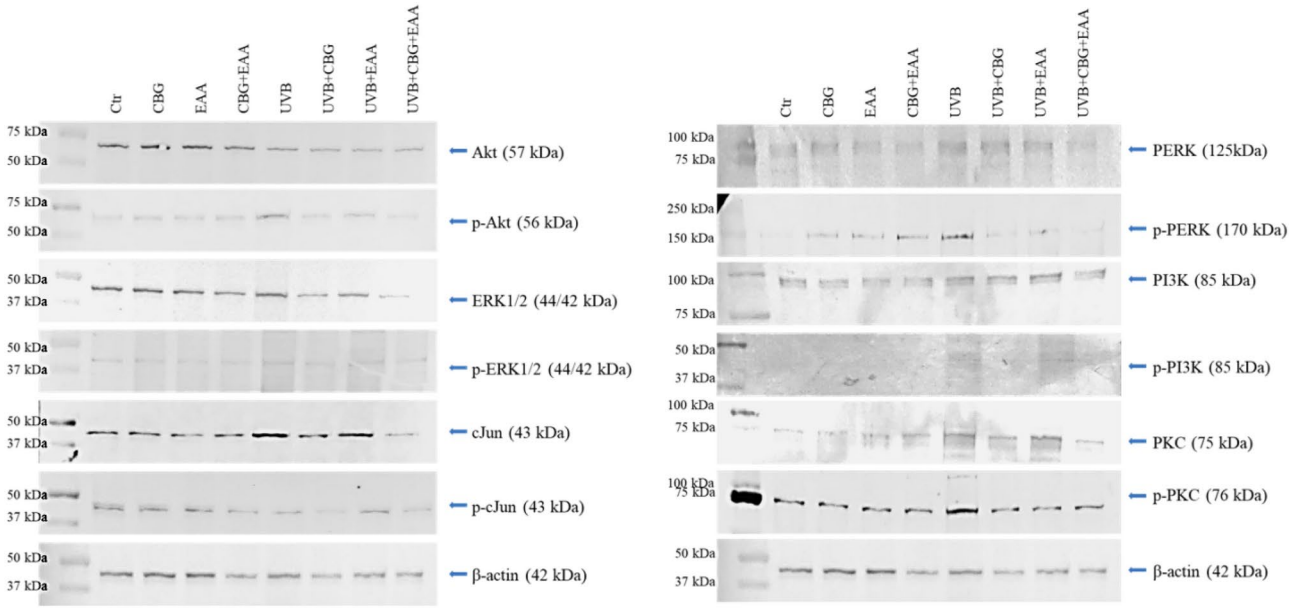


Fig. 5. Western blot analysis of kinases (Akt, ERK1/2, cJun, PERK, PI3K, PKC) and their phosphorylated forms in keratinocytes exposed to UVB and treated with cannabigerol [CBG] or 3-O-ethyl ascorbic acid [EAA].

The results of this study have further shown that UVB induces the expression of several kinases important for cell functioning. These include, among others, IKKα and IKKβ catalytic subunits of the IKK kinase, which following activation phosphorylates IκB causing its degradation. The IκB is the inhibitor of NFκB, thereby, its degradation favours NFκB nuclear translocation and induction of biosynthesis of pro-inflammatory cytokines[26]. At the same time, the UVB-increased levels of PKCα and PKCδ, accompanied by increased expression of their potential phosphorylation target p38 protein, are indicators of p38-dependent apoptosis induction in UVB-damaged cells[27]. On the other hand, an increase in the level of PKCα and PKCδ, which enhance the synthesis

