



Protective effects of cannabidiol on the membrane proteins of skin keratinocytes exposed to hydrogen peroxide via participation in the proteostasis network

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ABSTRACT

Hydrogen peroxide (H₂O₂) is widely used in clinical practice due to its antiseptic properties and its ability to heal wounds. However, due to its involvement in the formation of ROS, H₂O₂ causes several side effects, including disorders of the metabolism of skin cells and the development of chronic inflammation mediated by oxidative stress. Therefore, this study evaluated the effects of cannabidiol (CBD), a phytocannabinoid known for its antioxidant and anti-inflammatory properties, on the proteome of keratinocyte membranes exposed to H₂O₂. Overall, the hydrogen peroxide caused the levels of several proteins to increase, while the treatment with CBD prevented these changes. Analysis of the protein-protein interaction network showed that the significant changes mainly involved proteins with important roles in the proteasomal activity, protein folding processes (regulatory subunit of the proteasome 26S 6A, beta proteasome subunit type 1, chaperonin 60 kDa), protein biosynthesis (40S ribosomal proteins S16, S2 and ubiquitin-S27a), regulation of the redox balance (carbonyl reductase [NADPH] 1 and NAD(P)H [quinone] 1 dehydrogenase) and cell survival (14-3-3 theta protein). Additionally, CBD reduced the total amount of MDA, 4-HNE and 4-ONE-protein adducts. Therefore, we conclude that CBD partially prevents the changes induced by hydrogen peroxide by reducing oxidative stress and maintaining proteostasis networks. Moreover, our results indicate that combination therapy with CBD may bring a promising approach in the clinical use of hydrogen peroxide by preventing its pro-oxidative and pro-inflammatory effect through potential participation of CBD in membrane mediated molecular signaling.

1. Introduction

Hydrogen peroxide (H₂O₂) is the main endogenous oxidant involved in cellular redox regulation and intracellular signalling [1]. On the other hand, in solution, it is also used to disinfect the skin. Topically applied H₂O₂ reveals antibacterial and antifungal properties and is involved in the wound healing process [2,3]. It has also been indicated that the use of a concentrated solution of hydrogen peroxide (33%) shows a significant reduction in the size of multiple lesions in non-melanoma skin cancer [4]. However, in addition to the beneficial local action, hydrogen peroxide easily penetrates the epidermis through the stratum corneum and transforming into hydroxyl radicals can promote keratinocytes apoptosis or necrosis [3]. And, depending on the used concentration, it can pass even deeper into the dermis and cause further oxidative

damages and metabolic alteration in skin cells together with activation of signal transducer and activator of transcription 3 (STAT3) [5]. It has been also demonstrated that hydrogen peroxide is mainly locating in the membranes of skin cells [6,7], especially keratinocytes [8]. Thus, it may contribute to the intensification of pro-oxidative conditions in keratinocytes [8]. This favours oxidative structural modifications of proteins, lipids and nucleic acids, and therefore changes in their functionality, including redox-sensitive transcription factor signalling - nuclear factor associated with erythroid factor 2 (Nrf2) and nuclear factor kappa B (NF-κB) [9]. Hence, hydrogen peroxide may even contribute to chronic inflammation or cell death, as well as affect the innate immune system [1–3,9].

For the above reasons, there is a real need to develop therapies supporting the beneficial effects of hydrogen peroxide on the

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metabolism of skin cells, while eliminating the harmful effects. One of the promising chemical cooperators for hydrogen peroxide is cannabidiol (CBD), the major non-psychoactive phytocannabinoid of *Cannabis sativa* L. with antioxidant and anti-inflammatory effects through interactions with endocannabinoid components and membrane receptors [10]. This is all the more important as CBD, which has lipophilic character, tends to accumulate in cell membranes, especially when the cells are exposed to UV radiation, including in the sunlight as well as to hydrogen peroxide [8,11]. Our previous research as well as data from the literature clearly show that CBD can protect various cells, including skin cells, by preventing damage from oxidative stress resulting from exposure to H₂O₂ [8,12–14]. The antioxidant activity of CBD results mainly from the reduction of the excessive generation of oxidants, which promotes, among other, the inhibition of ROS-dependent lipid peroxidation [8,11,15]. As a consequence, the level of highly reactive products of polyunsaturated fatty acids oxidative fragmentation such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) as well as the level of their adducts with protein is reduced [16–18]. Moreover, recent studies indicate that CBD supports the maintenance of proteostasis under oxidative conditions by regulating molecular signalling in the biosynthesis and degradation of proteins under different conditions, *in vivo* and *in vitro* [17,19,20]. In addition, CBD can modify cell viability by altering the permeability of mitochondrial membranes and calcium metabolism, as well as post-translational modifications and the level of apoptosis regulatory proteins such as Bcl-2 and Bax [19,21–25].

In addition, although there is no direct data in the literature, CBD may also potentiate the beneficial effects of H₂O₂ in clinical practice due to its potential anti-cancer [26], anti-microbial [27] and wound healing [28] effects. Moreover, in the current pandemic of acute respiratory syndrome 2 (SARS-CoV-2) coronavirus, it is pointed to a dramatic increase in the use of disinfectants, including various preparations of hydrogen peroxide. This creates potential risks such as skin damages, additional infections and alcohol poisoning [29]. Therefore, due to its antioxidant and anti-inflammatory role, CBD may also protect skin cells from the oxidative damages associated with H₂O₂ in disinfectant use.

Since hydrogen peroxide acts directly on skin cell membranes, while CBD tends to accumulate in keratinocyte membranes, this research focuses on the effect of H₂O₂ on membrane proteins, including those modified by products of phospholipid peroxidation. As stated above, CBD can help to protect skin cells from redox imbalance under the influence of hydrogen peroxide towards oxidative conditions, especially with long-term use of this compound. Therefore, the present study was also designed to evaluate the protective effect of CBD on the membrane proteome of keratinocytes exposed to H₂O₂.

2. Material and methods

2.1. Cell culture of experimental groups and membrane proteins isolation

Human epidermal keratinocytes (CDD 1102 KERTr, American Type Culture Collection, Virginia, USA) were cultured in standard Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 U/mL penicillin and 50 µg/mL streptomycin (used as a standard medium) in a humidified atmosphere of 5% CO₂ at 37 °C. The cells were grown to 70% confluence and were passaged at a ratio of 1:3. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was used to check the viability of keratinocytes [30]. After keratinocytes reached the required confluence (at least 90%), the four experimental groups were formed as described in Table 1. Used CBD treatment in this study, before and after H₂O₂ exposure, was chosen according to data from previous studies due to observed efficiency of this specific CBD treatment in maintaining of membrane integrity [8] and protein homeostasis [17] that was disrupted by hydrogen peroxide as well as another oxidative stressor, UV radiation.

Once the cell culture was completed, the keratinocytes from all the groups were scraped and centrifuged (300×g, 3 min). The pellets were

Table 1

Experimental groups of keratinocytes and their preparation for proteomic analysis. The selected concentrations of CBD were not toxic to the cultured cells and H₂O₂ concentration corresponded to 70% cell viability according to the MTT assay.

