

## Research Article

# Antioxidant and Anti-inflammatory Effect of Cannabidiol Contributes to the Decreased Lipid Peroxidation of Keratinocytes of Rat Skin Exposed to UV Radiation

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There is a great need for compounds with antioxidant and anti-inflammatory properties for protection against UV radiation, which is the most prooxidative physical factor that skin cells are exposed to everyday. Therefore, the aim of the study was to evaluate the mechanism of phytocannabinoid-cannabidiol (CBD) action *in vivo* on lipid metabolism in keratinocytes of rat skin exposed to UVA/UVB radiation. Our results show that CBD protects keratinocytes against the effects of UVA/UVB radiation by reducing lipid peroxidation products: 4-HNE and 8-isoPGF<sub>2α</sub>. In addition, CBD significantly increases the level of endocannabinoids, such as anandamide, 2-arachidonylglycerol, and palmitoylethanolamide, and the activation of their receptors CB1/2 or TRPV1. The above changes are due to the protective effect of CBD against the UVA/UVB-induced decrease in the level/activity of superoxide dismutase and the components of the thioredoxin and glutathione systems. CBD also increases the *in vivo* transcriptional activity of Nrf2 and the expression of its Bach1 inhibitor as well as preventing the UVA/UVB-induced increase in the expression of Nrf2 activators p21, p62, p38, and KAP1 and proinflammatory factors such as NFκB and TNFα. By counteracting oxidative stress and changes in lipid structure in keratinocytes, CBD prevents cellular metabolic disturbances, protecting the epidermis against UV damage.

## 1. Introduction

Ultraviolet (UV) rays from solar radiation that reach the earth's surface include UVA and UVB, which are the most common exogenous factors that cause damage to skin cells [1]. UVA and UVB radiation has various biological effects, but their joint effect is the increased generation of reactive oxygen species (ROS) and disturbances to cellular antioxidant capacity [2]. Moreover, UVA activates endogenous photosensitizers that can also lead to generation of ROS [3]. As a result, UV-induced changes lead to oxidative modifications of lipids, proteins, and DNA in skin cells, modulating intracellular signal transduction pathways that can lead to photoaging and development of skin cancer [4]. On the other hand, the described UVA or UVB action on skin cell has been

used in phototherapies of skin diseases, e.g., psoriasis, resulting in the treatment of both diseased skin cells and adjacent to them healthy cells [5].

Among some of the consequences of oxidative stress are modifications to phospholipid metabolism including increased ROS-dependent peroxidation with the formation of oxidative fragmentation and cyclization products, which appear to be of particular importance. The resulting electrophilic  $\alpha,\beta$ -unsaturated aldehydes and cyclic prostaglandin derivatives at low concentrations participate in the defense against microorganisms while at higher concentrations they can cause metabolic disorders [6]. In addition, oxidative stress promotes the increased activity of enzymes participating in the metabolism of membrane phospholipids, which results in the formation of further groups of lipid mediators

including endocannabinoids, which act mainly by activating G protein-dependent membrane receptors involved in the regulation of ROS and levels of pro/anti-inflammatory cytokines [7].

Taking all this into account, there is a significant need to find a compound, especially from groups of natural compounds, with antioxidant and anti-inflammatory properties that would protect epidermal cells in the human skin from UV radiation emitted by solar radiation that is encountered on a daily basis, as well as UV rays used in phototherapy for skin diseases. One potential candidate is the pharmacologically active—but nonpsychoactive—antioxidant and anti-inflammatory phytocannabinoid-cannabidiol (CBD, Figure 1) found in the *Cannabis sativa* L. plant [8–10]. Assessment of the antioxidant activity of CBD has shown that it can regulate the redox state directly by influencing redox components and indirectly interacting with other molecular targets related to redox balance [11].

CBD has been found to modify redox balance by changing the levels and activities of both prooxidative and antioxidant compounds [12]. The antioxidant activity of CBD has been demonstrated through its effect on the activity of Nrf2 transcription factor that is responsible for the expression of cytoprotective proteins including antioxidant enzymes [13]. Among other things, CBD has been found to increase the mRNA levels and enzymatic activity of superoxide dismutase isoenzymes in the mouse with diabetic cardiomyopathy type I and in human cardiomyocytes exposed to 3-nitropropionic acid or streptozotocin [14] and also increases the activity of glutathione peroxidase (GSH-Px) in human keratinocytes irradiated with UVB *in vitro* [15].

Through modulation of these cellular effectors, CBD mitigates the effects of oxidative stress. Moreover, due to its lipophilic nature, CBD accumulates in cell membranes [10], having a particularly effective antioxidant effect on membrane components. CBD treatment has been found to reduce lipid peroxidation, estimated through levels of 4-hydroxynonenal (4-HNE) in C57BL/6J mouse liver and malondialdehyde (MDA) in mouse neuronal cells (HT22) under reperfusion conditions [16, 17]. It has been shown to prevent 4-HNE-protein and MDA-protein adduct levels in fibroblasts irradiated with UV [18]. In this way, CBD protects lipids and proteins involved in cellular signaling pathways from oxidative effects [19, 20]. The purpose of this study was to evaluate the mechanism of CBD action with respect to redox imbalance in keratinocytes of rat skin exposed to chronic UVA or UVB radiation.

## 2. Materials and Methods

**2.1. Materials: Rat Experiment.** Experiments were carried out on male nude rats (Hsd:RH-Foxn1<sup>rn</sup>) at the age of 8–9 weeks (body weight 260–302 g) purchased from Vivari (Vivari s.c., Warsaw, Poland). Animals are characterized by skin hairlessness and thymic aplasia, which ensures obtaining keratinocyte fraction from the epidermis unpolluted with lymphocytes. Rats were kept under standardized conditions, 12 h light/12 h dark cycles, and fed pellets containing a mixture of various dietary components such as proteins, fiber,

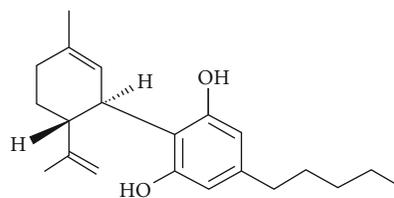


FIGURE 1: The chemical structure of cannabidiol.

and minerals [21]. All experimental activities with rats were approved by the Local Ethics Committee for Animal Experiments in Olsztyn (Resolution No. 37/2019 of April 26, 2019).

