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

Sinemyiz Atalay [a], Agnieszka Gęgotek [a], Pedro Domingues [b], Elżbieta Skrzydlewska [a,*]

[a] Department of Analytical Chemistry, Medical University of Bialystok, Mickiewicza 2D, 15-222, Bialystok, Poland
[b] Mass Spectrometry Center, Department of Chemistry, University of Aveiro, 3810-193, Aveiro, Portugal

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ABSTRACT

Hydrogen peroxide (H_2O_2) 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, H_2O_2 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 H_2O_2. 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 (H_2O_2) 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 H_2O_2 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

* Corresponding author.
E-mail addresses: sinemyiz.atalay@umb.edu.pl (S. Atalay), agnieszka.gegotek@umb.edu.pl (A. Gęgotek), p.domingues@ua.pt (P. Domingues), elzbieta.skrzydlewska@umb.edu.pl (E. Skrzydlewska).

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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 H_2O_2 [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 H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2.

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% CO_2 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 H_2O_2 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 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].

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 H_2O_2 concentration corresponded to 70% cell viability according to the MTT assay.

		Method
Experimental groups	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_2O_2 exposed cells (**H_2O_2**)	Keratinocytes were cultured in medium containing 200 μM hydrogen peroxide for 24 h
	Cells treated with CBD before and after H_2O_2 exposure (**CBD+H_2O_2+CBD**)	Keratinocytes were cultured in a medium containing 4 μM CBD for 24 h before and 24 h after 200 μM H_2O_2 exposure (for 24 h).

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 Gęgotek [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

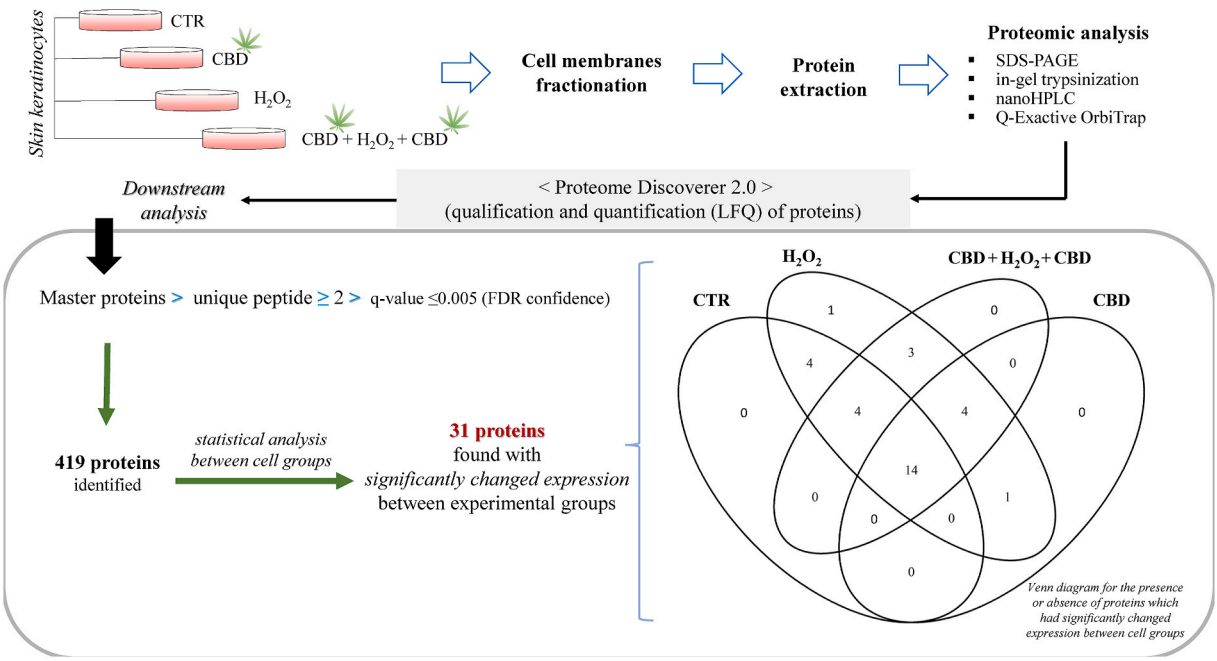

Fig. 1. A general view of the experimental steps and the Venn diagram of proteins with significantly changed expression in total membrane fractions of skin keratinocytes (from the control group (**CTR**), CBD treated group (**CBD**), H_2O_2 exposed cell group (**H_2O_2**) and the cell group treated with CBD before and after H_2O_2 exposure (**CBD+H_2O_2+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 β5 (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% H_2O_2, 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% H_2O_2) 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-κB 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

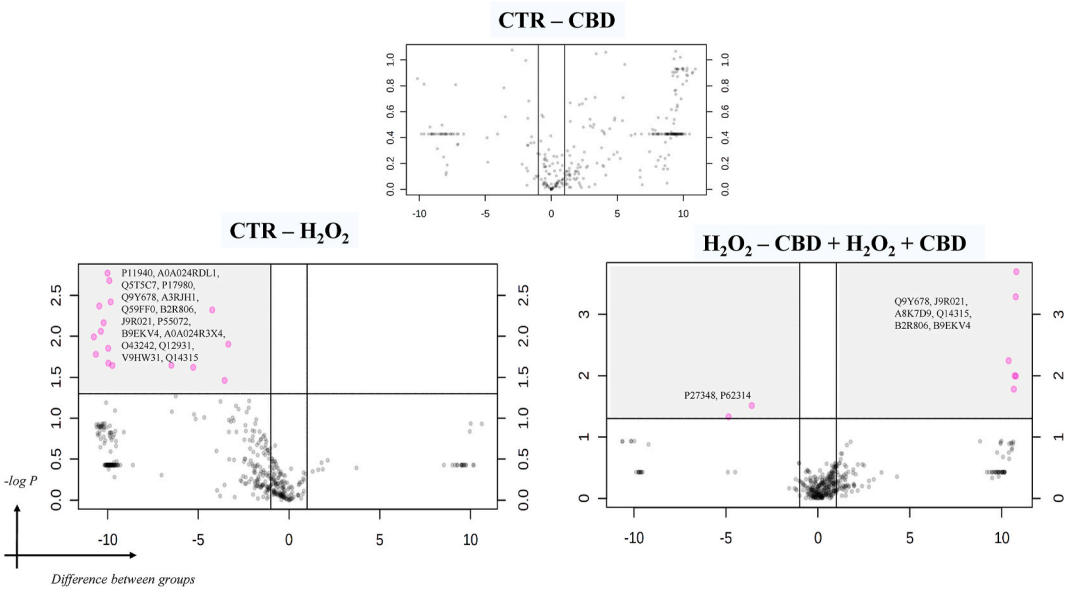

Fig. 2. Volcano plots comparing the effect of CBD (4 μM) and H_2O_2 (200 μM) on membrane proteome of keratinocytes from the control group (**CTR**), CBD treated group (**CBD**), H_2O_2 exposed cell group (**H_2O_2**) and cell group treated with CBD before and after H_2O_2 exposure (**CBD+H_2O_2+CBD**),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