Experimental groups	Method
Control group (CTR)	Keratinocytes were cultured in standard medium
CBD treated cells (CBD)	Keratinocytes were cultured in medium containing 4 µM CBD for 48 h (h)
H ₂ O ₂ exposed cells (H ₂ O ₂)	Keratinocytes were cultured in medium containing 200 µM hydrogen peroxide for 24 h
Cells treated with CBD before and after H ₂ O ₂ exposure (CBD+H ₂ O ₂ +CBD)	Keratinocytes were cultured in a medium containing 4 µM CBD for 24 h before and 24 h after 200 µM H ₂ O ₂ exposure (for 24 h).

resuspended in Tris-buffered saline (TBS) containing protease inhibitor cocktail (Sigma-Aldrich, P8340) (pH 8.0) for the membrane fractionation process. The cell suspensions were then centrifuged (15,000×g, 10 min), their pellets, containing membrane fractions, were resuspended in TBS containing 1% Triton X-100 and sonicated on ice, for 5 s. After the sonication step, the proteins of the membrane fractions were obtained by centrifugation (15,000×g, 10 min) for use in proteomic analysis. To normalize the assay performance to the same amount of protein, the total protein concentrations were assessed using the Bradford assay [31].

2.2. Proteomic analysis of membrane fractions

The qualitative and semi-quantitative analysis of the proteomes of the membrane fractions of all the groups of keratinocytes was performed by liquid chromatography - tandem mass spectrometry (LC-MS/MS) as described previously by Gegotek [32]. The general view of the experimental steps is shown in Fig. 1, while the comprehensive description of the analysis is given below:

a. **Electrophoretic separation of proteins:** Samples containing 30 µg of protein were mixed with Laemmli buffer containing 5% 2-mercaptoethanol (in a volume ratio of 1:2), heated at 100 °C for 7 min, and separated using 10% Tris-Glycine SDS-PAGE gels.

b. **Coomassie staining and gel cutting:** Gels were fixed in a 4:1:5 mixture of methanol:acetic acid:water for 1 h and stained overnight with Coomassie Brilliant Blue R-250. The lines were cut from the gels and sliced into eight sections (supplementary figure 1).

c. **Alkylation, reduction and in-gel digestion:** The proteins in each slice were reduced using 10 mM dithiothreitol (DTT) and alkylated with 50 mM iodoacetamide. Next, they were incubated overnight at 37 °C with trypsin (Promega, Madison, WI, USA) for in-gel protein digestion.

d. **Separation of peptides using nano-HPLC:** After extraction (from gel) and drying peptide mixtures, they were dissolved in 5% acetonitrile with 0.1% formic acid. The final peptide mixtures were separated using an Ultimate 3000 high-performance liquid chromatography (HPLC) system (Dionex, Idstein, Germany) on a 150 mm × 0.075 mm PepMap RSLC capillary analytical C18 column with 2 µm particle size (Dionex, LC Packings) at a flow rate of 0.300 µL/min. Samples were mobilized through the column by application of eluent A and B (eluent A: 5% acetonitrile + 0.1% formic acid; eluent B: 90% acetonitrile + 0.03% formic acid) over a time gradient (started at 3 min and increased to 60% eluent B for 40 min). The peptides were analysed using a Q Exactive HF mass spectrometer calibrated and operated in positive and data-dependent mode with an electrospray ionization source (ESI) (Thermo Fisher Scientific, Bremen, Germany).

e. **Protein identification, grouping, and label-free quantification:** The

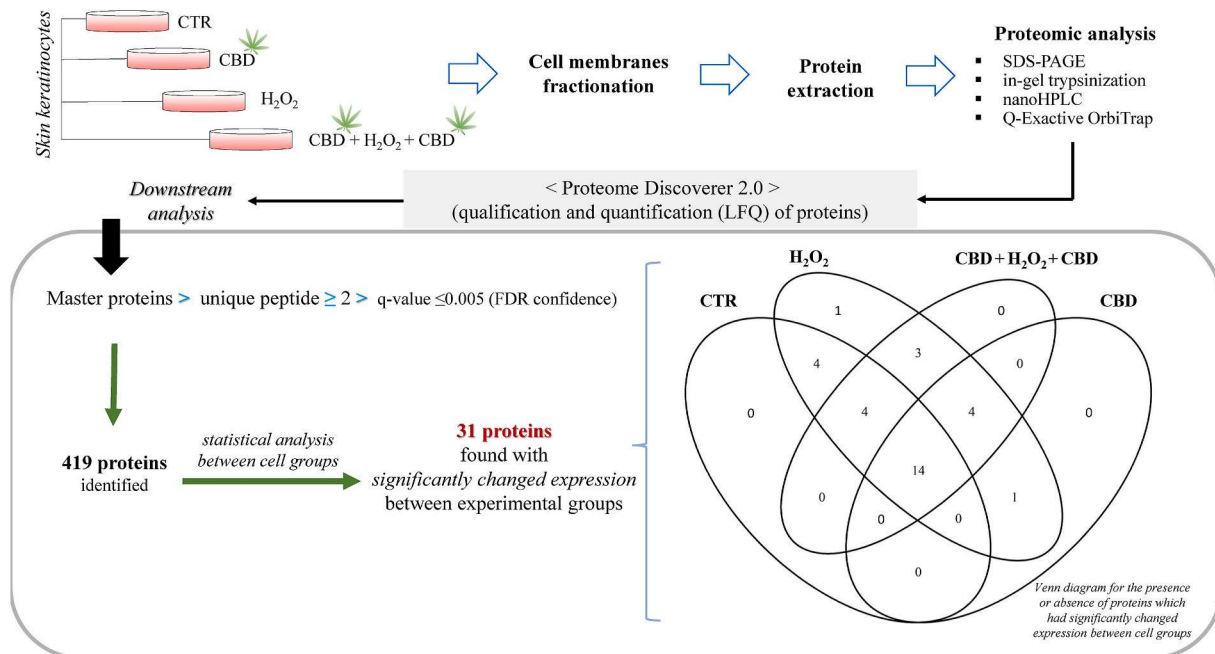


Fig. 1. A general view of the experimental steps and the Venn diagram of proteins with significantly changed expression in total membrane fractions of skin keratinocytes (from the control group (CTR), CBD treated group (CBD), H₂O₂ exposed cell group (H₂O₂) and the cell group treated with CBD before and after H₂O₂ exposure (CBD+H₂O₂+CBD), as explained in Table 1).

raw data generated by LC-MS/MS were analysed using Proteome Discoverer Software 2.0 (Thermo Fisher Scientific, Bremen, Germany) and MS Amanda (MS Amanda algorithm, license Thermo Scientific, a registered trademark of the University of Washington, Seattle, WA, USA). For protein identification, the following search parameters were used: peptide mass tolerance 10 ppm; MS/MS mass tolerance 0.02 Da; mass precision 2 ppm; up to two missed cleavages allowed; minimal peptide length of six amino acids; and cysteine carbamidomethylation and carboxymethylation, methionine oxidation, MDA-lysine, 4-HNE-cysteine/lysine/histidine and 4-oxo-2-nonenal (4-ONE)-cysteine/lysine/histidine adduct formation set as a dynamic modification. The data were searched against the UniProtKB-SwissProt database (taxonomy: *Homo sapiens*). For the semi-quantitative analysis, protein label-free quantification was performed according to the signal intensities of precursor ions.