The rats were divided into the following groups of six:

- (1) Control group: rats were treated with nontoxic hydrophilic petrolatum applied topically on the back for 20 min every 12 h for 4 weeks
- (2) CBD group: rats were treated with CBD (2.5% [22]; *w/w* in petrolatum) applied on the back for 20 min every 12 h for 4 weeks
- (3) UVA group: the skin of the backs of the rats was irradiated with UVA in increasing dose from 0.5 to 5 J/cm<sup>2</sup> every 48 h for 4 weeks; individual radiation doses were distributed as follows: 3 times 0.5 J/cm<sup>2</sup>, 3 times 1 J/cm<sup>2</sup>, 3 times 1.75 J/cm<sup>2</sup>, 3 times 2.5 J/cm<sup>2</sup>, and 2 times 5 J/cm<sup>2</sup>
- (4) UVA+CBD group: the skin of the backs of the rats was irradiated with UVA every 48 h as in the UVA group, and every 12 h, the backs of the rats were treated with CBD as in the CBD group
- (5) UVB group: the skin of the backs of the rats was irradiated with UVB in increasing doses from 0.02 to 2 J/cm<sup>2</sup> every 48 h for 4 weeks; individual radiation doses were distributed as follows: 2 times 0.02 J/cm<sup>2</sup>, 2 times 0.05 J/cm<sup>2</sup>, 2 times 0.1 J/cm<sup>2</sup>, 2 times 0.2 J/cm<sup>2</sup>, 2 times 0.5 J/cm<sup>2</sup>, 2 times 1 J/cm<sup>2</sup>, and 2 times 2 J/cm<sup>2</sup>
- (6) UVB+CBD group: the skin of the backs of the rats was irradiated with UVB every 48 h as in the UVB group, and every 12 h, the backs of the rats were additionally treated with CBD as in the CBD group

The scheme of experimental schedule is shown in Figure 2.

Animals were irradiated using the lamp with UVA/UVB emitter (Cosmedico, Stuttgart, Germany) that are applied for the treatment of human skin diseases. To maintain a required dose of radiation and constant conditions of the experiment, the plastic combs, around 2 cm long, were used, which also provide skin protection against overheating and burns.

At the end of the experiment, the animals were anesthetized with inhaled isoflurane and sacrificed by heart excision. The skin from the back of the animals was immediately placed into phosphate-buffered saline (PBS) with protease inhibitor cocktail and incubated on ice for 1 h. Next, samples

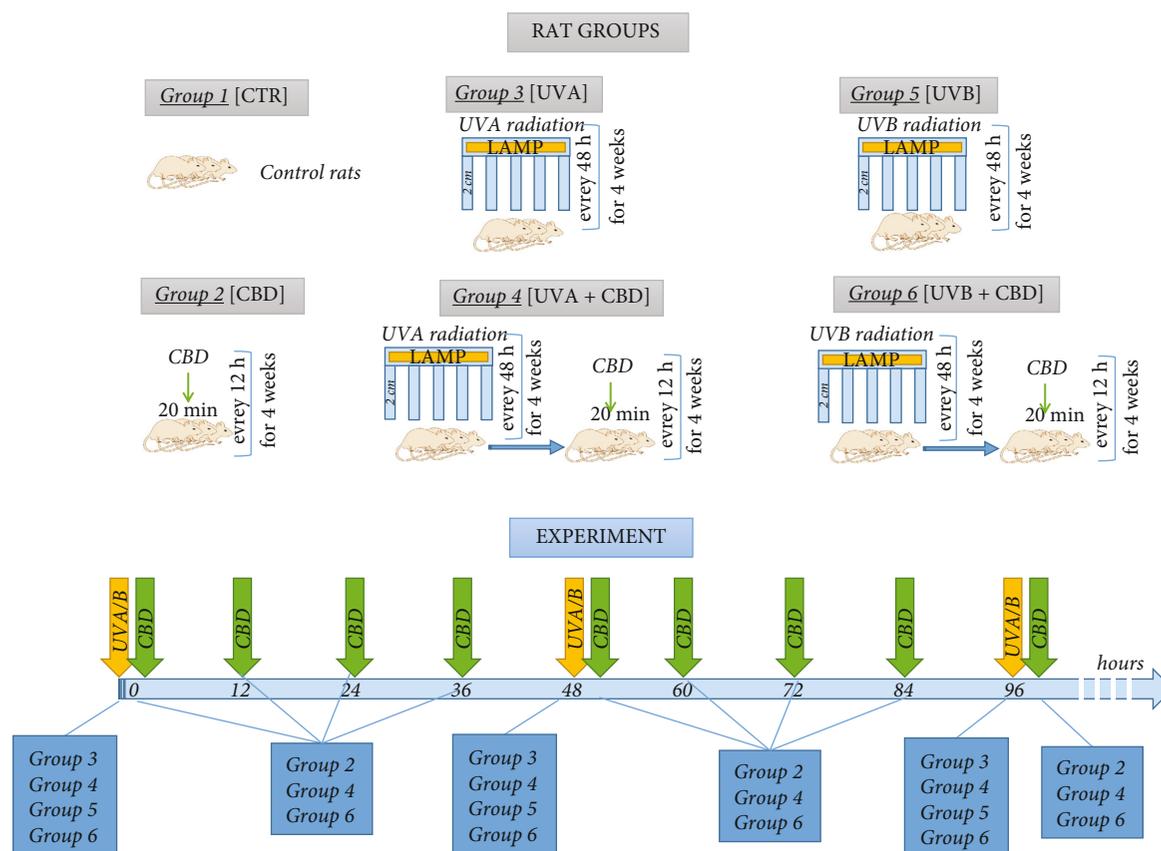


FIGURE 2: The scheme of experimental schedule showing animal group ( $n = 6$ ) treatment and distribution of activities over time.

were fragmented and then incubated overnight at 4°C in 1 mg/mL dispase to separate the layer of epidermal cells from the dermis [23]. The epidermis was digested for 20 min with 2.5% trypsin to release keratinocyte clear fraction. Cells obtained after 3 min of centrifugation at  $300 \times g$  were resuspended in PBS with proteasome inhibitor cocktail. Keratinocytes were then sonicated and subjected to analysis. The total protein content in cell lysates was measured by the Bradford method [24].

## 2.2. Methods

**2.2.1. Determination of Lipid Peroxidation.** 8-isoPGF<sub>2α</sub> was extracted from lysates of keratinocytes using solid phase extraction (SPE) and identified using ultraperformance liquid chromatography tandem mass spectrometry (LCMS 8060, Shimadzu, Kyoto, Japan) [25]. A separation was performed in solvent gradient from 40% to 100% of ACN. For all samples, the internal standard (8-isoPGF<sub>2α</sub>-d4) was added, and they were analyzed in negative-ion mode using MRM mode. Transitions of the precursor to the product ion were as follows:  $m/z$  353.2 → 193.1 for 8-isoPGF<sub>2α</sub> and 357.2 → 197.1 for 8-isoPGF<sub>2α</sub>-d4. The level of 8-isoPGF<sub>2α</sub> is expressed in ng/mg protein.

4-Hydroxynonenal (4-HNE) was determined as a O-PFB-oxime-TMS derivative using gas chromatography coupled to mass spectrometry (GC-MS/MS, Agilent Tech-

nologies, Santa Clara, CA, USA) [26]. 4-HNE was derivatized by adding O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride. After 24 h incubation at room temperature, the samples were deproteinized with methanol and the obtained O-PFB-oxime derivatives were extracted with hexane. The hexane layer was evaporated and N,O-bis(trimethylsilyl)trifluoroacetamide in 1% trimethylchlorosilane was added. 1 μL aliquots were loaded onto the column. The following ions were monitored:  $m/z$  242.0 for 4-HNE-PFB-TMS and  $m/z$  245.0 for IS derivatives. The level of 4-HNE is expressed as pmol/mg protein.