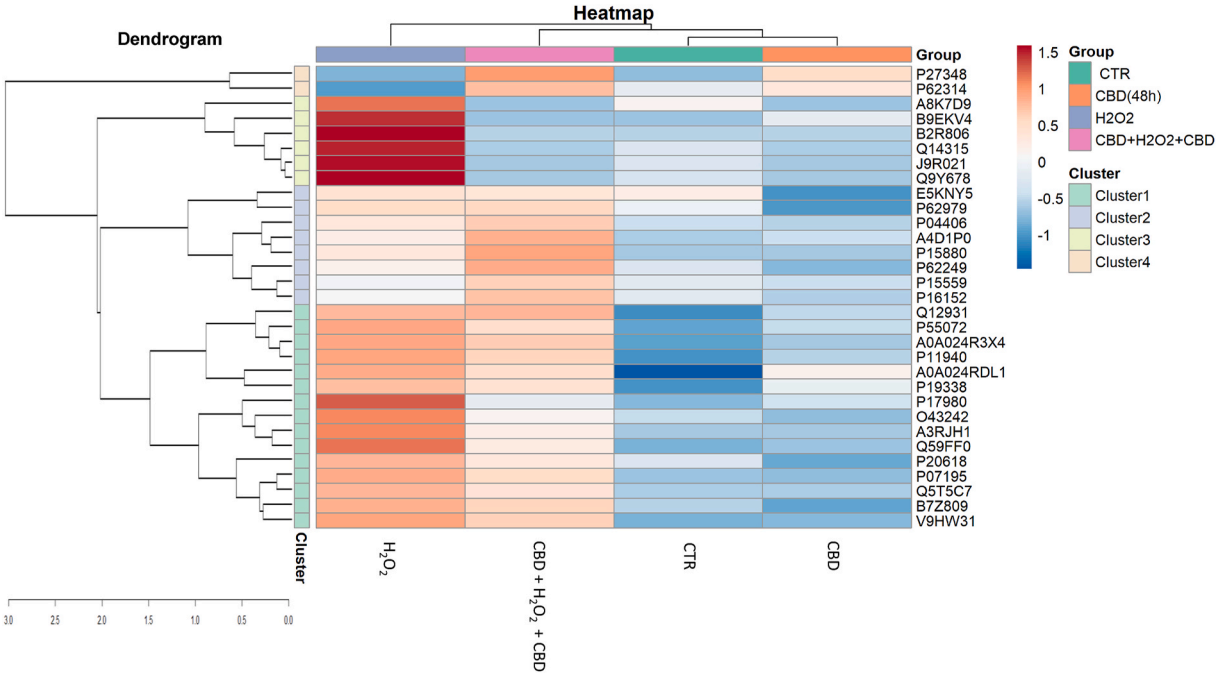

Fig. 3. Heatmap, clustering and dendrogram of membrane proteins with significantly changed expression in keratinocytes of the control group (**CTR**), CBD treated group (**CBD**), H_2O_2 exposed cell group (**H_2O_2**) and cell group treated with CBD before and after H_2O_2 exposure (**CBD+H_2O_2+CBD**), as explained in Table 1.

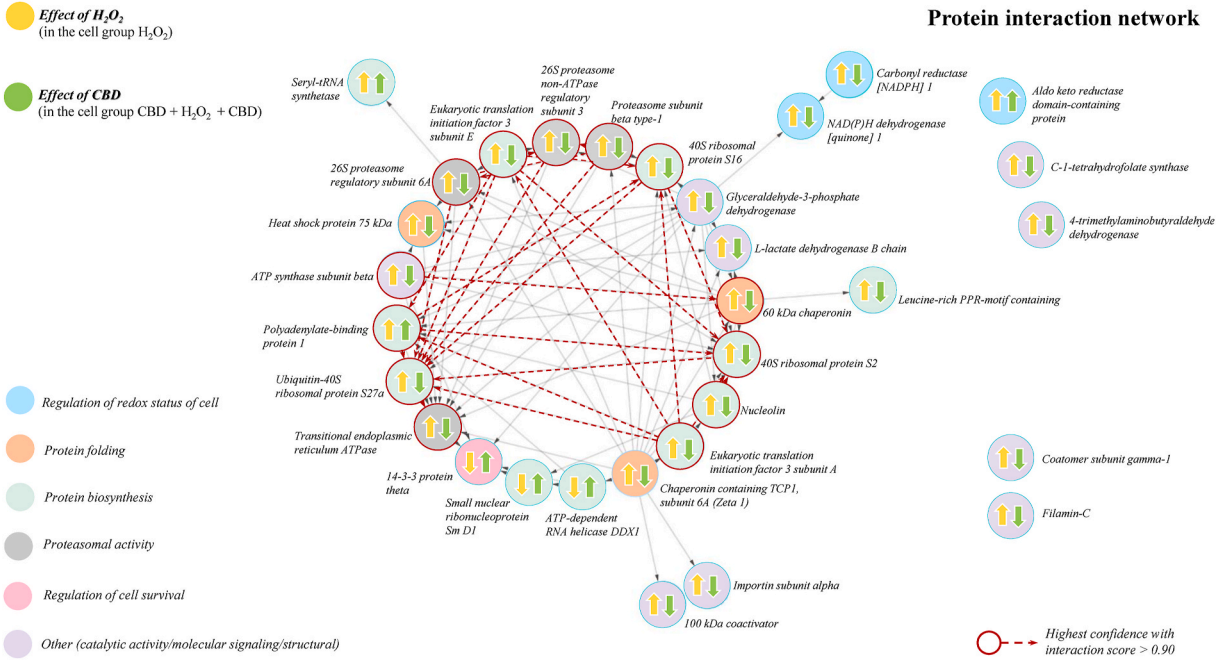

Fig. 4. Protein-protein interaction network, molecular functions of proteins together with the effect of CBD/H_2O_2 on protein levels (according to fold-changes, supplementary table 2) for membrane proteins with significantly changed expression level of keratinocytes from the control group (**CTR**), CBD treated group (**CBD**), 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.

associated with regulation of cellular response caused by H_2O_2, 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 H_2O_2, however, treatment with CBD reduced these levels in H_2O_2-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 H_2O_2 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 H_2O_2 strongly increased the formation of these adducts in biological membranes, while