2.3. Analysis of protein expression by western blot

In order to validate the results obtained in the MS/MS experiment, an analysis of the expression of proteins selected from among the most significantly altered was performed using the Western blot technique [33]. Electrophoretically separated proteins as presented in point a. were transferred to nitrocellulose membranes, blocked with 5% skim milk and incubated overnight with primary antibodies (host: mouse) against heat shock protein (HSP) (Abcam; Cambridge, MA, USA) and proteasome subunits: LMP7 and $\beta 5$ (Enzo Life Sciences, Inc., Farmingdale, NY, USA) and beta-actin (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1:1000. After incubation and washing with TBS-T, the membranes were incubated for 2 h with polyclonal alkaline phosphatase secondary antibodies against mouse (Sigma-Aldrich; St. Louis, MO, USA). Protein bands were visualized using the BCIP/NBT liquid substrate system (Sigma-Aldrich; St. Louis, MO, USA) and each band intensity was estimated using VersaDoc System and QuantityOne software (Bio-Rad Laboratories Inc., Hercules, CA, USA). The results were expressed as a percentage of the expression determined in the control cells.

2.4. Measurement of total 4-HNE-protein adducts level by ELISA

The levels of lipid peroxidation products-protein adducts obtained by MS/MS experiment were validate by measurement of total 4-HNE-protein adducts using the ELISA method [34]. Samples were loaded into ELISA plate wells (Nunc Immuno Maxisorp, Thermo Scientific, Waltham, MA, USA) and incubated at 4 °C overnight with 4-HNE-protein conjugate primary antibody (Invitrogen; Waltham, MA, USA). After washing, the plate was incubated for 30 min with peroxidase blocking solution (3% H₂O₂, 3% skim milk) at RT. The goat anti-mouse secondary antibody solution (Dako, Carpinteria, CA, USA) was then added to each well for 1 h. Next, chromogen substrate solution (0.1 mg/mL TMB, 0.012% H₂O₂) was added to each well for 40 min. The reaction was stopped by addition of 2 M sulfuric acid. Absorption was read at 450 nm with the reference filter set to 620 nm. Levels of total 4-HNE-protein adducts were expressed as a percentage of the expression determined in the control cells.

2.5. Statistical analysis

Samples from each experimental group of keratinocytes were analysed in three independent experiments. For results obtained by Western blot or ELISA analysis to determine statistically significant differences between the study groups, standard statistical method was used (one-way analysis of variance, ANOVA). P-value <0.05 was considered as a statistically significant. The proteins, identified by Proteome Discoverer (master proteins with ≥ 2 unique peptides and q-value ≤ 0.005 - false discovery rate confidence) were considered for statistical analysis. Perseus was used for Z-score normalization and log-transformation of individual protein intensities from label-free quantification (Perseus 1.6.5.0) [35]. Quality control and biostatistical analysis including one-way ANOVA, dendrogram and volcano plot assessments were applied using the open-source software MetaboAnalyst (MetaboAnalyst 5.0) [36]. Heatmaps, clustering analysis and Venn diagram were created using R (RStudio version 1.4.1106) [37] with the packages “tidyverse” [38], “pheatmap” [39], “dendextend” [40] and “VennDiagram” [41]. Open-source database STRING (STRING 11.0) [42] was used for protein

annotation and obtained data compared against UNIPROT database [43]. For analysis of protein-protein interaction networks and assessment of biological pathways, STRING database and Cytoscape (Cytoscape 3.8.2) [44] were used in coordination.

3. Results

A total of 419 proteins were identified in the total membrane fractions of the experimental keratinocyte groups. The list of identified proteins, along with their name, ID, sequence coverage, number of associated peptides, number of unique peptides and average intensity levels are shown in [supplementary table 1](#). We found that 31 of the proteins identified had a significantly altered expression in all experimental groups ([Fig. 1](#), [supplementary table 2](#)). The Venn diagram of these proteins is shown in [Fig. 1](#), with an overview of the main steps of the experiment. Treatment with CBD (in both hydrogen peroxide exposed and unexposed keratinocytes membrane) did not significantly altered expression of any proteins ([Fig. 1](#)). At the same time, we also did not find any significantly changed protein as specific for the control group. We observed that exposure to H_2O_2 significantly changed the expression of 22 proteins compared to control cells, while treatment with CBD significantly changed the level of 18 of these proteins ([Fig. 1](#)). Moreover, changes in the level of 14 proteins including chaperons and antioxidant proteins were found in all experimental groups.

On the other hand, the dendrogram of the top 25 proteins (with the smallest q-value) showed an apparent hierarchical clustering of membrane samples from keratinocyte cell groups ([supplementary figure 2](#)). As shown in [supplementary figure 2](#), membrane samples from the H_2O_2 group were clustered independently from the control samples as well as all CBD treated groups. Moreover, the volcano plots comparing the experimental groups showed a noticeable difference in the membrane proteome between the CTR and H_2O_2 cell groups as well as the H_2O_2 and $CBD+H_2O_2+CBD$ cell groups. Together with this, there were no significant differences between the CTR and CBD cell groups ([Fig. 2](#)).

Heatmap obtained using mean intensities of significantly changed proteins for each experimental cell groups ([Fig. 3](#)) showed that exposure of cells to H_2O_2 caused an apparent increase in the expression of some membrane proteins (vs. CTR), with the exception of two proteins, 14-3-3 protein theta (P27348) and small nuclear ribonucleoprotein Sm D1 (P62314). Furthermore, the heatmap indicated that the CBD treatment

prevented these changes caused by H_2O_2 . At the same time, the use of CBD did not induce a significant change of the membrane proteome of keratinocytes not exposed to H_2O_2 . The hierarchical dendrogram of the heatmap (including all significantly changed proteins and creating by using average intensities for each of the main experimental cell groups) showed a correlation with that of the dendrogram (top 25 proteins, [supplementary figure 2](#)): we observed that the H_2O_2 group was separated from the other cell groups and the CTR and CBD groups also were clustered close to each other ([Fig. 3](#)).

Assessment of the biological pathways and molecular functions of significantly altered proteins showed that treatment with CBD significantly changed the level of membrane proteins involved in the regulation of the cell redox status, cell survival, protein biosynthesis, folding and proteasomal activity as well as NF- κ B signalling (in association with proteasomal activity) in the cellular environment affected by H_2O_2 ([Fig. 4](#)). Protein interactions found with high confidence (STRING interaction score > 0.90) indicated that significantly changed proteins, which participated in protein biosynthesis/proteasomal activity/protein folding processes, in keratinocyte membranes were found with a strong interaction ([Fig. 4](#)). Based on the changes in protein levels between experimental cell groups ([supplementary table 2](#)), we found that exposure to H_2O_2 induced the expression of all these proteins while the treatment with CBD reduced the expression of all, except the small nuclear ribonucleoprotein Sm D1 (P11940) and 14-3-3 protein theta (P27348). The proteins with increased expression were 26S proteasome regulatory subunit 6A (P17980), 26S proteasome non-ATPase regulatory subunit 3 (O43242), proteasome subunit beta type-1 (P20618), ubiquitin-40S ribosomal protein S27a (P62979), 60 kDa chaperonin (A0A024R3X4), chaperonin containing TCP1, subunit 6A (Zeta 1) (A0A024RDL1), 40S ribosomal protein S2 (P15880), 40S ribosomal protein S16 (P62249), eukaryotic translation initiation factor 3 subunit A (J9R021) and E (B2R806), polyadenylate-binding protein 1 (P11940), transitional endoplasmic reticulum ATPase (P55072), ATP synthase subunit beta (V9HW31) and nucleolin (P19338) ([supplementary table 2](#), [Fig. 4](#)). Additionally, the results confirming changes in proteasome subunits and chaperon (HSP) expression were confirmed by Western blot analysis ([Fig. 5](#)).