**2.2.2. Determination of Endocannabinoids.** Endocannabinoids (anandamide (AEA), 2-arachidonylglycerol (2-AG), and palmitoylethanolamide (PEA)) levels were determined using LC-MS (LCMS 8060, Shimadzu) [27]. SPE was used for the extraction of analyzed compounds. The chromatographic conditions used for the separation of endocannabinoids were as follows: 1 min initial isocratic elution with 70% ACN in water containing 0.1% ( $v/v$ ) formic acid as ionizing agent, a linear gradient 70–80% ACN in 5 min; 5–15 min gradient 80–88% ACN, followed by 100% ACN reached after 0.5 min and kept until 25 min. Endocannabinoids were analyzed in MRM mode with the use of AEA-d8 and 2-AG-d8 as internal standards. The following precursors to product ion transitions were used:  $m/z$  348.3 → 62.1 for AEA,  $m/z$  379.3 → 287.2 for 2-AG,  $m/z$  300.3 → 62.0 for PEA,

356.3 → 63.1 for AEA-d8,  $m/z$  387.0 → 295.0 for 2-AG-d8, and 330.20 → 66.15 for OEA-d4. Endocannabinoid levels are expressed in nmoles or pmoles/mg of protein.

**2.2.3. Determination of Antioxidant Enzyme Activity.** Superoxide dismutase (Cu,Zn-SOD (EC.1.15.1.1)) activity was examined according to the method by Misra and Fridovich [28] as modified by Sykes et al. [29], which allows for the measurement of cytosolic Cu,Zn-SOD activity. The amount of the enzyme that inhibits 50% of epinephrine oxidation was shown as one unit of enzyme activity and normalized by total protein content. Results were expressed in units per mg of protein.

Glutathione peroxidase (GSH-Px (EC.1.11.1.6)) activity was estimated based on spectrophotometric measurement of NADPH to NADP<sup>+</sup> conversion per min at pH 7.4 [30]. The amount of enzyme catalyzing this oxidation was shown as one unit of enzyme activity and normalized by total protein content. Results were expressed in units per mg of protein.

Glutathione reductase (GSSG-R (EC.1.6.4.2)) activity was measured based on spectrophotometric measurement reduction of NADP<sup>+</sup> to NADPH at 340 nm [31]. The amount of enzyme that catalyzes the reduction of 1  $\mu$ mol NADP<sup>+</sup> min<sup>-1</sup> at pH 7.4 was shown as one unit of enzyme activity and normalized by total protein content. Results were expressed in units per mg of protein.

Thioredoxin reductase (TrxR (EC.1.8.1.9)) activity was estimated spectrophotometrically using a commercial assay (Sigma-Aldrich, St. Louis, MO, USA) according to the included instructions [32]. Results were expressed in micro-units of enzyme activity per milligram of protein.

**2.2.4. Determination of Nonenzymatic Antioxidant Level.** Glutathione (GSH) level was measured using capillary electrophoresis (CE). To prepare samples for measurement, cells were sonicated in 62.5% (v/v) ACN. The separation of supernatant components was performed on a capillary with 40 cm effective length and was operated at 27 kV with UV detection at 200 ± 10 nm [33]. The GSH level was estimated using a calibration curve range 1–120 nmol/L ( $r^2$  0.9985) and normalized by total protein content.

Thioredoxin (Trx) level was quantified using ELISA [34]. The bottoms of a 96-well plate for ELISA analysis were covered with samples and incubated overnight at 4°C with primary antibody against thioredoxin (Abcam, Cambridge, MA, USA). As a labeled antibody, goat anti-rabbit secondary antibody (Dako, Carpinteria, CA, USA) was applied. After washing, 3,30,5,50-tetramethylbenzidine in a concentration of 0.1 mg/mL was used as a chromogen substrate. Absorption was read at 450 nm. To determine the thioredoxin level, a calibration curve range 1–5 mg/L with  $r^2$  0.9979 was used. Thioredoxin levels were normalized by total protein content.

**2.2.5. Determination of Protein Expression.** Expression of cellular (cytosolic or membrane fraction) proteins was determined by western blot analysis [35]. Samples were denatured by dissolving in Laemmli buffer with 5% 2-

mercaptoethanol and boiling for 10 min. Electrophoretically, separation was conducted on 10% gels containing SDS. After separation, proteins were transferred onto nitrocellulose membrane, blocked with 5% skim milk, and overnight incubated with primary antibodies against phospho-Nrf2 (pSer40), Keap1, TNF $\alpha$ , NF $\kappa$ B (p52 and p65), HO-1, p38, MAPK, PGAM5 (Sigma-Aldrich), Bach1, KAP1, p21, p62, CB1, CB2, and TRPV1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (antibody concentration 1 : 1000). The analysis of  $\beta$ -actin and Na<sup>+</sup>,K<sup>+</sup>ATPase (antibodies against these proteins were from Sigma-Aldrich, St. Louis, MO, USA) was used to determine the loading controls. The visualization of bands for specific proteins was done using the BCIP/NBT Liquid Substrate System (Sigma-Aldrich). The intensity of bands was quantitated using the Versa Doc System and Quantity One software (Bio-Rad Laboratories Inc., CA) and normalized by respective loading controls. All images from the western blot analyses are presented in Supplementary file S1.

**2.2.6. Protein Modifications.** Protein oxidative modifications were assessed according to the levels of carbonyl groups and adducts of 4-HNE-protein. Carbonyl groups were estimated spectrophotometrically (370 nm) using 2,4-dinitrophenylhydrazine [36]. The level of carbonyl groups is expressed in nmol per mg of protein.

The level of 4-HNE-protein adducts was quantified using ELISA [37]. Plates coated with samples were incubated overnight at 4°C with primary antibody against 4-HNE-His murine monoclonal antibody (anti-4-HNE-His murine monoclonal antibody, clone 4-HNE 1g4). A labeled goat anti-mouse antibody (Dako) was used as a secondary antibody. After washing, 3,30,5,50-tetramethylbenzidine in a concentration 0.1 mg/mL was used as a chromogen substrate. Absorption was read at 450 nm. To determine 4-HNE-protein adduct level, a calibration curve range 1–7  $\mu$ mol/L with  $r^2$  0.9982 was used. 4-HNE-protein adduct levels were normalized by total protein content.

**2.2.7. Statistical Analysis.** All obtained data are expressed as mean ± SD and analyzed by one-way analysis of variance followed by a post hoc Tukey test using Statistica software (Statistica 13.3, StatSoft Polska, Poland). All values of  $p \leq 0.05$  were considered significant, and only these results were discussed in detail.