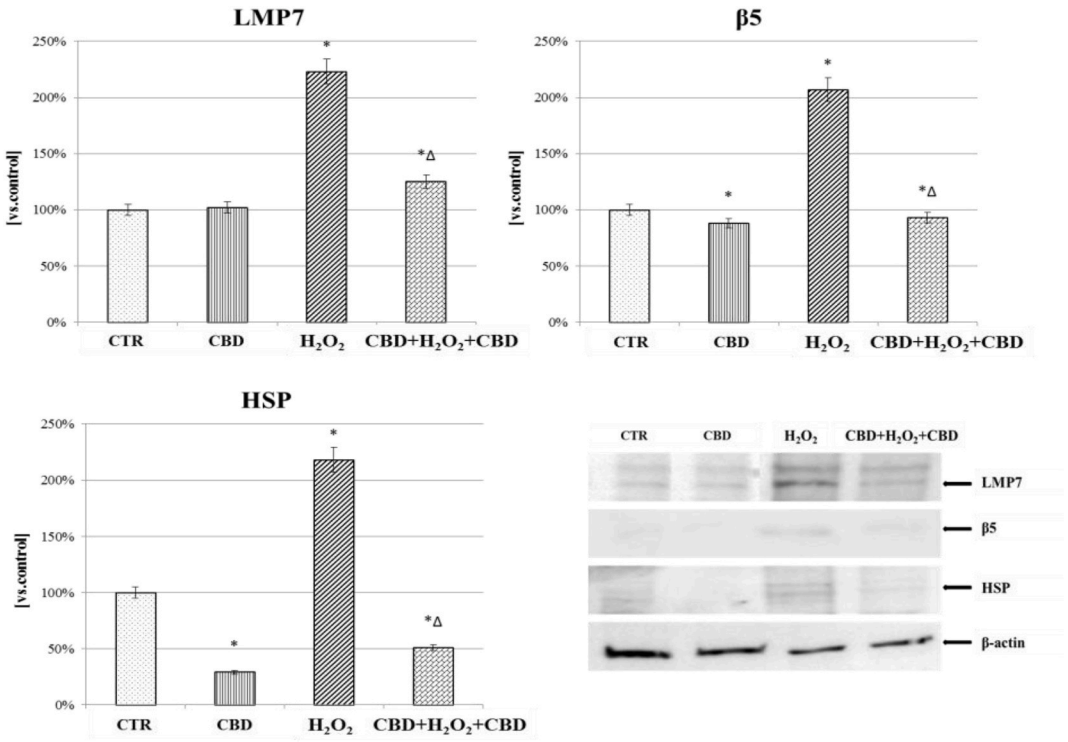

Fig. 5. Changes in the expression of proteasome subunits (LMP7 and β5) and heat shock protein (HSP) expression in membrane fractions from the control group (**CTR**), CBD treated group (**CBD**), H_2O_2 exposed cell group (**H_2O_2**) and cell group treated with CBD before and after H_2O_2 exposure (**CBD+H_2O_2+CBD**) estimated by Western blot analysis. Mean values ± SD of three independent samples and statistically significant differences for $p \leq 0.05$ are presented: (*) is used for differences vs. CTR; (**Δ**) is used for differences between the group H_2O_2 and CBD+H_2O_2+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 H_2O_2 had the highest level of MDA-protein adducts compared to the level of

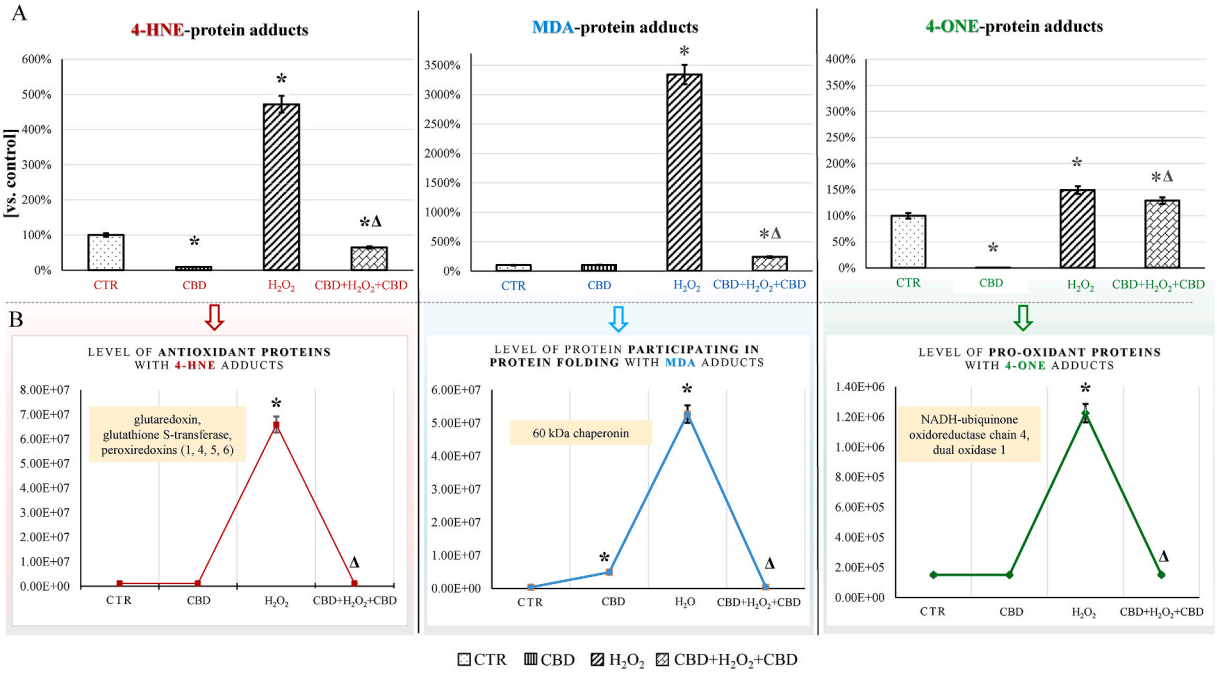

Fig. 6. Changes in the average level of 4-HNE/MDA/4-ONE-protein adducts for membrane fractions from the control group (**CTR**), CBD treated group (**CBD**), H_2O_2 exposed cell group (**H_2O_2**) and cell group treated with CBD before and after H_2O_2 exposure (**CBD+H_2O_2+CBD**). A) shows the average 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 $p \leq 0.05$ are presented: (*) is used for differences vs. CTR; (**Δ**) is used for differences between the group H_2O_2 and CBD+H_2O_2+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, H0YK19) 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

H_2O_2, 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 H_2O_2, 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, H_2O_2 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, H_2O_2 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 the antioxidant properties of CBD to counter skin damage caused by the pro-oxidative microenvironment chemically induced by H_2O_2. 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 H_2O_2-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 Ca^{2+} 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 Ca^{2+} 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 H_2O_2-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-κB 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 H_2O_2. 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 H_2O_2. 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 H_2O_2. 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 H_2O_2. 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 H_2O_2 (Fig. 7).

4.2. Effect of CBD on protein biosynthesis

Our study also showed that exposure to H_2O_2 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 H_2O_2-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 H_2O_2. 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 H_2O_2.