Moreover, the protein-protein interaction network as well as fold changes, calculated using the average protein intensity, also showed that CBD treatment altered the expression of oxidoreductase proteins

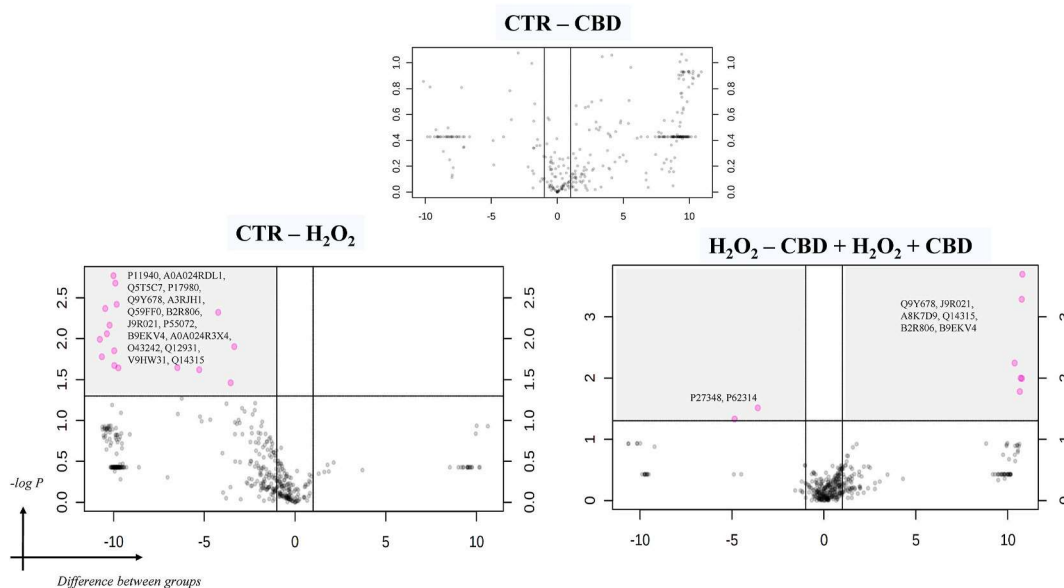


Fig. 2. Volcano plots comparing the effect of CBD (4 μ M) and H_2O_2 (200 μ M) on membrane proteome of keratinocytes from the control group (CTR), CBD treated group (CBD), H_2O_2 exposed cell group (H_2O_2) and cell group treated with CBD before and after H_2O_2 exposure ($CBD+H_2O_2+CBD$), as explained in [Table 1](#). Also, proteins IDs are given which are significantly changed between keratinocyte cell groups.

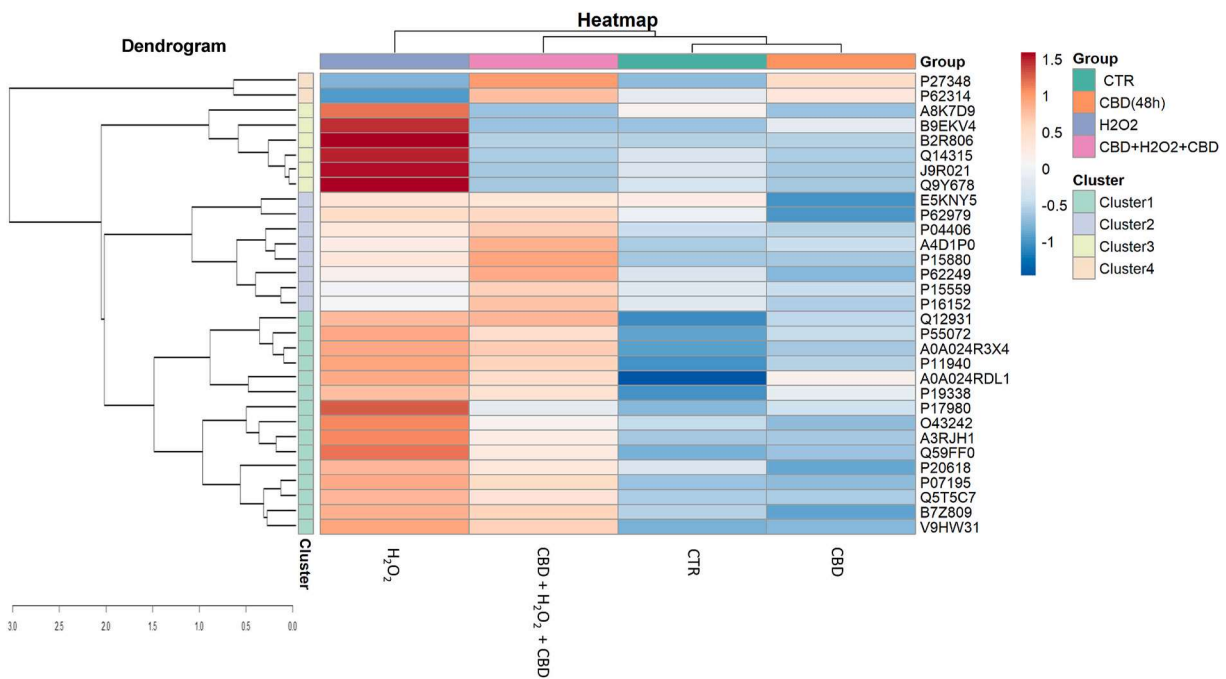


Fig. 3. Heatmap, clustering and dendrogram of membrane proteins with significantly changed expression in keratinocytes of the control group (CTR), CBD treated group (CBD), H₂O₂ exposed cell group (H₂O₂) and cell group treated with CBD before and after H₂O₂ exposure (CBD+H₂O₂+CBD), as explained in Table 1.