### 3. Results

**3.1. CBD Protects against UV-Induced Lipid Metabolism.** The obtained results indicate that long-term topical application of CBD to the skin of nude rats led to significant changes in the metabolism of membrane phospholipids of keratinocytes that under the experimental conditions were the most exposed skin cells to both types of applied factors (physical (UVA/UVB) and chemical(CBD)). This was observed for enzyme-dependent as well as ROS-dependent metabolism. Both UV radiation types and CBD modulated the functioning of the endocannabinoid system through changes in levels of the main endocannabinoids such as AEA, 2-AG, and the

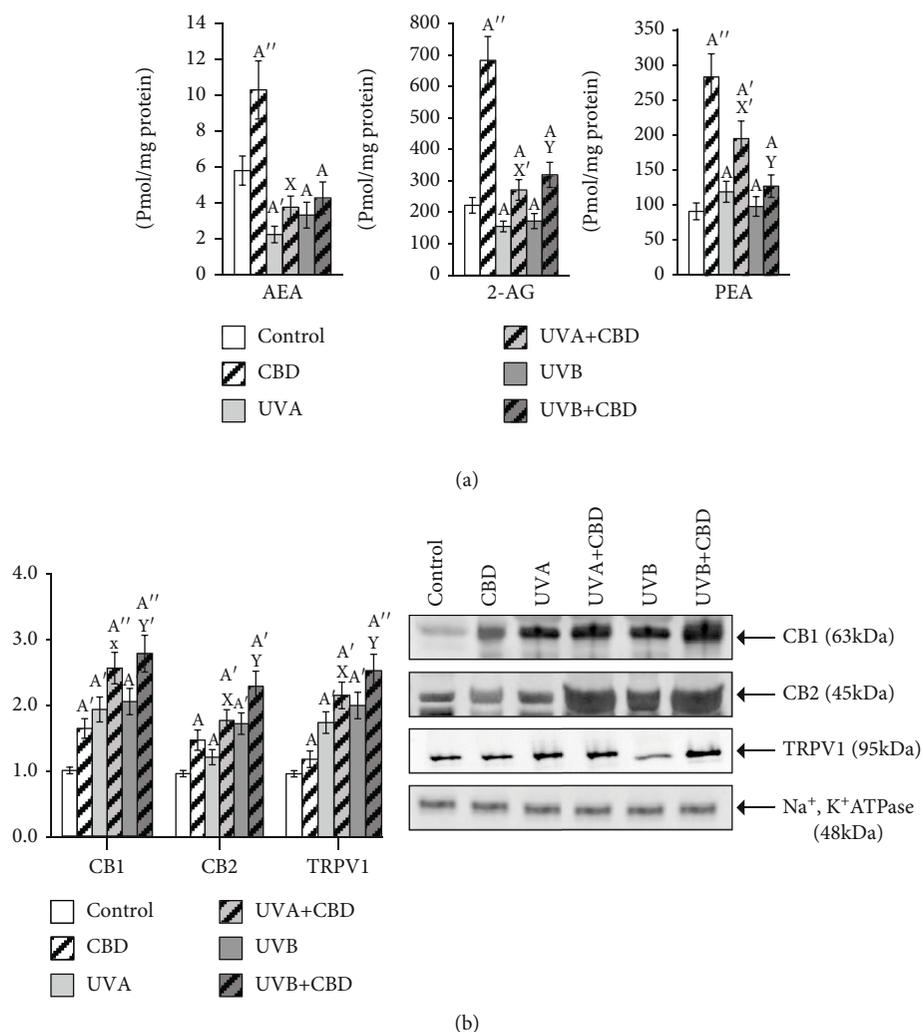


FIGURE 3: (a) Endocannabinoid level and their (b) receptors' expression in the keratinocytes of the skin of control rats, as well as rats' skin for 4 weeks exposed every 48 hours to UVA (dose increased from 0.5 to 5 J/cm<sup>2</sup>) or UVB radiation (dose increased from 0.02 to 2 J/cm<sup>2</sup>) and/or every 12 hours to the action of CBD (2.5% *w/w*). The mean values for six rats in each group  $\pm$  SD are shown as follows: a—differences vs. control group,  $p < 0.05$  (a',  $p < 0.01$ ; a'',  $p < 0.005$ ); x—differences vs. UVA-treated group,  $p < 0.05$  (x',  $p < 0.01$ ; x'',  $p < 0.005$ ); y—differences vs. UVB-treated group,  $p < 0.05$  (y',  $p < 0.01$ ; y'',  $p < 0.005$ ).

anandamide analogue PEA, as well as the expression of receptors that they are agonists of, including the cannabinoid receptors (CB1 and CB2) and the vanilloid receptor TRPV1 (Figure 3). UV radiation and CBD treatment caused opposing changes in levels of the evaluated endocannabinoids. While both types of radiation significantly lowered levels of AEA and 2-AG and increased PEA, CBD significantly enhanced their levels, with several fold increases observed for 2-AG and PEA. Consequently, CBD used together with UV radiation further modulated levels of these endocannabinoids, with both reducing levels of AEA and 2-AG and increasing PEA. Expression levels of the G protein-dependent receptors CB1, CB2, and TRPV1 were increased under the influence of UVA radiation and, to a greater extent, UVB radiation for CB2 and TRPV1. CBD also increased the expression of the receptors in both control and UV-irradiated keratinocytes.

Regardless of enzyme-dependent changes in phospholipid metabolism, ROS-dependent phospholipid metabolism was altered under the influence of UV radiation and CBD treatment. UVA and UVB radiation increased levels of the phospholipid oxidative cyclization product 8-isoPGF<sub>2 $\alpha$</sub>  and the product of oxidative fragmentation 4-HNE (Figure 4). On the other hand, CBD significantly prevented increases in the levels of these compounds, especially after UVB radiation.

UVA/UVB radiation also favored the structural modifications of proteins induced by interactions with a reactive aldehyde—4-HNE—as well as by ROS-dependent generation of carbonyl groups on protein structures, with UVB activity being much more effective. CBD applied to the skin of rats, especially those treated with UVB, decreased the level of 4-HNE-protein adducts. On the other hand, carbonyl groups were significantly reduced by CBD in all groups