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 phytocannabinoid modulation of both redox balance as well as inflammation factors in keratinocytes exposed to H_2O_2. We found that H_2O_2 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 H_2O_2. 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 H_2O_2. However, it is known that glutaredoxin 2 and glutathione S-transferase 4-4 are relatively resistant to modifications

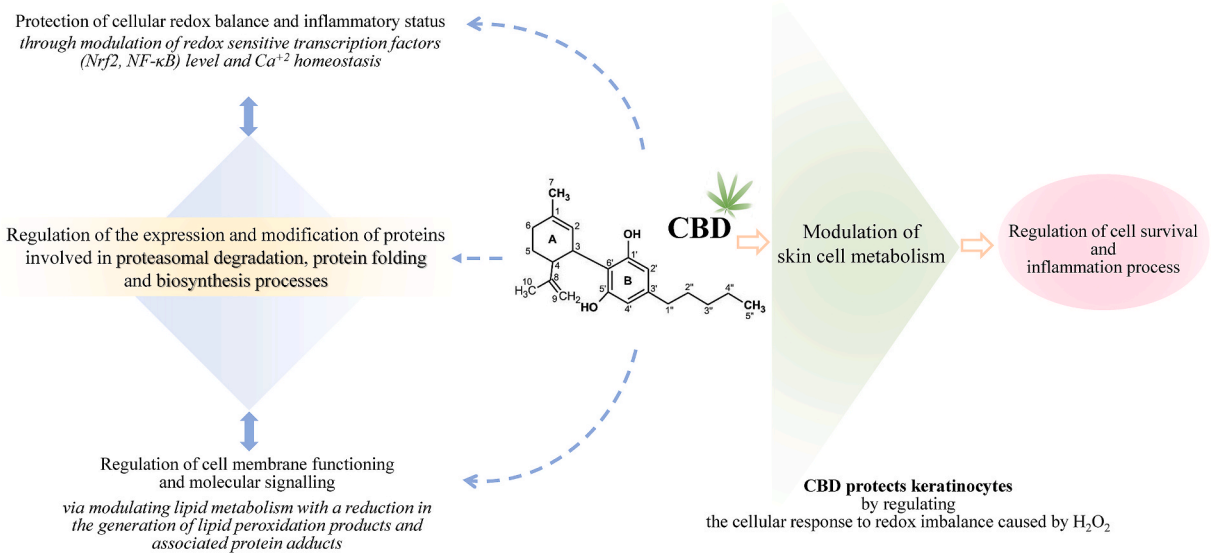

Fig. 7. A network of potential CBD activity in generating a cellular response caused by redox imbalance induced by exposure to H_2O_2 in skin keratinocytes.

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 IFNγ and TNFα [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 H_2O_2 action. Moreover, CBD may maintain the beneficial oxidative signaling in keratinocytes exposed to H_2O_2.

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 H_2O_2 (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 H_2O_2. 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 H_2O_2 [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 H_2O_2, 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.

References

[1] H. Sies, Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: oxidative eustress, Redox Biol 11 (2017) 613–619, https://doi.org/10.1016/j.redox.2016.12.035.
[2] G. Zhu, Q. Wang, S. Lu, Y. Niu, Hydrogen peroxide: a potential wound therapeutic target? Med. Princ. Pract. 26 (2017) 301–308, https://doi.org/10.1159/000475501.
[3] E.C. Murphy, A.J. Friedman, Hydrogen peroxide and cutaneous biology: translational applications, benefits, and risks, J. Am. Acad. Dermatol. 81 (2019) 1379–1386, https://doi.org/10.1016/j.jaad.2019.05.030.
[4] N. Mundi, K. Jordan, P. Doyle, C. Moore, 33% hydrogen peroxide as a Neoadjuvant treatment in the surgical excision of non-melanoma skin cancers: a case series, J. Otolaryngol. - Head Neck Surg. 49 (2020) 33, https://doi.org/10.1186/s40463-020-00433-6.
[5] T. Bito, K. Izu, Y. Tokura, Evaluation of toxicity and Stat3 activation induced by hydrogen peroxide exposure to the skin in healthy individuals, J. Dermatol. Sci. 58 (2010) 157–159, https://doi.org/10.1016/j.jdermsci.2010.03.013.
[6] M. Nita, A. Grzybowski, The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults, Oxid. Med. Cell. Longev. 2016 (2016), 3164734, https://doi.org/10.1155/2016/3164734.
[7] S. Nocchi, S. Björklund, B. Svensson, J. Engblom, T. Ruzgas, Electrochemical monitoring of native catalase activity in skin using skin covered oxygen electrode, Biosens. Bioelectron. 93 (2017) 9–13, https://doi.org/10.1016/j.bios.2017.01.001.
[8] S. Atalay, I. Dobrzyńska, A. Gęgotek, E. Skrzydlewska, Cannabidiol protects keratinocyte cell membranes following exposure to UVB and hydrogen peroxide, Redox Biol 36 (2020), 101613, https://doi.org/10.1016/j.redox.2020.101613.
[9] N. Di Marzo, E. Chisci, R. Giovannoni, The role of hydrogen peroxide in redox-dependent signaling: homeostatic and pathological responses in mammalian cells, Cells 7 (2018) 156, https://doi.org/10.3390/cells7100156.
[10] S. Atalay, I. Jarocka-karpowicz, E. Skrzydlewskas, Antioxidative and anti-inflammatory properties of cannabidiol, Antioxidants 9 (2020) 21, https://doi.org/10.3390/antiox9010021.
[11] I. Jarocka-Karpowicz, M. Biernacki, A. Wroński, A. Gęgotek, E. Skrzydlewska, Cannabidiol effects on phospholipid metabolism in keratinocytes from patients with Psoriasis Vulgaris, Biomolecules 10 (2020) 367, https://doi.org/10.3390/biom10030367.
[12] J. Chen, C. Hou, X. Chen, D. Wang, P. Yang, X. He, J. Zhou, H. Li, Protective effect of cannabidiol on hydrogen peroxide-induced apoptosis, inflammation and