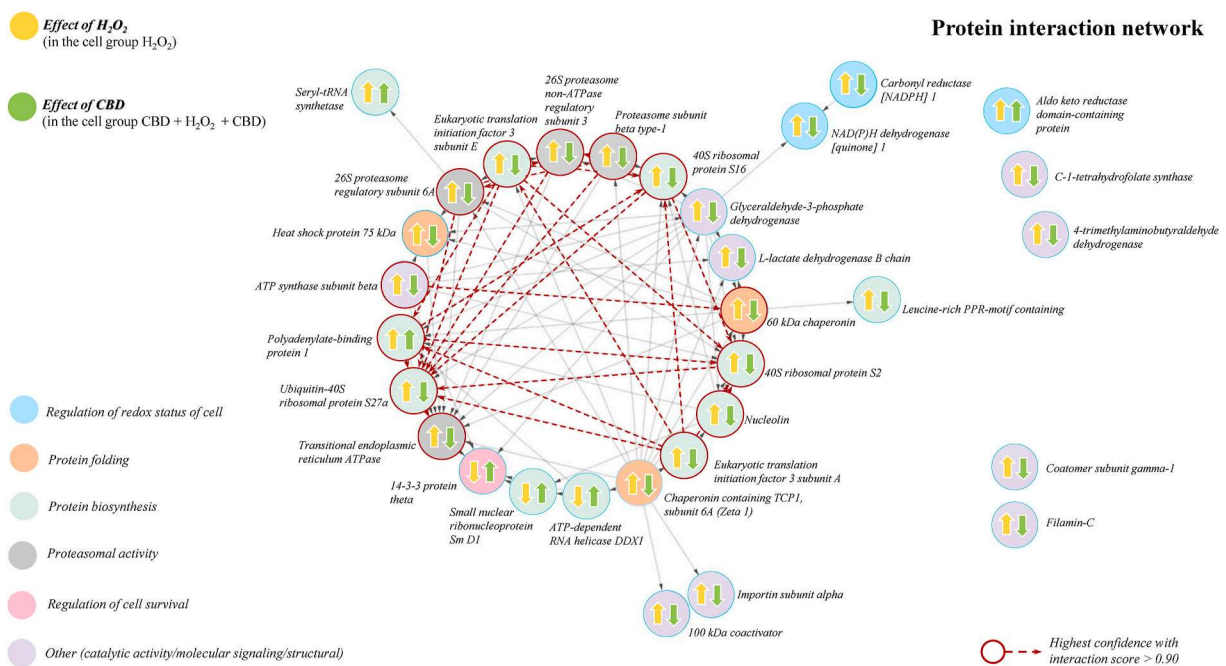


Fig. 4. Protein-protein interaction network, molecular functions of proteins together with the effect of CBD/H₂O₂ on protein levels (according to fold-changes, supplementary table 2) for membrane proteins with significantly changed expression level of keratinocytes from the control group (CTR), CBD treated group (CBD), H₂O₂ exposed cell group (H₂O₂) and cell group treated with CBD before and after H₂O₂ exposure (CBD+H₂O₂+CBD), as explained in Table 1.

associated with regulation of cellular response caused by H₂O₂, carbonyl reductase [NADPH]1 (P16152) and NAD(P)H dehydrogenase [quinone] 1 (P15559). The level of these proteins in keratinocyte membranes increased after exposure to H₂O₂, however, treatment with CBD reduced these levels in H₂O₂-treated cells (supplementary table 2, Fig. 4). Other proteins with oxidoreductase activity (protein containing the aldo-keto reductase domain, A4D1P0) were also identified with significantly elevated levels but no potential interaction with other proteins was found (supplementary table 2, Fig. 4). In addition, 14-3-3 protein theta

(P27348) was observed with a significantly reduced level of expression in the membrane of the H₂O₂ treated cell group compared to the CTR group: However, treatment with CBD increased this level more than 10-fold (supplementary table 2).

We also found that CBD, as a lipophilic molecule, located mainly in biological membranes, influences the modification of membrane proteins by the products of lipid peroxidation. Comparative analysis of 4-HNE/MDA/4-ONE adducts with proteins showed that H₂O₂ strongly increased the formation of these adducts in biological membranes, while

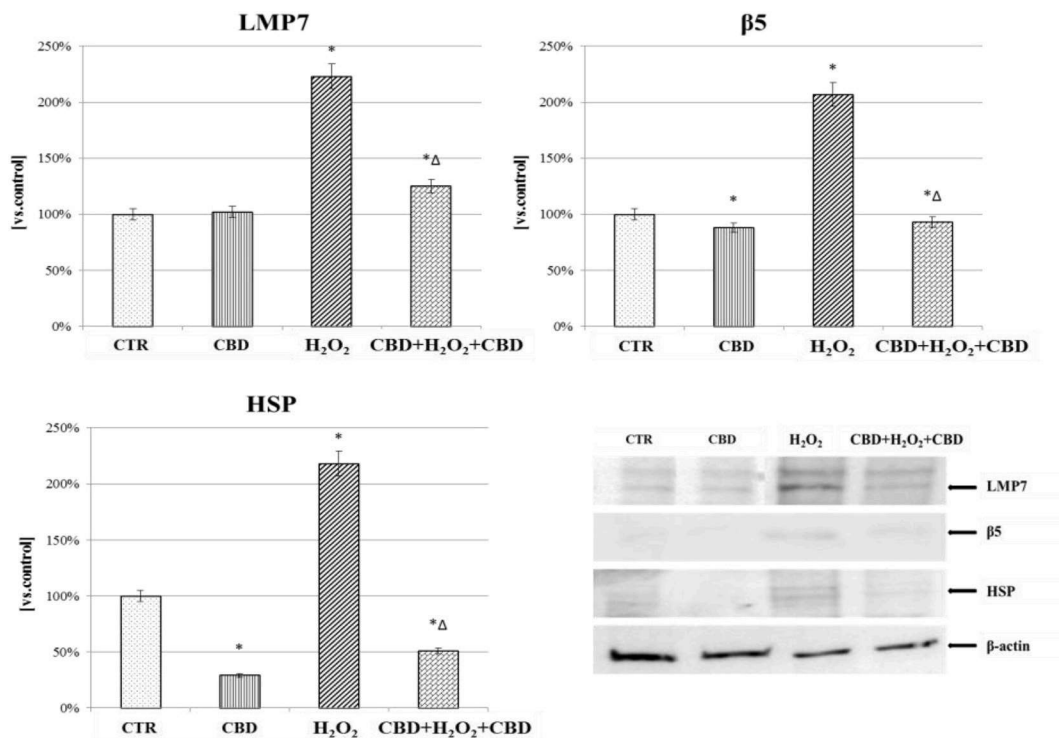


Fig. 5. Changes in the expression of proteasome subunits (LMP7 and β5) and heat shock protein (HSP) expression in membrane fractions from the control group (CTR), CBD treated group (CBD), H₂O₂ exposed cell group (H₂O₂) and cell group treated with CBD before and after H₂O₂ exposure (CBD+H₂O₂+CBD) estimated by Western blot analysis. Mean values ± SD of three independent samples and statistically significant differences for p ≤ 0.05 are presented: (·) is used for differences vs. CTR; (Δ) is used for differences between the group H₂O₂ and CBD+H₂O₂+CBD.

treatment with CBD significantly reduced their levels (Fig. 6). These proteomic results were partially confirmed by the 4-HNE-protein adducts level analysis assessed by ELISA and the results were presented in

the supplementary figure 3.