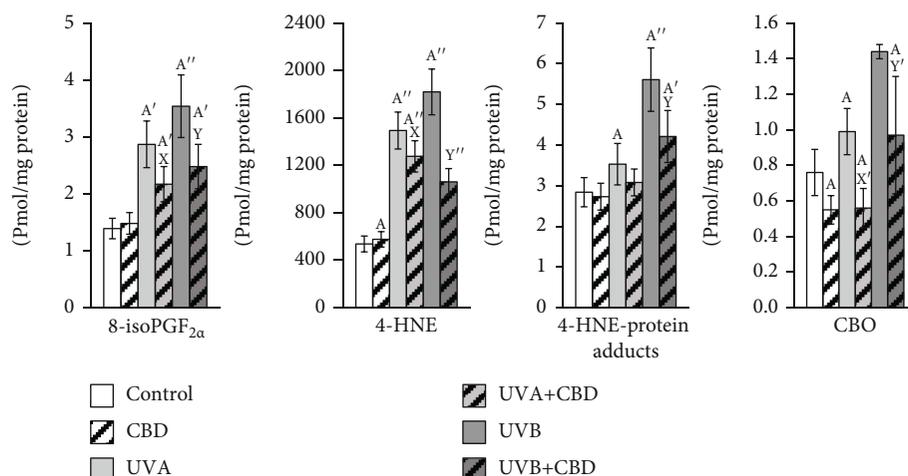


FIGURE 4: The level of 8-isoPGF<sub>2α</sub>, 4-HNE, 4-HNE-protein adducts, and protein carbonyl groups (CBO) in the keratinocytes of the skin of control rats, as well as rats' skin for 4 weeks exposed every 48 hours to UVA (dose increased from 0.5 to 5 J/cm<sup>2</sup>) or UVB radiation (dose increased from 0.02 to 2 J/cm<sup>2</sup>) and/or every 12 hours to the action of CBD (2.5% w/w). The mean values for six rats in each group ± SD are shown as follows: a—differences vs. control group, *p* < 0.05 (a', *p* < 0.01; a'', *p* < 0.005); x—differences vs. UVA-treated group, *p* < 0.05 (x', *p* < 0.01; x'', *p* < 0.005); y—differences vs. UVB-treated group, *p* < 0.05 (y', *p* < 0.01; y'', *p* < 0.005).

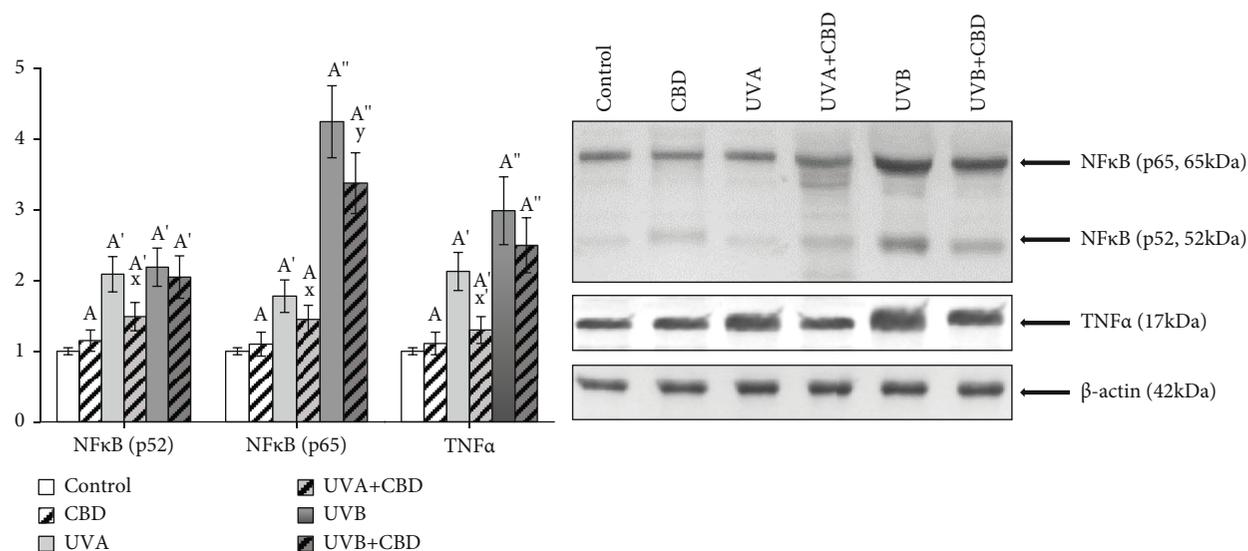


FIGURE 5: The level of NFκB subunits (p52 and p65) as well as product of its transcriptional activity (TNFα) in the keratinocytes of the skin of control rats, as well as rats' skin for 4 weeks exposed every 48 hours to UVA (dose increased from 0.5 to 5 J/cm<sup>2</sup>) or UVB radiation (dose increased from 0.02 to 2 J/cm<sup>2</sup>) and/or every 12 hours to the action of CBD (2.5% w/w). The mean values for six rats in each group ± SD are shown as follows: a—differences vs. control group, *p* < 0.05 (a', *p* < 0.01; a'', *p* < 0.005); x—differences vs. UVA-treated group, *p* < 0.05 (x', *p* < 0.01; x'', *p* < 0.005); y—differences vs. UVB-treated group, *p* < 0.05 (y', *p* < 0.01; y'', *p* < 0.005).

of keratinocytes, i.e., both in the control and from skin irradiated with UVA or UVB.

**3.2. CBD Anti-inflammatory Effect on UV-Irradiated Skin Cells.** UV radiation and CBD treatment were also found to influence inflammatory processes in the skin of nude rats. UV radiation alone changed total expression levels of the proinflammatory transcription factor NFκB and the cytokine TNFα, which is a product of its transcriptional activity, in keratinocytes. For both NFκB nuclear factor subunits and TNFα, it was observed that irradiation of rats' skin with UVA or UVB radiation significantly increased the expression

of these proteins, with a more significant increase in the expression of the p65 subunit and TNFα after UVB compared to UVA (Figure 5). Application of CBD to the skin of control rats increased the level of all measured proteins, while CBD applied to irradiated skin prevented a significant increase in their levels. This was especially notable for the p65 subunit of NFκB.

**3.3. CBD Antioxidant Action in Cells Exposed to UV Radiation.** The CBD effect on lipid metabolism and proinflammatory signaling is also reflected in the activity of the antioxidant system. CBD led to increased activity of most