oxidative stress in nucleus pulposus cells, Mol. Med. Rep. 14 (2016) 2321–2327, https://doi.org/10.3892/mmr.2016.5513.
[13] V. Di Giacomo, A. Chiavaroli, L. Recinella, G. Orlando, A. Cataldi, M. Rapino, V. Di Valerio, M. Ronci, S. Leone, L. Brunetti, L. Menghini, G. Zengin, A. Gunes, H. H. Abdallah, C. Ferrante, Antioxidant and neuroprotective effects induced by cannabidiol and cannabigerol in rat CTX-TNA2 astrocytes and isolated cortexes, Int. J. Mol. Sci. 21 (2020) 3575, https://doi.org/10.3390/ijms21103575.
[14] J. Kim, J.Y. Choi, J. Seo, I.S. Choi, Neuroprotective effect of cannabidiol against hydrogen peroxide in hippocampal neuron culture, Cannabis Cannabinoid Res 6 (2021) 40–47, https://doi.org/10.1089/can.2019.0102.
[15] A. Jastrząb, I. Jarocka-Karpowicz, A. Markowska, A. Wroński, A. Gęgotek, E. Skrzydlewska, Antioxidant and anti-inflammatory effect of cannabidiol contributes to the decreased lipid peroxidation of keratinocytes of rat skin exposed to UV radiation, Oxid. Med. Cell. Longev. 2021 (2021), 6647222, https://doi.org/10.1155/2021/6647222.
[16] A. Gęgotek, S. Atalay, P. Domingues, E. Skrzydlewska, The differences in the proteome profile of cannabidiol-treated skin fibroblasts following UVA or UVB irradiation in 2D and 3D cell cultures, Cells 8 (2019) 995, https://doi.org/10.3390/cells8090995.
[17] S. Atalay, A. Gęgotek, E. Skrzydlewska, Protective effects of cannabidiol on the membrane proteome of uvb-irradiated keratinocytes, Antioxidants 10 (2021) 402, https://doi.org/10.3390/antiox10030402.
[18] M. Biernacki, M.M. Brzóska, A. Markowska, M. Gałażyn-Sidorczuk, B. Cylwik, A. Gęgotek, E. Skrzydlewska, Oxidative stress and its consequences in the blood of rats irradiated with UV: protective effect of cannabidiol, Antioxidants 10 (2021) 821, https://doi.org/10.3390/antiox10060821.
[19] S. Atalay, A. Gęgotek, A. Wroński, P. Domigues, E. Skrzydlewska, Therapeutic application of cannabidiol on UVA and UVB irradiated rat skin. A proteomic study, J. Pharmaceut. Biomed. Anal. 192 (2021), 113656, https://doi.org/10.1016/j.jpba.2020.113656.
[20] R. Dash, M.C. Ali, I. Jahan, Y.A. Munni, S. Mitra, M.A. Hannan, B. Timalsina, D. F. Oktaviani, H.J. Choi, I.S. Moon, Emerging potential of cannabidiol in reversing proteinopathies, Ageing Res. Rev. 65 (2021), 101209, https://doi.org/10.1016/j.arr.2020.101209.
[21] H.Y. Wu, C.H. Huang, Y.H. Lin, C.C. Wang, T.R. Jan, Cannabidiol induced apoptosis in human monocytes through mitochondrial permeability transition pore-mediated ROS production, Free Radic. Biol. Med. 124 (2018) 311–318, https://doi.org/10.1016/j.freeradbiomed.2018.06.023.
[22] M. Olivas-Aguirre, L. Torres-López, J.S. Valle-Reyes, A. Hernández-Cruz, I. Pottosin, O. Dobrovinskaya, Cannabidiol directly targets mitochondria and disturbs calcium homeostasis in acute lymphoblastic leukemia, Cell Death Dis. 10 (2019) 779, https://doi.org/10.1038/s41419-019-2024-0.
[23] S. Jeong, M.J. Jo, H.K. Yun, D.Y. Kim, B.R. Kim, J.L. Kim, S.H. Park, Y.J. Na, Y. A. Jeong, B.G. Kim, H. Ashktorab, D.T. Smoot, J.Y. Heo, J. Han, S. Il Lee, H. Do Kim, D.H. Kim, S.C. Oh, D.H. Lee, Cannabidiol promotes apoptosis via regulation of XIAP/Smac in gastric cancer, Cell Death Dis. 10 (2019) 846, https://doi.org/10.1038/s41419-019-2001-7.
[24] L.S. May-Zhang, V. Yermalitsky, J.T. Melchior, J. Morris, K.A. Tallman, M.S. Borja, T. Pleasent, V. Amarnath, W. Song, P.G. Yancey, W.S. Davidson, M.F. Linton, S. S. Davies, Modified sites and functional consequences of 4-oxo-2-nonenal adducts in HDL that are elevated in familial hypercholesterolemia, J. Biol. Chem. 294 (2019) 19022–19033, https://doi.org/10.1074/jbc.RA119.009424.
[25] A. Sainz-Cort, C. Müller-Sánchez, E. Espel, Anti-proliferative and cytotoxic effect of cannabidiol on human cancer cell lines in presence of serum, BMC Res. Notes 13 (2020) 389, https://doi.org/10.1186/s13104-020-05229-5.
[26] E.S. Seltzer, A.K. Watters, D. MacKenzie Jr., L.M. Granat, D. Zhang, Cannabidiol (CBD) as a promising anti-cancer drug, Cancers 12 (2020) 3203, https://doi.org/10.3390/CANCERS12113203.
[27] M.A.T. Blaskovich, A.M. Kavanagh, A.G. Elliott, B. Zhang, S. Ramu, M. Amado, G. J. Lowe, A.O. Hinton, D.M.T. Pham, J. Zuegg, N. Beare, D. Quach, M.D. Sharp, J. Pogliano, A.P. Rogers, D. Lyras, L. Tan, N.P. West, D.W. Crawford, M.L. Peterson, M. Callahan, M. Thurn, The antimicrobial potential of cannabidiol, Commun. Biol. 41 (2021) 4, https://doi.org/10.1038/s42003-020-01530-y (2021) 7.
[28] L.R. Copeland-Halperin, L.C. Herrera-Gomez, J.R. LaPier, N. Shank, J.H. Shin, The effects of Cannabis: implications for the surgical patient, Plast. Reconstr. Surg. Glob. Open. 9 (2021), e3448, https://doi.org/10.1097/GOX.0000000000003448.
[29] D. Ghafoor, Z. Khan, A. Khan, D. Ualiyeva, N. Zaman, Excessive use of disinfectants against COVID-19 posing a potential threat to living beings, Curr. Res. Toxicol. 2 (2021) 159–168, https://doi.org/10.1016/J.CRTOX.2021.02.008.
[30] Y. Rai, R. Pathak, N. Kumari, D.K. Sah, S. Pandey, N. Kalra, R. Soni, B. S. Dwarakanath, A.N. Bhatt, Mitochondrial biogenesis and metabolic hyperactivation limits the application of MTT assay in the estimation of radiation induced growth inhibition, Sci. Rep. 8 (2018) 1531, https://doi.org/10.1038/s41598-018-19930-w.
[31] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.
[32] A. Gęgotek, P. Domingues, A. Wroński, P. Wójcik, E. Skrzydlewska, Proteomic plasma profile of psoriatic patients, J. Pharmaceut. Biomed. Anal. 155 (2018) 185–193, https://doi.org/10.1016/j.jpba.2018.03.068.
[33] S. Eissa, L.S. Seada, Quantitation of bcl-2 protein in bladder cancer tissue by enzyme immunoassay: comparison with Western blot and immunohistochemistry, Clin. Chem. 44 (1998) 1423–1429.
[34] D. Weber, L. Milkovic, S.J. Bennett, H.R. Griffiths, N. Zarkovic, T. Grune, Measurement of HNE-protein adducts in human plasma and serum by ELISA-Comparison of two primary antibodies, Redox Biol 1 (2013) 226–233, https://doi.org/10.1016/J.REDOX.2013.01.012.