Our results show that the membranes of cells exposed to H₂O₂ had the highest level of MDA-protein adducts compared to the level of

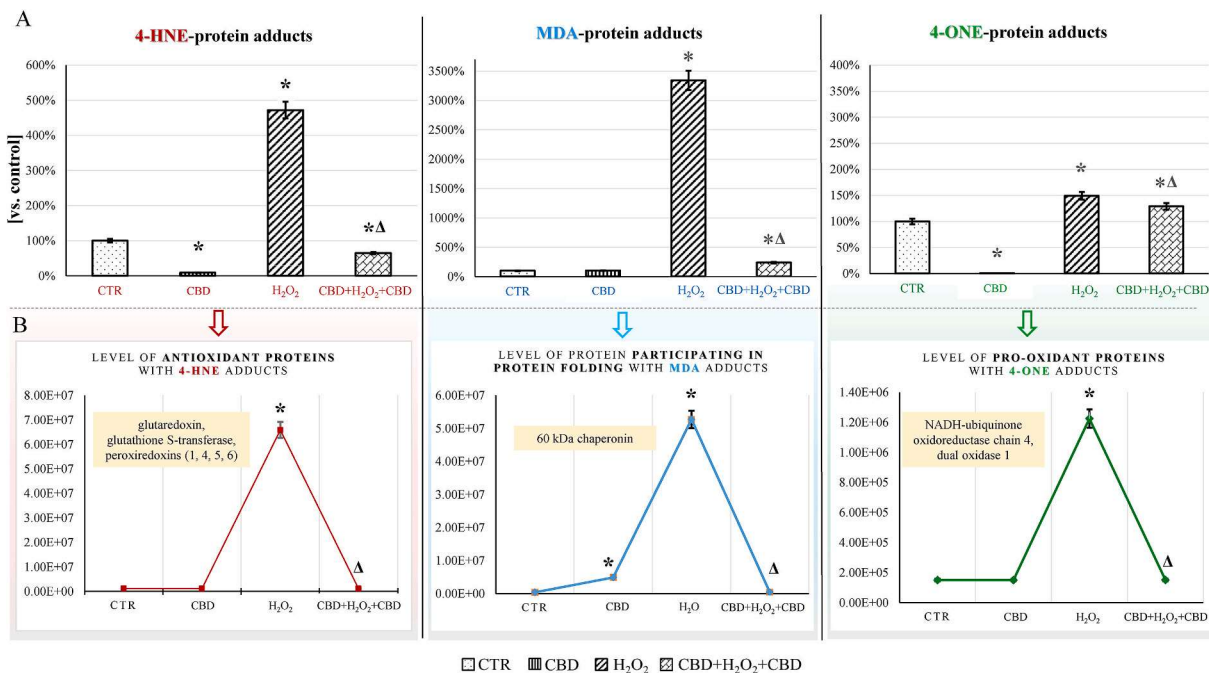


Fig. 6. Changes in the average level of 4-HNE/MDA/4-ONE-protein adducts for membrane fractions from the control group (CTR), CBD treated group (CBD), H₂O₂ exposed cell group (H₂O₂) and cell group treated with CBD before and after H₂O₂ exposure (CBD+H₂O₂+CBD). A) shows the average of all identified 4-HNE/MDA/4-ONE-protein adducts (separately for each adduct types). B) shows the average of identified adducts of antioxidant/chaperonin/pro-oxidant proteins with 4-HNE/MDA/4-ONE. Mean values ± SD of three independent samples and statistically significant differences for p ≤ 0.05 are presented: (·) is used for differences vs. CTR; (Δ) is used for differences between the group H₂O₂ and CBD+H₂O₂+CBD.

protein adducts with 4-HNE/4-ONE. Consequently, CBD therapy most effectively reduced the high level of MDA-protein adducts. CBD also significantly decreased the levels of 4-HNE and 4-ONE-membrane protein adducts (Fig. 6). Detailed analysis of the modified proteins revealed that these changes altered proteins involved in the regulation of redox balance and the protein folding process. Treatment of keratinocytes with CBD was found to significantly lower the average level of adducts of the following proteins: glutaredoxin (A0A024RAM2), glutathione S-transferase (Q59EJ5), peroxireoxin-1,4,5,6 (Q06830, A6NJJ0, P30044, P30041) with 4-HNE; 60 kDa chaperonin (B3GQS7) with MDA; chain 4 of NADH-ubiquinone oxidoreductase and dual oxidase-1 (A0A059RKZ9, HOYK19) with 4-ONE (Fig. 6). In addition, it is observed that the identified antioxidant/chaperone/pro-oxidative proteins, mentioned above, differed in the average adduct, specific to these proteins, formation versus overall adduct formation of all identified proteins (Fig. 6). For example, while the CBD-treated cells showed no significant change from the control cell group for all identified MDA-protein adducts, the level of MDA-60 kDa chaperonin adducts showed a significant change in the CBD group.

It should be noted that the formation of lipid peroxidation products-protein adducts was also observed for many different proteins (Fig. 6A) other than the antioxidant/chaperonins/pro-oxidative proteins in Fig. 6B. All identified 4-HNE/MDA/4-ONE-protein adducts were listed in [supplementary table 3](#).

4. Discussion

H₂O₂, used in medicine because of its antiseptic properties (against bacteria, yeasts, fungi and viruses) [3] and promoting wound healing [2], has numerous therapeutic advantages in the treatment of certain skin diseases, such as acne [45], skin infections [46], actinic keratosis [47], seborrheic keratosis [48], squamous cell carcinoma [4]. Moreover, as a second messenger H₂O₂, used alone or with other compounds or nanoparticles, can also increase the sensitization of abnormal cells (such as keratinocytes in seborrheic keratosis) and ultimately the effectiveness of photodynamic therapy [49–51]. On the other hand, as it is pointed out, H₂O₂ can also generate serious side effects. It can damage the skin, causing epidermal necrosis and lead to chronic inflammation by shifting the redox balance towards oxidation resulting from the overproduction of ROS [2,3,52]. For this reason, H₂O₂ may also intensify cell membrane disruption and pore formation in the phospholipid bilayer [53]. To take advantage of the positive effects of hydrogen peroxide and prevent side effects, we have tested the use of the antioxidant properties of CBD to counter skin damage caused by the pro-oxidative microenvironment chemically induced by H₂O₂. Our data indicate that CBD modulation of proteins involved in proteasomal degradation, protein folding, and protein biosynthesis may be one of the key points in the regulation of cellular redox balance and keratinocyte inflammation under oxidative stress.

4.1. The effect of CBD on proteasomal degradation and protein folding in a H₂O₂-mediated oxidative microenvironment

The potential protective role of CBD in the proteostasis network, which includes the unfolded protein system and the heat-shock proteins responses, ubiquitin-proteasome system, regulation of Ca²⁺ influx as well as inflammatory pathways, is of great interest [20,54]. Under oxidative conditions, the status of proteostasis can alter the cellular response, via functional changes in the proteome that regulates the processes of protein synthesis, folding, transport, and degradation [55]. Many recent studies highlight the protective role of CBD associated with its effect on the proteasomal system. CBD has been shown to lower the level of apoptotic suppressor - X-linked inhibitor apoptosis (XIAP) through its ubiquitination and proteasomal degradation [23]. In addition, CBD has been shown to induce proteasomal degradation of the Nrf2 transcription repressor - BACH1 protein, thus increasing the level of the

antioxidant - heme oxygenase 1 [56]. The activity of the proteasomal system maintained by CBD by regulating intracellular levels of Ca²⁺ causes the removal of oxidized and misfolded proteins in neurodegenerative diseases [20]. Moreover, CBD regulates the level of expression of proteasome subunits (α and β type) and the formation of adducts with 4-HNE [17]. Regardless of the above actions, the results of this study demonstrating a network of protein-protein interactions show that treatment with CBD can induce a significant change in the level of membrane proteins potentially involved in the molecular signaling in proteostasis network. These changes involve protein folding (60 kDa chaperonin, heat shock protein 75 kDa, chaperonin containing TCP1 subunit 6A) and proteasomal activity (proteasome subunit beta type-1, 26S proteasome regulatory subunit 6A, 26S proteasome non-ATPase regulatory subunit 3, transitional endoplasmic reticulum ATPase). Data from the literature and the results of this study highlight the role of protein folding and proteasomal degradation in determining the cellular response modulated by CBD under oxidative stress.