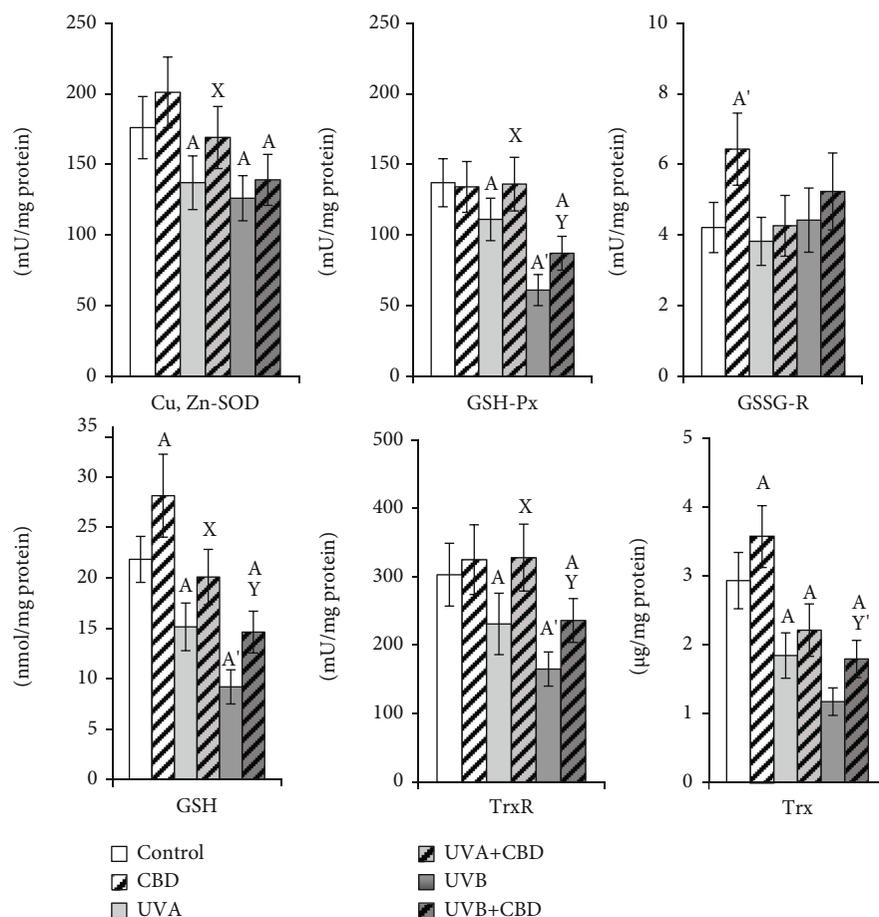


FIGURE 6: The activity of antioxidant enzymes (Cu,Zn-SOD, GSH-Px, GSSG-R, and TrxR) and the level of nonenzymatic antioxidants (GSH, Trx) in the keratinocytes of the skin of control rats, as well as rats' skin for 4 weeks exposed every 48 hours to UVA (dose increased from 0.5 to 5 J/cm<sup>2</sup>) or UVB radiation (dose increased from 0.02 to 2 J/cm<sup>2</sup>) and/or every 12 hours to the action of CBD (2.5% w/w). The mean values for six rats in each group  $\pm$  SD are shown as follows: a—differences vs. control group,  $p < 0.05$  (a',  $p < 0.01$ ; a'',  $p < 0.005$ ); x—differences vs. UVA-treated group,  $p < 0.05$  (x',  $p < 0.01$ ; x'',  $p < 0.005$ ); y—differences vs. UVB-treated group,  $p < 0.05$  (y',  $p < 0.01$ ; y'',  $p < 0.005$ ).

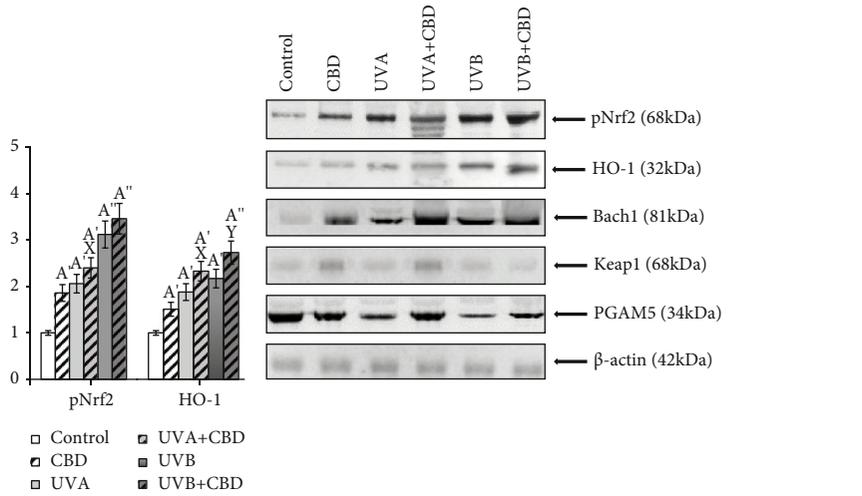
antioxidant parameters in keratinocytes including GSSG-R and TrxR and levels of GSH and Trx (Figure 6). Exposure of rats' skin to UVA radiation resulted in decreased activities and levels of antioxidant parameters in keratinocytes, including a statistically significant decrease in GSH-Px, Cu,Zn-SOD, and TrxR activity as well as GSH and Trx level. On the other hand, UVB radiation reduced the activity of Cu,Zn-SOD, GSH-Px, and TrxR and levels of GSH and Trx, while for effectors of the GSH and Trx-dependent systems these changes were much more pronounced. When CBD was applied to the skin of rats exposed to UVA or UVB radiation, a significant increase in Cu,Zn-SOD, GSH-Px, and TrxR activity and GSH and Trx level was observed.

CBD treatment and UVA/UVB radiation not only caused changes in the activity and level of antioxidants but also modified the expression of the phosphorylated Nrf2 transcription factor, which is responsible for the biosynthesis of antioxidant proteins and the effectiveness of which depends on the expression of its activators and inhibitors located both in the cytoplasm and in the cell nucleus (Figure 7). Irradiation of rats' skin with UVA or UVB increased the expression of p-Nrf2 and the product

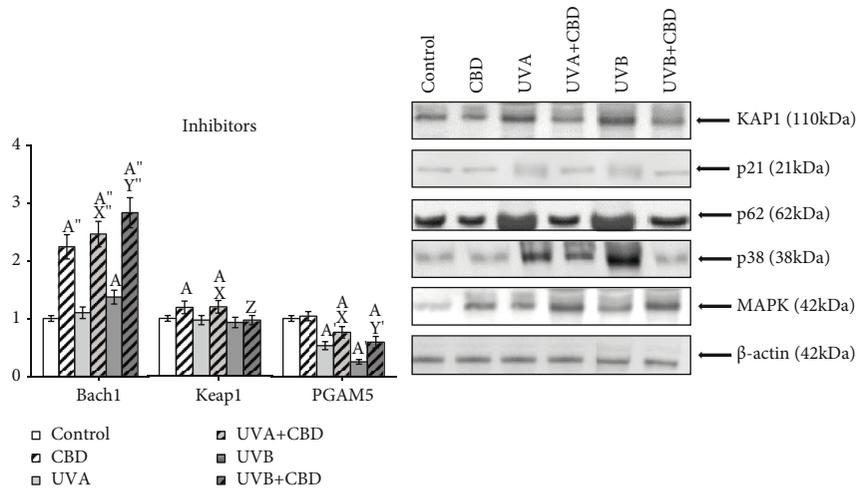
of its transcriptional activity—heme oxygenase 1 (HO-1). CBD also increased the expression of Nrf2 in the keratinocytes of control rats as well as in keratinocytes from skin exposed to both UVA and UVB radiation.

With respect to Nrf2 inhibitory proteins, levels of the Nrf2 nuclear inhibitor Bach1 increased after skin was irradiated with UVB rays. Application of CBD to the skin caused a further, significant increase in its levels in the keratinocytes of control rats, as well as in rats' skin exposed to UVA/UVB radiation. In addition, expression of the cytosolic Nrf2 inhibitor Keap1 was increased by CBD treatment, albeit to a much lesser extent than Bach1.