[35] S. Tyanova, T. Temu, P. Sinitcyn, A. Carlson, M.Y. Hein, T. Geiger, M. Mann, J. Cox, The Perseus computational platform for comprehensive analysis of (prote) omics data, Nat. Methods 13 (2016) 731–740, https://doi.org/10.1038/nmeth.3901.
[36] J. Chong, D.S. Wishart, J. Xia, Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis, Curr. Protoc. Bioinforma. 68 (2019) e86, https://doi.org/10.1002/cpbi.86.
[37] RStudio Team, RStudio: Integrated Development for R. RStudio, PBC, Bost. MA, 2020. http://www.rstudio.com/. (Accessed 16 June 2021).
[38] H. Wickham, M. Averick, J. Bryan, W. Chang, L. McGowan, R. François, G. Grolemund, A. Hayes, L. Henry, J. Hester, M. Kuhn, T. Pedersen, E. Miller, S. Bache, K. Müller, J. Ooms, D. Robinson, D. Seidel, V. Spinu, K. Takahashi, D. Vaughan, C. Wilke, K. Woo, H. Yutani, Welcome to the tidyverse, J. Open Source Softw. 4 (2019) 1686, https://doi.org/10.21105/joss.01686.
[39] Raivo Kolde, pheatmap: Pretty Heatmaps, R Package Version 1.0.12, 2019. https://cran.r-project.org/package=pheatmap.
[40] T. Galili, dendextend: an R package for visualizing, adjusting and comparing trees of hierarchical clustering, Bioinformatics 31 (2015) 3718–3720, https://doi.org/10.1093/bioinformatics/btv428.
[41] Hanbo Chen, VennDiagram: Generate High-Resolution Venn and Euler Plots. R Package Version 1.6.20, 2018. https://cran.r-project.org/package=VennDiagram. (Accessed 16 June 2021).
[42] D. Szklarczyk, A.L. Gable, D. Lyon, A. Junge, S. Wyder, J. Huerta-Cepas, M. Simonovic, N.T. Doncheva, J.H. Morris, P. Bork, L.J. Jensen, C. Von Mering, STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets, Nucleic Acids Res. 47 (2019) D607–D613, https://doi.org/10.1093/nar/gky1131.
[43] A. Bateman, M.J. Martin, S. Orchard, M. Magrane, R. Agivetova, S. Ahmad, E. Alpi, E.H. Bowler-Barnett, R. Britto, B. Bursteinas, H. Bye-A-Jee, R. Coetzee, A. Cukura, A. Da Silva, P. Denny, T. Dogan, T.G. Ebenezer, J. Fan, L.G. Castro, P. Garmiri, G. Georghiou, L. Gonzales, E. Hatton-Ellis, A. Hussein, A. Ignatchenko, G. Insana, R. Ishtiaq, P. Jokinen, V. Joshi, D. Jyothi, A. Lock, R. Lopez, A. Luciani, J. Luo, Y. Lussi, A. MacDougall, F. Madeira, M. Mahmoudy, M. Menchi, A. Mishra, K. Moulang, A. Nightingale, C.S. Oliveira, S. Pundir, G. Qi, S. Raj, D. Rice, M. R. Lopez, R. Saidi, J. Sampson, T. Sawford, E. Speretta, E. Turner, N. Tyagi, P. Vasudev, V. Volynkin, K. Warner, X. Watkins, R. Zaru, H. Zellner, A. Bridge, S. Poux, N. Redaschi, L. Aimo, G. Argoud-Puy, A. Auchincloss, K. Axelsen, P. Bansal, D. Baratin, M.C. Blatter, J. Bolleman, E. Boutet, L. Breuza, C. Casals-Casas, E. de Castro, K.C. Echioukh, E. Coudert, B. Cuche, M. Doche, D. Dornevil, A. Estreicher, M.L. Famiglietti, M. Feuermann, E. Gasteiger, S. Gehant, V. Gerritsen, A. Gos, N. Gruaz-Gumowski, U. Hinz, C. Hulo, N. Hyka-Nouspikel, F. Jungo, G. Keller, A. Kerhornou, V. Lara, P. Le Mercier, D. Lieberherr, T. Lombardot, X. Martin, P. Masson, A. Morgat, T.B. Neto, S. Paesano, I. Pedruzzi, S. Pilbout, L. Pourcel, M. Pozzato, M. Pruess, C. Rivoire, C. Sigrist, K. Sonesson, A. Stutz, S. Sundaram, M. Tognolli, L. Verbregue, C.H. Wu, C.N. Arighi, L. Arminski, C. Chen, Y. Chen, J.S. Garavelli, H. Huang, K. Laiho, P. McGarvey, D. A. Natale, K. Ross, C.R. Vinayaka, Q. Wang, Y. Wang, L.S. Yeh, J. Zhang, UniProt: the universal protein knowledgebase in 2021, Nucleic Acids Res. 49 (2021) D480–D489, https://doi.org/10.1093/nar/gkaa1100.
[44] P. Shannon, A. Markiel, O. Ozier, N.S. Baliga, J.T. Wang, D. Ramage, N. Amin, B. Schwikowski, T. Ideker, Cytoscape: a software Environment for integrated models of biomolecular interaction networks, Genome Res. 13 (2003) 2498–2504, https://doi.org/10.1101/gr.1239303.
[45] G. Fabbrocini, L. Panariello, Efficacy and tolerability of a topical gel containing 3% hydrogen peroxide, 1.5% salicylic acid and 4% D-panthenol in the treatment of mild-moderate acne - PubMed, G. Ital. Dermatol. Venereol. (2016) 287–291. https://pubmed.ncbi.nlm.nih.gov/26761768/. (Accessed 16 June 2021).
[46] M. Alsterholm, N. Karami, J. Faergemann, Antimicrobial activity of topical skin pharmaceuticals - an in vitro study, Acta Derm. Venereol. 90 (2010) 239–245, https://doi.org/10.2340/00015555-0840.
[47] L. Resnick, H. Rabinovitz, D. Binninger, M. Marchetti, H. Weissbach, Topical sulindac combined with hydrogen peroxide in the treatment of actinic keratoses, J. Drugs Dermatol. JDD 8 (2009) 29–32. https://europepmc.org/article/med/19180893. (Accessed 16 June 2021).
[48] L.S. Baumann, A. Blauvelt, Z.D. Draelos, S.E. Kempers, M.P. Lupo, J. Schlessinger, S.R. Smith, D.C. Wilson, M. Bradshaw, E. Estes, S.D. Shanler, Safety and efficacy of hydrogen peroxide topical solution, 40% (w/w), in patients with seborrheic keratoses: results from 2 identical, randomized, double-blind, placebo-controlled, phase 3 studies (A-101-SEBK-301/302), J. Am. Acad. Dermatol. 79 (2018) 869–877, https://doi.org/10.1016/j.jaad.2018.05.044.
[49] R.N. Manifold, C.D. Anderson, Increased cutaneous oxygen availability by topical application of hydrogen peroxide cream enhances the photodynamic reaction to topical 5-aminolevulinic acid-methyl ester, Arch. Dermatol. Res. 303 (2011) 285–292, https://doi.org/10.1007/s00403-011-1128-x.
[50] Z.W. Wang, D. Su, X.Q. Li, J.J. Cao, D.C. Yang, J.Y. Liu, A H2O2-responsive boron dipyrromethene-based photosensitizer for imaging-guided photodynamic therapy, Molecules 24 (2019) 32, https://doi.org/10.3390/molecules24010032.
[51] T.T. Khalil, R. Bazzi, S. Roux, M. Fromm, The contribution of hydrogen peroxide to the radiosensitizing effect of gold nanoparticles, Colloids Surf. B Biointerfaces 175 (2019) 606–613, https://doi.org/10.1016/j.colsurfb.2018.12.041.
[52] A. Van Der Vliet, Y.M.W. Janssen-Heininger, Hydrogen peroxide as a damage signal in tissue injury and inflammation: murderer, mediator, or messenger? J. Cell. Biochem. 115 (2014) 427–435, https://doi.org/10.1002/jcb.24683.