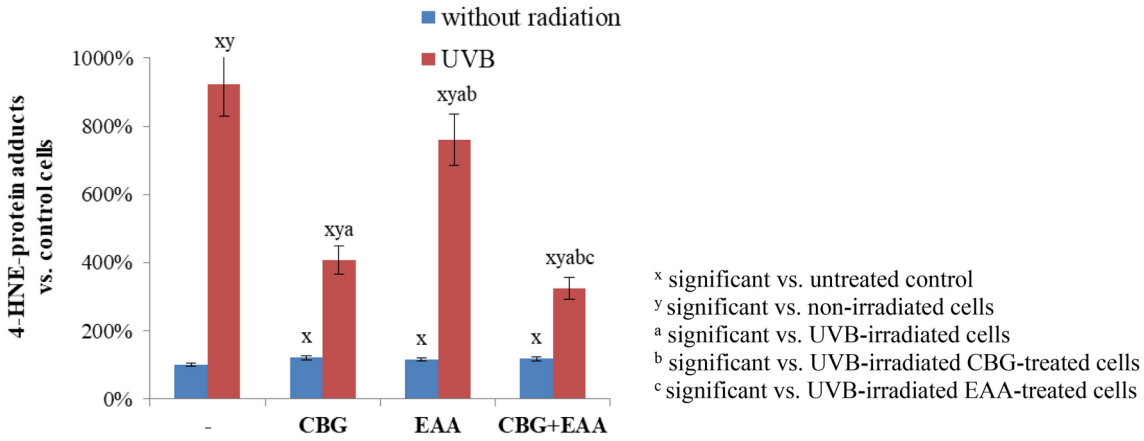


Fig. 6. The level of total 4-HNE–protein adducts in keratinocytes exposed to UVB afterward treated with cannabigerol [CBG] or 3-*O*-ethyl ascorbic acid [EAA] or with both compounds used together [CBG + EAA]. Mean values ± SD are presented with respective significance ($p < 0.05$).