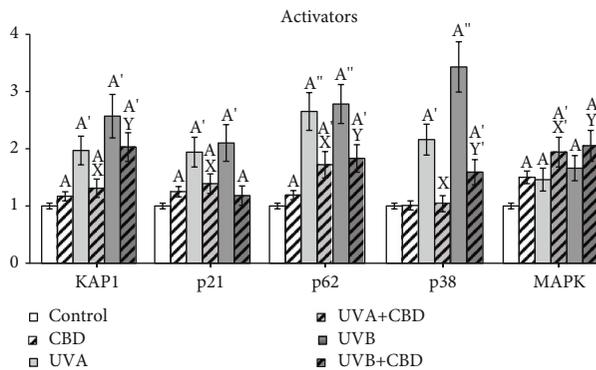
In contrast to the responses of the Nrf2 inhibitory proteins, levels of the PAGM5 protein were significantly lowered by UV exposure to the skin, and CBD partially prevented this change. Exposure of rats' skin to the prolonged action of CBD also increased the level of Nrf2 activators, with the exception of the p38 protein. On the other hand, UVA- or UVB-irradiated skin exhibited increased expression of all examined relevant activator proteins, with the greatest change observed for p62 and the least change for MAPK. However, application of CBD to the rats' skin exposed to



(a)



(b)



(c)

FIGURE 7: The expression of phospho-Nrf2 (pNrf2) and product of its transcriptional activity (HO-1) as well as its inhibitors (Keap1 and Bach1) and activators (KAP1, p21, p62, p38, and MAPK) in the keratinocytes of the skin of control rats, as well as rats' skin for 4 weeks exposed every 48 hours to UVA (dose increased from 0.5 to 5 J/cm<sup>2</sup>) or UVB radiation (dose increased from 0.02 to 2 J/cm<sup>2</sup>) and/or every 12 hours to the action of CBD (2.5% w/w). The mean values for six rats in each group  $\pm$  SD are shown as follows: a—differences vs. control group,  $p < 0.05$  (a',  $p < 0.01$ ; a'',  $p < 0.005$ ); x—differences vs. UVA-treated group,  $p < 0.05$  (x',  $p < 0.01$ ; x'',  $p < 0.005$ ); y—differences vs. UVB-treated group,  $p < 0.05$  (y',  $p < 0.01$ ; y'',  $p < 0.005$ ).

UVA or UVB radiation significantly prevented the increased expression of these activators, except for MAPK, for which both types of radiation increased its expression.

#### 4. Discussion

CBD, as a lipophilic compound with low absorption after oral administration and resulting in a relatively low bioavailability, is suggested to be administered as a transdermal therapy [38]. This is especially important in case when the therapy is aimed directly at skin cells with metabolic disorders caused by external physical factors or disease processes. Then, topical application provides a direct therapeutic effect of CBD. Moreover, it has been shown that transdermal administration of CBD extends the duration of action of the drug compared to other administration routes [39].

The results of this study show that long-term exposure to UVA or UVB radiation induces a proinflammatory reaction and reduces the antioxidant capacity of epidermal keratinocytes in rats. Similar metabolic alterations have been observed in *in vitro* studies in which metabolic changes in keratinocytes from both healthy people and people with psoriasis were evaluated following UV irradiation [20].

Inflammation in the skin is known to result from abnormal immune cell responses. Interaction between epidermal keratinocytes and blood cells (lymphocytes and dendritic cells) plays a key role in the skin's immune response through regulation of cytokines and chemokines involved in growth and inflammation [40]. The results of this study confirm the observed *in vitro* regulation of the inflammatory response in keratinocytes after exposure to UVA/UVB radiation, which includes the involvement of the endocannabinoid system and increased levels of the NF $\kappa$ B transcription factor and its activity product—the cytokine TNF $\alpha$  [41, 42].

Long-term topical application (on the skin) of CBD has been shown to significantly increase levels of the most important endocannabinoids, such as AEA, 2-AG, and PEA. These phospholipid metabolites affect the homeostasis of epidermal cells, including keratinocytes, through mechanisms both dependent and independent of receptors, including the cannabinoid receptors (CB1/2) [20, 43, 44]. The results of this study indicate that both UV and CBD activate both types of cannabinoid receptors as well as the TRPV1 receptor. It is known that activation of CB1 receptors leads to intensification of the proinflammatory and oxidative state through increased production of TNF $\alpha$  and ROS, while activation of CB2 enhances the reverse reaction [45]. CBD has been shown to be a weak agonist of CB1 and CB2 receptors in humans, mice, and rats [46], but it has also been suggested that under some conditions CBD may show inverse agonism to CB2 receptors [10]. However, it should be noted that CBD not only acts directly on the receptors but also indirectly, increasing levels of endocannabinoids, which are agonists of the G protein-related receptors [47]. Moreover, both CBD and endocannabinoids, especially AEA, activate the TRPV1 receptor [45, 48]. Knowing that ROS and products of lipid peroxidation significantly change the physiological activity of TRPV1 through oxidation of its thiol groups [49], it can be suggested that CBD and AEA binding of TRPV1 not only

activates it but also protects it and ensures its proper functioning. Consequently, through direct agonist-receptor interaction with TRPV1, CBD additionally reduces oxidative stress. Regardless of the direct effects on receptors, AEA is known to negatively regulate 2-AG metabolism. Endocannabinoids have been shown to be synthesized in membranes; however, phosphatidylinositol degradation catalyzed by phospholipase C leads to the generation of a diacylglycerol precursor, the hydrolysis of which allows the formation of 2-AG [50]. The higher level of AEA observed after the use of CBD in the keratinocytes of the control rats should favor the reduction of 2-AG, but the significantly increased level of GSH, which is essential for the synthesis of 2-AG, strongly intensifies the biosynthesis of this endocannabinoid [51]. On the other hand, the lower level of AEA after UV and CBD treatment favors the synthesis of 2-AG. However, the greatly reduced levels of GSH, which is needed for the synthesis of 2-AG, consequently only lead to a slight increase in the level of this endocannabinoid. Thus, the obtained results indicate a more effective regulation of 2-AG levels by GSH than by AEA, especially in the presence of CBD.

Both the endocannabinoids and endocannabinoid mimetic palmitoylethanolamide (PEA), a derivative of fatty acids, whose level is significantly increased after UV irradiation and CBD application to rats' skin, were earlier indicated to be able to counteract the production of the cytokine MCP2, which activates NF $\kappa$ B signaling [52, 53]. PEA is an anti-inflammatory mediator that acts mainly by direct activation of PPAR- $\alpha$  receptors [54]. As such, PEA could be a potential therapeutic target of CBD that can be used in phototherapy. Moreover, it is known that CBD activates PPAR- $\gamma$ /GPR55 receptors or increases DNA methylation in human keratinocytes in order to inhibit their proliferation [55, 56].