[53] T.Y. Wang, M.D.J. Libardo, A.M. Angeles-Boza, J.P. Pellois, Membrane oxidation in cell delivery and cell killing applications, ACS Chem. Biol. 12 (2017) 1170–1182, https://doi.org/10.1021/acschembio.7b00237.
[54] M. Bouchecareilh, W.E. Balch, Proteostasis: A New Therapeutic Paradigm for Pulmonary Disease, in: Proc. Am. Thorac. Soc., Proc Am Thorac Soc, 2011, pp. 189–195, https://doi.org/10.1513/pats.201008-055MS.
[55] A.J. Sala, L.C. Bott, R.I. Morimoto, Shaping proteostasis at the cellular, tissue, and organismal level, J. Cell Biol. 216 (2017) 1231–1241, https://doi.org/10.1083/jcb.201612111.
[56] L. Casares, V. García, M. Garrido-Rodríguez, E. Millán, J.A. Collado, A. García-Martín, J. Peñarando, M.A. Calzado, L. de la Vega, E. Muñoz, Cannabidiol induces antioxidant pathways in keratinocytes by targeting BACH1, Redox Biol 28 (2020), 101321, https://doi.org/10.1016/j.redox.2019.101321.
[57] J. feng Chen, Q. He, M. hui Dai, W. Kong, HSP75 inhibits TGF-β1-induced apoptosis by targeting mitochondria in human renal proximal tubular epithelial cells, Biochem. Biophys. Res. Commun. 515 (2019) 64–71, https://doi.org/10.1016/j.bbrc.2019.05.119.
[58] X. Wu, J. Guo, Y. Chen, X. Liu, G. Yang, Y. Wu, Y. Tian, N. Liu, L. Yang, S. Wei, H. Deng, W. Chen, The 60-kDa heat shock protein regulates energy rearrangement and protein synthesis to promote proliferation of multiple myeloma cells, Br. J. Haematol. 190 (2020) 741–752, https://doi.org/10.1111/bjh.16569.
[59] D.Y. Wang, K. Kamuda, G. Montoya, P. Mesa, The TRiC/CCT Chaperonin and its Role in Uncontrolled Proliferation, in: Adv. Exp. Med. Biol., Springer, 2020, pp. 21–40, https://doi.org/10.1007/978-3-030-40204-4_2.
[60] P.C. Ikwegbue, P. Masamba, B.E. Oyinloye, A.P. Kappo, Roles of heat shock proteins in apoptosis, oxidative stress, human inflammatory diseases, and cancer, Pharmaceuticals 11 (2018) 2, https://doi.org/10.3390/ph11010002.
[61] S. Kumar, J. O'Malley, A.K. Chaudhary, J.R. Inigo, N. Yadav, R. Kumar, D. Chandra, Hsp60 and IL-8 axis promotes apoptosis resistance in cancer, Br. J. Canc. 121 (2019) 934–943, https://doi.org/10.1038/s41416-019-0617-0.
[62] X.L. Li, Y.L. Wang, J. Zheng, Y. Zhang, X.F. Zhang, Inhibiting expression of HSP60 and TLR4 attenuates paraquat-induced microglial inflammation, Chem. Biol. Interact. 299 (2019) 179–185, https://doi.org/10.1016/j.cbi.2018.12.013.
[63] S. Swaroop, A. Mahadevan, S.K. Shankar, Y.K. Adlakha, A. Basu, HSP60 critically regulates endogenous IL-1β production in activated microglia by stimulating NLRP3 inflammasome pathway, J. Neuroinflammation 15 (2018) 177, https://doi.org/10.1186/s12974-018-1214-5.
[64] S. Hallal, B.P. Russell, H. Wei, M.Y.T. Lee, C.W. Toon, J. Sy, B. Shivalingam, M. E. Buckland, K.L. Kaufman, Extracellular vesicles from neurosurgical aspirates identifies chaperonin containing TCP1 subunit 6A as a potential glioblastoma biomarker with prognostic significance, Proteomics 19 (2019), e1800157, https://doi.org/10.1002/pmic.201800157.
[65] S.R. Slone, N. Lavalley, M. McFerrin, B. Wang, T.A. Yacoubian, Increased 14-3-3 phosphorylation observed in Parkinson's disease reduces neuroprotective potential of 14-3-3 proteins, Neurobiol. Dis. 79 (2015) 1–13, https://doi.org/10.1016/j.nbd.2015.02.032.
[66] K. Pennington, T. Chan, M. Torres, J. Andersen, The dynamic and stress-adaptive signaling hub of 14-3-3: emerging mechanisms of regulation and context-dependent protein–protein interactions, Oncogene 37 (2018) 5587–5604, https://doi.org/10.1038/s41388-018-0348-3.
[67] M. Hashemi, A. Zali, J. Hashemi, S. Oraee-Yazdani, A. Akbari, Down-regulation of 14-3-3 zeta sensitizes human glioblastoma cells to apoptosis induction, Apoptosis 23 (2018) 616–625, https://doi.org/10.1007/s10495-018-1476-5.
[68] C. Martín-Sierra, P. Laranjeira, M.R. Domingues, A. Paiva, Lipoxidation and cancer immunity, Redox Biol 23 (2019), 101103, https://doi.org/10.1016/j.redox.2019.101103.
[69] Á. Viedma-Poyatos, P. González-Jiménez, O. Langlois, I. Company-Marín, C. M. Spickett, D. Pérez-Sala, Protein lipoxidation: basic concepts and emerging roles, Antioxidants 10 (2021) 295, https://doi.org/10.3390/antiox10020295.
[70] A. Rodriguez, D. Von Salzen, B.A. Holguin, R.A. Bernal, Complex destabilization in the mitochondrial chaperonin Hsp60 leads to disease, Front. Mol. Biosci. 7 (2020) 159, https://doi.org/10.3389/fmolb.2020.00159.
[71] T.J.A. Höhn, T. Grune, The proteasome and the degradation of oxidized proteins: Part III-Redox regulation of the proteasomal system, Redox Biol 2 (2014) 388–394, https://doi.org/10.1016/j.redox.2013.12.029.
[72] M.S. Khilji, D. Verstappen, T. Dahlby, M.C. Burstein Prause, C. Pihl, S.E. Bresson, T. H. Bryde, P.A. Keller Andersen, K. Klindt, D. Zivkovic, M.P. Bousquet-Dubouch, B. Tyrberg, T. Mandrup-Poulsen, M. Tomasz Marzec, The intermediate proteasome is constitutively expressed in pancreatic beta cells and upregulated by stimulatory, low concentrations of interleukin 1 β, PloS One 15 (2020), e0222432 https://doi.org/10.1371/journal.pone.0222432.