CBD has been found to reduce the level of H₂O₂-induced expression of the following proteins in keratinocyte membranes (mainly in mitochondrial membranes and extracellular vesicles): 60 kDa chaperonin, 75 kDa heat shock protein, chaperonin containing the TCP1 6A subunit. All these proteins participate in the process of protein folding and homeostasis [57–59]. It is known that oxidative stress up-regulates 60 kDa chaperonin in order to protect the cell against protein aggregation [60]. Moreover, in addition to its role in mitochondrial proteostasis and anti-apoptotic action in tumour [61], the mitochondrial 60 kDa chaperonin also induces a pro-inflammatory response in cells by enhancing the IKK/NF- κ B activation as well as the expression of TNF- α , interleukin-1 beta and 6 [62,63]. In contrast, the 75 kDa heat shock protein may participate in an anti-apoptotic action [57], while a chaperone containing the TCP1 6A subunit is associated with poor survival of patients with grade IV glioblastoma [64]. CBD, by restoring proteostasis and reversing the pro-inflammatory response, can protect skin keratinocytes from death resulting from oxidative stress induced by H₂O₂. Also, the pro-survival regulation of cellular responses may be supported by the regulatory action of CBD causing increased levels of theta protein 14-3-3 [65,66] which are decreased by H₂O₂. Literature data also indicate an association between down-regulation of protein 14-3-3 and apoptosis caused by ROS production including hydrogen peroxide [67]. The pro-survival effect of CBD can also be associated with the prevention of the formation of non-physiological modifications in proteins by reducing the formation of protein adducts with lipid peroxidation products and, consequently, the protein cross-linking and aggregation. The results of this study showed a reduced level of MDA/4-HNE/4-ONE-protein adducts in keratinocyte membranes induced by CBD, which was significantly enhanced by H₂O₂. It is known that protein lipoxidation induces their instability and, consequently, cross-linking and aggregation [68], which, due to the reduced efficiency of proteasomal degradation, can lead to cell death. Thus, the reduction of above adducts formation by CBD indicates that this phytocannabinoid can prevent the failure of the proteolytic process in removing these modified proteins. Therefore, this may suggest a bidirectional action of CBD, preventing the intensification of adducts formation as well as further aggregation of these proteins by protecting the 60 kDa chaperonin. This may significantly improve the efficiency of their proteolytic degradation and, consequently, prevent pathological accumulation in keratinocytes. Despite the lack of direct data on the effect of 60 kDa chaperonin on MDA-protein adducts formation, it is known that 4-HNE/4-ONE modifications can lead to loss of activity of the chaperone proteins Hsp70 and Hsp90 [69]. Thus, a reduction in MDA-protein adduct formation may suggest a protective effect of CBD against the 60 kDa chaperonin. Such action of CBD can promote the maintenance of mitochondrial and cellular keratinocyte homeostasis [70].

In addition, our data showed that CBD significantly reduced the levels of all identified proteins involved in proteasomal degradation (beta proteasome subunit type 1, proteasome regulatory subunit 26S 6A,

proteasome regulatory subunit 26S other than ATPase 3, transient endoplasmic reticulum ATPase) whose levels were elevated due to exposure of keratinocytes to H₂O₂. Data from the literature confirm that under oxidative stress the levels of all proteins involved in proteasomal activity are increased in order to remove the modified proteins as well as to regulate the activity of NF-κB [71–73]. Thus, the results obtained in this study indicating the reducing level of these proteins by CBD indirectly confirm the reduction in the intensity of oxidative stress and the maintenance of protein homeostasis, but also its protective effect against chronic inflammation mediated by oxidative stress.

In addition to CBD's demonstrated effects on protein folding and degradation, it is known that CBD can modulate molecular signalling through the expression of redox-sensitive transcription factors such as Nrf2 and NF-κB [19,74] and gene expressions such as superoxide dismutase [19]. Also CBD can alter the expression and modification of proteins participating in the regulation of cell survival and differentiation processes such as Bcl-2 and protein S100 [19]. The ubiquitin-dependent proteasomal degradation of the activators or inhibitors of the above proteins, but in particular of the transcription factors, is a critical molecular mechanism behind the biological function of these proteins [75–78]. Therefore, it may be suggested that molecular signalling in ubiquitin-related proteasomal degradation and protein folding could be one of the central molecular mechanisms responsible for the protective activity of CBD under the oxidative stress mediated by H₂O₂ (Fig. 7).

4.2. Effect of CBD on protein biosynthesis

Our study also showed that exposure to H₂O₂ induced levels of proteins that play key roles in the regulation of translation, mRNA stability and ribosome structure, such as eukaryotic translation initiation factor 3 A and E subunits; 40S ribosomal proteins S16, S2 and ubiquitin-S27a; polyadenylate-binding protein 1 and nucleolin. CBD has been found to inhibit the H₂O₂-increased expression of all of these proteins, with the exception of the polyadenylate 1 binding protein. The regulation of protein synthesis under oxidative conditions can vary depending on the concentration of ROS. It is known that under a low or medium concentration of ROS, the translation process of certain proteins, such as the antioxidant transcription factor Nrf2, may be induced for adaptation to the oxidative microenvironment [19,76], while high concentrations of ROS completely damage this process [79]. Moreover, data from the literature indicate that the polyadenylate-binding protein 1 may play a role in mRNA stability [80]. In our study, CBD increased the level of

polyadenylate-binding protein 1 in the membrane of keratinocytes exposed to H₂O₂. Therefore, the suggested protective role of CBD in maintaining protein homeostasis may also apply to CBD's effect on mRNA stability. Moreover, it is known that the ribosomal protein 27a can participate in the NF-κB activity [81] while the inhibition of nucleolin is associated with an anti-inflammatory effect [82]. The action of CBD in lowering the level of these two proteins may also indicate the anti-inflammatory effect of CBD in keratinocytes exposed to H₂O₂.