Since inflammatory and redox pathways interact, the anti-inflammatory effects of CBD are accompanied by antioxidant action. *In vitro* studies have indicated that CBD directly reduces oxidative stress induced by UV radiation in keratinocytes, preventing ROS generation by reducing the activity of enzymes responsible for formation [20, 41, 57]. Moreover, it was found that CBD, in addition to directly lowering levels of oxidants, also affects the antioxidant capacity of skin cells [58]. This study shows that the *in vivo* antioxidant activity of CBD influences the regulation of antioxidant proteins down to the level of transcription because it activates the transcription factor Nrf2, which is sensitive to changes in redox conditions and which is responsible for the biosynthesis of antioxidant proteins [59], as observed in keratinocytes of rat skin irradiated with UVA/UVB and being treated with CBD [60]. The obtained results indicate that chronic skin irradiation of rats increases the expression of the transcription factor, and the use of CBD during the experiment additionally enhances Nrf2 expression. This is due to the dysfunction of both the inhibitors and activators of Nrf2. The transcriptional activity of Nrf2 depends on its binding by the cytosolic Keap1 inhibitor, which promotes its proteasomal degradation [61]. However, only a slight increase in Keap1 levels after exposure to CBD cannot significantly alter Nrf2 degradation. On the other hand, CBD induces a multiple increase in Bach1 levels, as already demonstrated in

earlier studies [62], which should inhibit the transcriptional activity of Nrf2 in the nucleus. However, this was not observed as both the expression of Nrf2 and its transcription products were significantly elevated after exposure to UV, especially UVA, as well as CBD. This may be due to the increased expression of Nrf2-activating proteins. We demonstrated that *in vivo*, UVA/UVB radiation increased levels of Nrf2 activators, including p21, KAP1, p38, and p62 in epidermal cells, while the use of CBD, in most cases, reduced their expression. However, in an autophagy-dependent manner, p62 activates Nrf2 that positively regulates expression of the p62 gene [61] which leads to Keap1 inactivation. Moreover, the combined effects of UV and CBD tend to reduce PGAM5 phosphatase expression, which can interact with both Nrf2 and Keap1. Due to regulation by various inhibitors and activators, increased expression of the target Nrf2 gene supports stress resistance in irradiated cells by inducing changes in metabolic pathways involved in cellular defense by reducing the level of cytotoxic electrophiles [63–65]. One of the basic activities of Nrf2 is the regulation of the expression of the HO-1 gene. HO-1 and its metabolites have previously been shown to have significant anti-inflammatory effect mediated by Nrf2 [59]. Therefore, it may be suggested that the increased expression of HO-1 induced by UV irradiation, in addition to CBD therapy, may induce an anti-inflammatory effect by inhibiting NF $\kappa$ B nuclear translocation [15]. The results of this study confirm a reduction in the level of NF $\kappa$ B and product of its transcriptional activity the cytokine TNF $\alpha$ , in the keratinocytes of the skin exposed to UV radiation and treated with CBD compared to keratinocytes exposed to UV radiation only. Modifications of the expression of antioxidant genes cause changes in the level and, consequently, in the activity of the protein products of these genes. Changes in the efficiency of enzymes and peptides are also the result of oxidative modifications of their structure or modification by lipid peroxidation products, which is confirmed by the reduced level of 4-HNE-protein adducts and protein carbonyl groups as a result of applying CBD to the skin of rats irradiated with UVA/UVB. Consequently, CBD increases the activity of antioxidant enzymes—GSH-Px, GSSG-R, TrxR, and Cu,Zn-SOD—and nonenzymatic antioxidants—GSH and Trx. Data from the literature indicate that CBD also increases mRNA level and superoxide dismutase activity in other pathological conditions such as diabetic cardiomyopathy in a mouse model [14]. This is presumably due to CBD increasing the biological activity of Nrf2 to promote the effectiveness of antioxidant enzymes [62]. Similar to the present study, other reports also indicate that multiple doses of CBD enhance the activity of GSH-Px and GSSG-R in inflammatory conditions [14, 66]. Additionally, the direction of changes in glutathione peroxidase activity and GSH level in the keratinocytes of rats irradiated with UVB and treated with CBD observed in this study is similar to the results of *in vitro* experiment using CBD to treat human keratinocytes irradiated with UVB [15, 20]. Under oxidation conditions generated by UV radiation, changes in enzyme activity may result from oxidative modifications of proteins, especially those containing aromatic and sulfuric amino acids [67], and the

reduction of these changes resulting from the use of CBD. The explanation for this may be the high affinity of CBD for cysteine that is also present in GSH-Px and Trx, resulting in a protective effect [10]. CBD supports the antioxidant enzyme activity [62], which prevents against the reduction level of micronutrients, including Sn or Zn, under oxidative stress [67]. The aforementioned microelements are essential for the proper biological activity of enzymes, especially responsible for antioxidant response, such as superoxide dismutase or glutathione peroxidase [68].

Moreover, it was found that modifications to the protein structure, revealed by the appearance of carbonyl groups, may result in a decrease in the biological activity of proteins assessed in the keratinocytes of rats exposed to UV radiation. The significant protective effect of CBD is evidenced by a significant reduction in the level of carbonyl groups in protein molecules and 4-HNE-protein adducts. CBD, by reducing oxidative stress, simultaneously counteracts the reduction of glutathione peroxidase activity and levels GSH, which is a cosubstrate of this enzyme and is essential for the reduction of lipid peroxides [20, 69]. The effectiveness of CBD reduction of peroxidation of membrane phospholipids is additionally enhanced by its lipophilic character and the previously observed tendency to accumulate this phytocannabinoid in biological membranes [20]. Consequently, CBD counteracts the increase in the levels of arachidonic acid oxidative fragmentation products, i.e., reactive aldehydes, including 4-HNE, and oxidative cyclization products, including 8-isoPGF $_{2\alpha}$ . Earlier studies involving isolated keratinocytes exposed to UV radiation and treatment with CBD *in vitro* have also shown a reduction in lipid peroxidation estimated from MDA and 4-HNE levels [15, 41]. Since 4-HNE belongs to the group of  $\alpha,\beta$ -unsaturated aldehydes, it is an extremely reactive, electrophilic compound, and for this reason, it easily interacts with nucleophilic compounds in cells, including peptides and proteins [70]. It can therefore be suggested that CBD, by modulating oxidative stress in various ways, prevents oxidative modifications of lipids and proteins and thus prevents the participation of modified proteins in cell signaling pathways, thus reducing the pathophysiological effects of their action.

## 5. Conclusions

Consequently, by counteracting UV-induced modifications of lipids, as well as protein metabolism, CBD prevents disturbances in their biological activity, including changes in intracellular and intercellular signaling, which can lead to metabolic changes in keratinocytes and thus affect the physiology/pathophysiology of the epidermis and whole organism. Moreover, the known inhibitory effect of CBD on the proliferation of human keratinocytes due to exogenous factors suggests that it may have therapeutic potential in the treatment of skin diseases associated with skin cell hyperproliferation including psoriasis. This is all the more important as this effect can also be transferred to skin fibroblasts and blood.

## Data Availability

The western blot data used to support the findings of this study are included within the supplementary information file.

## Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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## Supplementary Materials

Supplementary file SF1: western blot analysis of the keratinocytes of the skin of control rats, as well as rats' skin for 4 weeks exposed every 48 hours to UVA (dose increased from 0.5 to 5 J/cm<sup>2</sup>) or UVB radiation (dose increased from 0.02 to 2 J/cm<sup>2</sup>) and/or every 12 hours to the action of cannabidiol (CBD; 2.5% w/w). (*Supplementary Materials*)

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