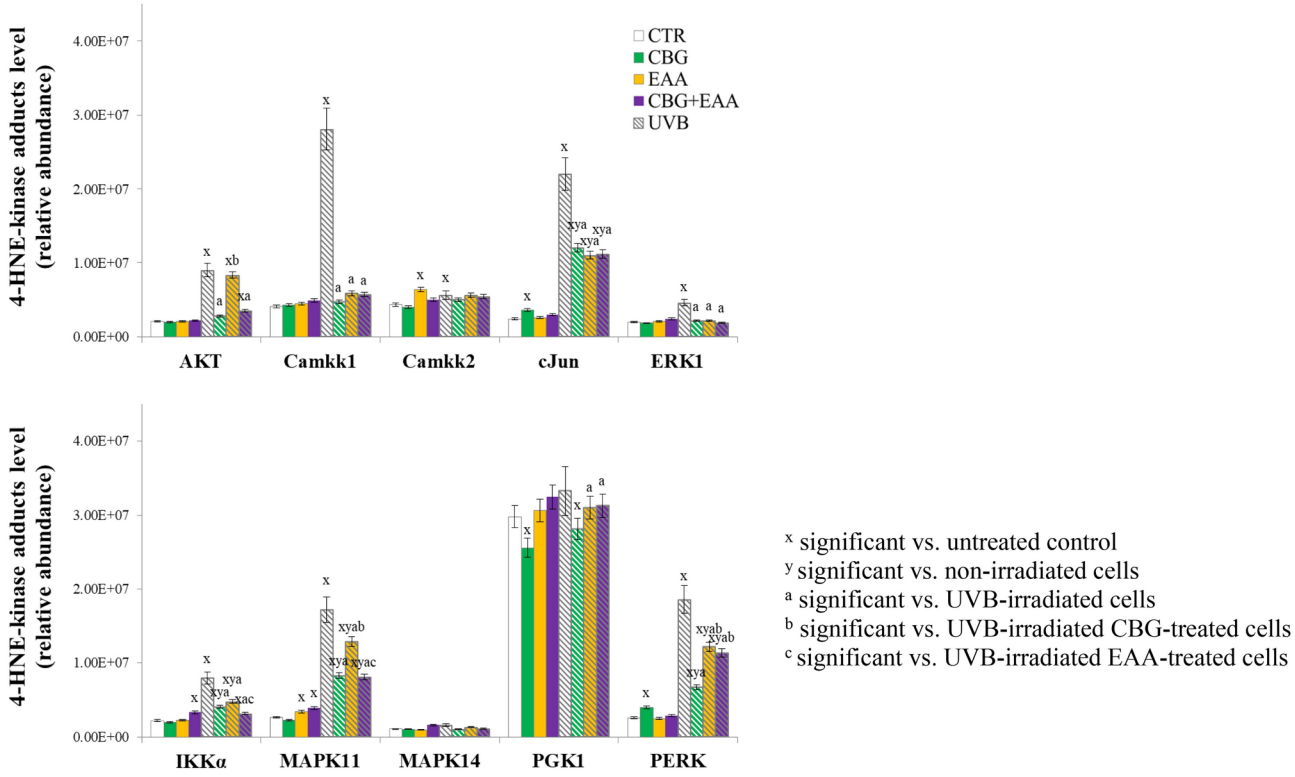


Fig. 7. The level of total 4-HNE–kinase adducts in keratinocytes exposed to UVB [60 mJ/cm^2] and treated with cannabigerol [CBG, 1 μM] or 3-*O*-ethyl ascorbic acid [EAA, 150 μM] and both compounds used together [1 μM CBG and 150 μM EAA]. Mean values ± SD are presented with statistically significant differences ($p < 0.05$).

of growth factors like TGF and VEGF, promotes cell proliferation under stress conditions increasing the risk of malignant transformation[28]. Moreover, UVB significantly enhances the levels of ERK1 and 2, which play important roles in protecting keratinocytes from death following UVB-induced oxidative stress[29]. On the contrary, the UVB-increased levels of NLK and the kinase responsible for its activation—MAP3K7 promote cell differentiation and maturation[30] that might protect skin cells from UVB and support the integrity of the skin barrier protecting the body[31].

Aiming to support the skin barrier function, new, especially natural compounds are constantly being sought to counteract the harmful effects of environmental factors like UV light. The results of our study indicate that CBG and EAA, especially if applied together can potentially fulfil expectations in the recovery of human

keratinocytes exposed directly to UVB in vitro, thus resembling previous results obtained by the proteomic analysis of the UVA-irradiated cells[18]. Namely, it was shown before that the mechanism of CBG action is based mainly on the activation of CB1/2 receptors, causing the induction of various signalling pathways including the MAPK activation[32]. Moreover, CBG acting through CB1 activation induces ERK1/2 phosphorylation with partial omission of the influence of the CB2 receptor[33]. In the case of EAA, its effect on cellular kinase activity was not clearly defined so far, however, because it is a derivative of vitamin C, its effect may be equally diverse depending on the type of kinase and the function it performs[34]. The results presented in this paper revealed that in the UVB-irradiated keratinocytes, both EAA and CBG significantly reduced the levels of kinases that were most strongly enhanced by UVB. It was observed that CBG has a stronger effect than EAA, probably because EAA acts directly as a strong ROS scavenger, while CBG induces cellular reactions dependent on CB1/2 receptors as mentioned above. Nevertheless, the results undoubtedly show that CBG and EAA used together have the strongest reversing effect on the changes induced by UVB irradiation on the proteome, notably the kinases profile of the keratinocytes, thus restoring the levels of kinases to normal, as detected for the cells not treated with UVB. This could be of great importance, especially considering the biological functions played by these kinases including signal transduction, and phosphate compound/lipid/nitrogen compound metabolic process, that are essential for the proper keratinocyte metabolism and skin functioning[35,36].

Kinase-dependent pathways regulation is often influenced by their structural modification, including adducts formation with lipid peroxidation products such as 4-HNE[37], a well-known molecule involved in redox signalling[37], which is affected by UVB radiation[2,38,39]. It has been found before that UVB irradiation also induces 4-HNE-protein adduct formation in skin cells[40]. The results of the current study indicate that cytoplasmic kinases are an important target for 4-HNE binding. This mainly applies to AKT, Camkk1, cJun, and PERK. So far, it has been described that 4-HNE forms adducts with PERK consequently inhibiting cell proliferation[41], while by addition to the Camkk1 4-HNE upregulates autophagy marker Beclin1[42]. The increased autophagy processes are accompanied by increased markers of apoptosis (Bad, p53, caspase-3/9), due to the inhibition of AKT through the interaction of 4-HNE with its enzymatic domain[43]. Simultaneously, 4-HNE-cJun adducts may also lead to cJun-dependent apoptosis[44].