[73] K. Song, S. Li, The role of ubiquitination in NF-kB signaling during virus infection, Viruses 13 (2021) 145, https://doi.org/10.3390/v13020145.
[74] A. Jastrząb, A. Gęgotek, E. Skrzydlewska, Cannabidiol regulates the expression of keratinocyte proteins involved in the inflammation process through transcriptional regulation, Cells 8 (2019) 827, https://doi.org/10.3390/cells8080827.
[75] S. Shimamoto, Y. Kubota, F. Yamaguchi, H. Tokumitsu, R. Kobayashi, Ca2+/S100 proteins act as upstream regulators of the chaperone-associated ubiquitin ligase chip (c terminus of hsc70-interacting protein), J. Biol. Chem. 288 (2013) 7158–7168, https://doi.org/10.1074/jbc.M112.436758.
[76] A. Gęgotek, E. Skrzydlewska, The role of transcription factor Nrf2 in skin cells metabolism, Arch. Dermatol. Res. 307 (2015) 385–396, https://doi.org/10.1007/s00403-015-1554-2.
[77] Y. Kravtsova-Ivantsiv, A. Ciechanover, The ubiquitin-proteasome system and activation of NF-κB: involvement of the ubiquitin ligase KPC1 in p105 processing and tumor suppression, Mol. Cell. Oncol. 2 (2015), e1054552, https://doi.org/10.1080/23723556.2015.1054552.
[78] N. Edison, Y. Curtz, N. Paland, D. Mamriev, N. Chorubczyk, T. Haviv-Reingewertz, N. Kfir, D. Morgenstern, M. Kupervaser, J. Kagan, H.T. Kim, S. Larisch, Degradation of bcl-2 by XIAP and ARTS promotes apoptosis, Cell Rep. 21 (2017) 442–454, https://doi.org/10.1016/j.celrep.2017.09.052.
[79] A. Ghosh, N. Shcherbik, Effects of oxidative stress on protein translation: implications for cardiovascular diseases, Int. J. Mol. Sci. 21 (2020) 2661, https://doi.org/10.3390/ijms21082661.
[80] M.J. Massimelli, V. Majerciak, M. Kruhlak, Z.-M. Zheng, Interplay between polyadenylate-binding protein 1 and kaposi's sarcoma-associated herpesvirus ORF57 in accumulation of polyadenylated nuclear RNA, a viral long noncoding RNA, J. Virol. 87 (2013) 243–256, https://doi.org/10.1128/jvi.01693-12.
[81] J.C.S. Vieira, G. de Oliveira, C.P. Braga, M. da Silva Fernandes, P.M. de Moraes, M. A.R. Buzalaf, L.C.S. de Oliveira, P. de Magalhães Padilha, Parvalbumin and ubiquitin as potential biomarkers of mercury contamination of amazonian Brazilian fish, Biol. Trace Elem. Res. 197 (2020) 667–675, https://doi.org/10.1007/s12011-020-02026-w.
[82] L.H. Mariero, M.K. Torp, C.M. Heiestad, A. Baysa, Y. Li, G. Valen, J. Vaage, K. O. Stensløkken, Inhibiting nucleolin reduces inflammation induced by mitochondrial DNA in cardiomyocytes exposed to hypoxia and reoxygenation, Br. J. Pharmacol. 176 (2019) 4360–4372, https://doi.org/10.1111/bph.14830.
[83] P. Nioi, J.D. Hayes, Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors, Mutat. Res. Fund Mol. Mech. Mutagen 555 (2004) 149–171, https://doi.org/10.1016/j.mrfmmm.2004.05.023.
[84] J.H. Kwon, J. Lee, J. Kim, V.A. Kirchner, Y.H. Jo, T. Miura, N. Kim, G.W. Song, S. Hwang, S.G. Lee, Y.I. Yoon, E. Tak, Upregulation of carbonyl reductase 1 by Nrf2 as a potential therapeutic intervention for ischemia/reperfusion injury during liver transplantation, Mol. Cells 42 (2019) 672–685, https://doi.org/10.14348/molcells.2019.0003.
[85] S. Saha, B. Buttari, E. Panieri, E. Profumo, L. Saso, An overview of Nrf2 signaling pathway and its role in inflammation, Molecules 25 (2020) 5474, https://doi.org/10.3390/molecules25225474.
[86] X. Zhang, K. Han, D.H. Yuan, C.Y. Meng, Overexpression of NAD(P)H: quinone oxidoreductase 1 inhibits hepatocellular carcinoma cell proliferation and induced apoptosis by activating AMPK/PGC-1α pathway, DNA Cell Biol. 36 (2017) 256–263, https://doi.org/10.1089/dna.2016.3588.
[87] H. Zhang, Y. Du, X. Zhang, J. Lu, A. Holmgren, Glutaredoxin 2 reduces both thioredoxin 2 and thioredoxin 1 and protects cells from apoptosis induced by auranofin and 4-Hydroxynonenal, Antioxidants Redox Signal. 21 (2014) 669–681, https://doi.org/10.1089/ars.2013.5499.
[88] S.S. Singhal, S.P. Singh, P. Singhal, D. Horne, J. Singhal, S. Awasthi, Antioxidant role of glutathione S-transferases: 4-Hydroxynonenal, a key molecule in stress-mediated signaling, Toxicol. Appl. Pharmacol. 289 (2015) 361–370, https://doi.org/10.1016/j.taap.2015.10.006.
[89] J.A. Arevalo, J.P. Vázquez-Medina, The role of peroxiredoxin 6 in cell signaling, Antioxidants 7 (2018) 172, https://doi.org/10.3390/antiox7120172.
[90] J.O. Onukwufor, B.J. Berry, A.P. Wojtovich, Physiologic implications of reactive oxygen species production by mitochondrial complex I reverse electron transport, Antioxidants 8 (2019) 285, https://doi.org/10.3390/antiox8080285.
[91] L. Meziani, M. Gerbé de Thoré, P. Hamon, S. Bockel, R. Andrade Louzada, C. Clemenson, R. Corre, W. Liu, C. Dupuy, M. Mondini, E. Deutsch, Dual oxidase 1 limits the IFNγ-associated antitumor effect of macrophages, J. Immunother. Cancer. 8 (2020) 622, https://doi.org/10.1136/jitc-2020-000622.
[92] X. Cheng, J.C. Smith, Biological membrane organization and cellular signaling, Chem. Rev. 119 (2019) 5849–5880, https://doi.org/10.1021/acs.chemrev.8b00439.