4.3. Protective role of CBD by reducing adduct formation mediated by lipid peroxidation of anti- and pro-oxidative proteins

The ability of CBD to regulate the synthesis or modification of proteins, and ultimately the proteostasis network, is related to this phyto-cannabinoid modulation of both redox balance as well as inflammation factors in keratinocytes exposed to H₂O₂. We found that H₂O₂ induces levels of proteins that regulate the redox balance of cells by modifying the activity of oxidoreductase carbonyl reductase [NADPH]1, NAD(P)H [quinone] 1 dehydrogenase and a protein containing an aldo-keto reductase domain. The use of CBD significantly reduces the level of carbonyl reductase [NADPH]1, NAD(P)H [quinone]1 dehydrogenase. Such modulation of the cellular redox state has also been observed in rats whose skin was chronically treated with CBD [19]. This may be the result of reduced ROS generation and, consequently, lipid peroxidation [8,15]. At the same time, the oxidoreductases mentioned are stimulated by the transcriptional action of Nrf2, the expression and activity of which increase in the acute phase of cellular defense against oxidative stress [76,83,84]. On the other hand, Nrf2 negatively regulates the NF-κB-mediated transcription of pro-inflammatory cytokine genes while the chronic intensification of Nrf2 activity could induce neoplastic transformation [76,85]. Thus, a decrease in levels of oxidoreductases through a decrease in Nrf2 activity may also indicate the cytoprotective effect of CBD by modulating the redox imbalance in keratinocytes associated with exposure to H₂O₂. Also, the reduced levels of NAD(P)H [quinone]1 dehydrogenase by CBD indicate a protective role of CBD against cell death induced by oxidative stress since NAD(P)H [quinone] 1 dehydrogenase, apart from oxidoreductase activity, can also induce apoptosis [86].

In addition, our data show that CBD partially prevents the excessive formation of 4-HNE adducts with antioxidant enzymes (glutaredoxin, glutathione S-transferase and peroxiredoxin 1, 4, 5 and 6) induced by the action of H₂O₂. However, it is known that glutaredoxin 2 and glutathione S-transferase 4-4 are relatively resistant to modifications

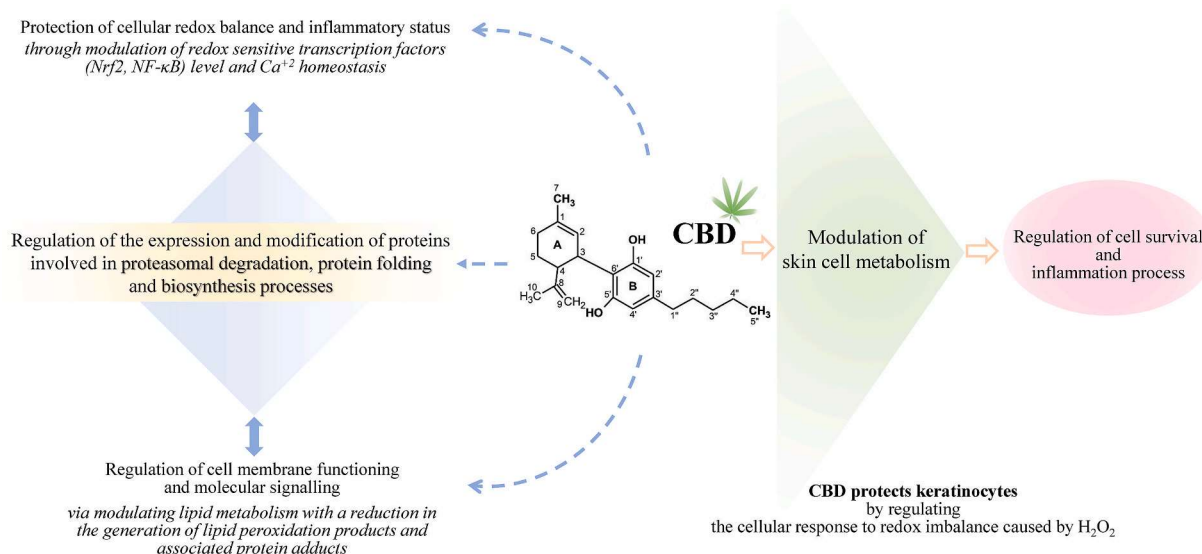


Fig. 7. A network of potential CBD activity in generating a cellular response caused by redox imbalance induced by exposure to H₂O₂ in skin keratinocytes.

induced by 4-HNE, due to their neutralizing role in maintaining cellular redox homeostasis and defence against oxidative stress [87,88]. Therefore, a lower level of their adducts with 4-HNE may indicate attenuation of oxidative stress by the action of CBD. A similar situation may also apply to peroxiredoxin, which, after creating adducts with 4-HNE, was characterized by a reduced biological activity [89].

Due to a relatively less knowledge of protein adducts with 4-ONE, it is difficult to interpret the consequences of an increase in their abundance, namely with pro-oxidant enzymes (chain 4 of NADH-ubiquinone oxidoreductase, dual oxidase-1). It is possible that the potential inhibition of NADH-ubiquinone oxidoreductase protects cells from damage associated with excessive generation of ROS, but may also attenuate beneficial oxidative signaling [90]. However, it is known, that dual oxidase 1 deficiency can also induce the release of pro-inflammatory cytokines such as IFN γ and TNF α [91]. The above suggestions are confirmed by studies of other authors, which have shown that the formation of 4-ONE-protein adducts is associated with the inhibition of pyruvate kinase and hsp90 [69] and high-density lipoproteins dysfunction [24]. However, since the formation of adducts promotes the inhibition of the activity of these enzymes, the use of CBD may protect keratinocytes from the oxidative stress and inflammation associated with H₂O₂ action. Moreover, CBD may maintain the beneficial oxidative signaling in keratinocytes exposed to H₂O₂.

Regardless of the above data, it should be noted that CBD tends to promote the formation of MDA-60kDa chaperonins adducts in the absence of hydrogen peroxide and reverses the total generation of 4-HNE and 4-ONE-protein adducts in keratinocytes treated with hydrogen peroxide. Overall, however, CBD prevents and reverses the cellular response induced by H₂O₂ (similar to previously demonstrated using UVB radiation as a stressor [17]). The differences observed in the direction of the changes can be considered as characteristic of a given protein and a type of stressor. For example, the increased level of pro-apoptotic NAD(P)H dehydrogenase in keratinocytes irradiated with UVB and treated with CBD [17] comparing to CBD-decreased level of this enzyme in keratinocytes exposed to H₂O₂. This effect can elicit a variety of cellular responses through differences in molecular signalling in biological pathways.

5. Conclusions

Our data indicate that CBD creates conditions conducive to the survival of keratinocytes treated with hydrogen peroxide by reducing inflammation and oxidative stress and its consequences, such as lipid peroxidation. This phytocannabinoid can directly affect the level of membrane proteins as well as modulate their level by reducing the level of adducts of these proteins with lipid peroxidation products generated in the cellular response to exposure to H₂O₂ [92]. By lowering the level of adducts and thus preventing protein aggregation, CBD maintains the functionality of chaperones and antioxidants and, consequently, the homeostasis of intracellular proteins, as has recently been demonstrated [17,20]. Therefore, it can be suggested that CBD protects keratinocytes by preventing the adverse effects of H₂O₂, including oxidative stress and chronic inflammation, which is important in the use of hydrogen peroxide in clinical practice.

Authorship contribution statement

SA: Acquisition of data, analysis and interpretation of data, drafting the article. **AG:** Drafting the article and revising it critically for important intellectual content. **PD:** Revising article critically for important intellectual content. **ES:** The conception and design of the study and revising article critically and finally approval of the version to be submitted.

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Declaration of competing interest

None. Authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2021.102074>.

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