Therefore, the decrease of the level of 4-HNE protein adducts induced using CBG and EAA after exposure of keratinocytes to UVB irradiation may be as important as are effects of these compounds on the cellular protein profile. This is especially relevant for the CBG/EAA-dependent reduction in the level of 4-HNE adducts with Camkk1, cJun, and PERK, which were significantly increased after UVB irradiation. Hence, it can be suggested that the CBG and EAA could prevent cell proliferation disorders and limit processes of autophagy and apoptosis that could cause damage to the skin tissue. In respect to that it should be mentioned that the experimental model on hairless mice exposed to the UVA light revealed that 4-HNE plays an important role in photoaging through binding with elastin, while such negative effects of UV light and 4-HNE can be attenuated by carbonyl scavengers[45]. On the other hand, psoriasis, characterized by hyperproliferation of the skin cells, is also associated with excessive formation of the 4-HNE-protein adducts even detectable in plasma mostly affecting proteins with catalytic activities[33]. Therefore, 4-HNE might be an important factor not only in local but also in systemic consequences of various diseases, which were recently reviewed in more detail[46].

The results of our study have shown that CBG and EAA reduced the level of 4-HNE-ERK1 adducts after UVB irradiation. Formation of the 4-HNE-ERK adducts may prevent the ERK dimerization, its phosphorylation, and activation[47,48], so this kinase cannot phosphorylate the Nrf2 molecule thus reducing biosynthesis of antioxidant proteins[49]. While most of these effects were similar for CBG or EAA, as well as for both compounds applied together, different effects were observed in the case of 4-HNE-AKT adducts, in case of which CBG reduced more effectively than EAA formation of these adducts upon their UVB-induced formation. This may be a consequence of CBG action on the AKT activity itself, as has been previously shown that CBG may significantly increase the phosphorylation of AKT[50], thereby favouring this kinase activity, causing selective autophagy induction in damaged or cells undergoing malignant transformation[51,52]. Therefore, CBG-induced phosphorylation of AKT could be s relevant like 4-HNE binding, as was observed for the UVB-irradiated cells treated with CBG alone, but also if treated with a mixture with EAA.

Furthermore, UVB irradiation triggers pro-inflammatory signalling by the NFκB activation based on increased IKKα/β expression. This signal transduction may be additionally reinforced in UVB irradiated cells by 4-HNE adducts created with IKKα, as was observed in this study, thus promoting IKK phosphorylation and, consequently, NFκB activation[37]. The formation of 4-HNE-IKK adducts was detected only for subunit α, which is known as an essential one in non-canonical NFκB activation in skin cells[53]. Because both CBG and EAA significantly reduced the level of 4-HNE-IKKα, these could silence pro-inflammatory signalling induced by UVB[18]. Such a potentially anti-inflammatory effect of CBG and EAA was greatest when these compounds were used together suggesting that combined use of CBG and EAA could produce the best effects for recovery of skin cells from ROS-dependent inflammation caused by the UVB light.

It should be mentioned that pleiotropic effects of 4-HNE are time- and concentration-dependent but can be different even at the same concentration range for different types of cells. Until early 90', 4-HNE was considered only to be the cytotoxic end-product of non-enzymatic lipid peroxidation, until the group of Hermann Esterbauer, who discovered 4-HNE sixty years ago, described that at low concentrations, 4-HNE can act as a growth factor, interacting with the humoral growth factors[63]. Such effects of 4-HNE manifested not before 24 h after treatment of cultured cells, even though it was found also that this aldehyde can regulate expression of immediate early genes (IEGs) within minutes after treatment[64]. One of the reasons for that is the high affinity of 4-HNE to bind to proteins, thus modifying their structure and function, in particular targeting various kinases and antioxidant enzymes[46]. Therefore, although 4-HNE is physiologically present in cells regulating growth, antioxidant capacities and apoptosis, its effects reflect the level of protein modifications[65]. Consequently, its cytotoxic effects can be determined even days after an increase of the 4-HNE protein adduct levels, while its

regulatory, even stimulating effects can be also observed days after a decrease of the cellular levels of the 4-HNE-protein adducts.

The results of our study showing that in keratinocytes prominent differences of the kinases and reduction of the total 4-HNE-protein modifications can be observed one day after treatment with CBG and EAA suggest that these substances may support the recovery of human keratinocytes exposed to UVB radiation, especially if applied together, while the time-dependence of their effects should be further studied.

Methods

Treatments of the cells

Human immortalized keratinocytes (CDD 1102 KERTr) obtained from the American Type Culture Collection (ATCC) were cultured according to the manufacturer's protocol. The growing medium based on Keratinocyte Serum-Free Medium (Gibco, Grand Island, NY) was supplemented with epidermal growth factor EGF (5 µg/l), foetal bovine serum (10%), and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin). Before exposure to UVB radiation cells were washed with culture temperature PBS (37 °C) to remove any remaining culture medium and in the cold buffer (PBS, 4 °C) to avoid possible heat increase while the cells were irradiated. The UVB irradiation at 312 nm was done using Bio-Link Crosslinker BLX 312 (Vilber Lourmat, Germany) in a total dose of 60 mJ/cm^2 chosen according to the 70% cell viability measured by the MTT assay[54]. The cells were irradiated for 25 s. at 15 cm distance from the 6 lamps with 6 W each, which corresponds to 4.08 mW/cm^2. The chosen dose (60 mJ/cm^2) was used according to the 70% viability of the cells measured by the MTT assay because the experiment was intended to induce significant changes in different signalling pathways and avoid substantial decay of the cells aiming to test if the substances tested would modify the effects of the UVB irradiation. After radiation cells were incubated for 24 h in a culture medium under standard conditions (humidified atmosphere of 5% CO_2 at 37 °C). To exam the effect of CBG (Orbis Cannabis Sp. z o.o., Warsaw, Poland) and EAA (TCI Europe, Zwijndrecht, Belgium), keratinocytes were incubated for 24 h in a medium containing 1 µM CBG or/and 150 µM EAA. All solutions were prepared in ethanol with the final ethanol concentration in the medium at 0.3%. In parallel, control cells were cultured without UVB irradiation in a medium containing 0.3% ethanol.

Following incubation all cell groups were collected by scraping into cold PBS and subjected to lysis by sonification on ice. Proteomic analyses were conducted in the supernatant obtained by samples centrifuge (15 min, 12.000 g, at 4 °C).

Protein digestion

The total protein content in all samples was measured using a Bradford assay[55], which allowed for further analysis of the volume of lysates containing 50 µg proteins. Before in-solution digestion, proteins were denatured by 8 M urea, reduced with 10 mM 1,4-dithiothreitol, and alkylated using 50 mM iodoacetamide. Following fourfold dilution, samples were digested overnight (37 °C) with trypsin (Promega, Madison, WI, USA) in a ratio of 1:50 (trypsin:proteins). To stop the reaction, 10% formic acid (FA) was added with the final concentration in the samples at 0.1%[56]. The obtained peptide mixture was dried under inert gas.

Proteomic analysis

A dried mixture of peptides was dissolved in 5% acetonitrile (ACN) with 0.1% FA and separated using the high-performance liquid chromatography system Ultimate 3000 (Dionex, Idstein, Germany) with a 50 mm × 75 µm PepMap RSLC capillary analytical C18 column (Dionex LC Packings, Dionex, Idstein, Germany) at a flow rate of 0.300 µl/min. Eluted peptides were analysed using a Q Exactive HF mass spectrometer with an electrospray ionization source (ESI) (Thermo Fisher Scientific, Bremen, Germany) operated in positive mode and data-dependent mode. Survey MS scans were conducted in the 200–2000 *m/z* range with a resolution of 120,000. In subsequent scans, the top ten most intense ions were isolated, fragmented, and analysed at 30,000 resolution. A 10 s dynamic exclusion window was applied, and an isolation window of 4 *m/z* and one microscan was used to collect suitable tandem mass spectra by Xcalibur software (Thermo Fisher Scientific, Bremen, Germany). Conditions of the peptide analysis have been described in detail previously[57].

Protein identification and label-free quantification

Raw data were analysed using Proteome Discoverer 2.0 (Thermo Fisher Scientific, Seattle, WA, USA). Input data were searched against the UniProtKB-SwissProt database (taxonomy: Homo sapiens, release 2024-02). Peptide mass tolerance at 10 ppm, MS/MS mass tolerance at 0.02 Da, up to two missed cleavages, cysteine carbamidomethylation, and methionine oxidation as dynamic modifications were used for protein identification. Additional dynamic modification of cysteine/lysine/histidine by 4-hydroxynonenal (4-HNE)[58] was set. Protein label-free quantification was performed according to the signal intensities of the precursor ions. The modified protein quantification was done based on the peak area analysis.

Determination of phosphorylated kinase expression

Western blot analysis of kinase expression was performed following 10% Tris–Glycine SDS-PAGE separation and protein transfer on the nitrocellulose membrane[59]. The blotted membrane was blocked with 5% skim milk for 1 h. Primary antibodies against ERK1/2, p-ERK1/2 (Thr202/Tyr204), cJun (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Akt, p-Akt (Ser473), PKC, p-PKC (Ser657) (Bioss Antibodies Inc., MA, USA), PERK, PI3K, p-cJun (Ser63), p-PI3K (Tyr458, Tyr199), p-PERK (Thr981) (Abcam, Cambridge, UK) and β-actin (Sigma-Aldrich, St. Louis, MO, USA), as well as secondary antibodies against mouse/rabbit (Sigma-Aldrich, St. Louis, MO, USA), were used at a concentration of 1:1000. Protein bands were visualized using the BCIP/NBT Liquid substrate system (Sigma-Aldrich, St. Louis, MO, USA) and were quantitated using the Versa Doc System and

Quantity One software (Bio-Rad Laboratories Inc., CA). The obtained results were expressed as a percentage of control cells.

Statistical analysis

Each cell variant was repeated in 3 independent biological replicates. Only proteins with identified at least three peptides longer than six amino acid residues and at least two unique peptides were taken for statistical analysis. To obtain the normal distribution, results from individual protein label-free quantification were log-transformed, auto-scaled (mean-centered and divided by the standard deviation of each variable), and normalized by the sum of the protein intensities obtained for each sample using open-source software MetaboAnalyst 5.0 (http://www.metaboanalyst.ca)[60]. The same software was used for biostatistical analysis, including t-test/univariate analysis one-way (ANOVA, Fisher's least significant differences (LSD), the false discovery rate (FDR) < 5%). Protein functions were determined using Protein ANalysis THrough Evolutionary Relationships Classification System (PANTHER 18.0)[61] while their clustering was done using STRING.11.5 based on Gene Ontology (GO) database[62].

Data availability

All data generated or analysed during this study are included in this published article and are available in details as supplementary data upon request sent to the first author (agnieszka.gegotek@umb.edu.). Because of technical reasons, as requested by the Manuscript Administration of the journal, these files were not included as Supplementary Information files.

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Author contributions

Conception/design of the work: A.G. and E.S.; Acquisition/analysis: A.G., I.J.-K., and A.R.; Interpretation of data: A.G. and I.J.-K.; Software/methodology: A.G., I.J.-K., and A.R.; Funding acquisition: E.S.; Visualization: A.G., A.R.; Writing—original draft: A.G. and E.S.; Writing—review and editing: N.Z. and E.S. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Competing interests

The authors declare no competing interests.

Additional information

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