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ORIGINAL PAPER

Protective Effects of the Synthetic Cannabinoids CP55,940 and JWH-015 on Rat Brain Mitochondria upon Paraquat Exposure

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Abstract The effects of cannabinoids in mitochondria after acute oxidative stress insult are not fully established. We investigated the ability of CP55,940 and JWH-015 to scavenge reactive oxygen species and their effect on mitochondria permeability transition (MPT) in either a mitochondria-free superoxide anion generation system, intact rat brain mitochondria or in sub-mitochondrial particles (SMP) treated with paraquat (PQ). Oxygen consumption, mitochondrial membrane potential ($\Delta\psi_m$) and MPT were determined as parameters of mitochondrial function. It is found that both cannabinoids effectively attenuate mitochondrial damage against PQ-induced oxidative stress by scavenging anion superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), maintaining $\Delta\psi_m$ and by avoiding Ca^{2+}-induced mitochondrial swelling. Understanding the mechanistic action of cannabinoids on mitochondria might provide new insights into more effective therapeutic approaches for oxidative stress related disorders.

Keywords CP55,940 · Cannabinoids · JWH-015 · Mitochondria · Oxidative stress · Paraquat

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Introduction

Cannabinoids are a group of structurally heterogeneous chemical compounds that mainly include the classical cannabinoids related to tetrahydrocannabinol (THC), the non-classical cannabinoids (e.g. CP55,940), the aminoalkylindoles (e.g. JWH-015), and the eicosanoids related to the endocannabinoids [1–3]. Cannabinoids bind generally to two G protein-coupled receptors, CB_1 and CB_2 receptors [4]. These receptors share a 44% identity (68% within the transmembrane domains) and are typically $G\alpha_{i/o}$ protein-coupled receptors widely expressed in the central nervous system, especially in basal ganglia [5] and peripheral tissues. In the last few years, cannabinoids have been extensively studied as potential neuroprotective agents in different neurodegenerative pathologies including Parkinson's disease (PD) [6, 7]. The idea behind this potential neuroprotection is based on the antioxidant, immunomodulatory, anti-inflammatory, and anti-excitotoxic properties. However, the molecular mechanisms of cannabinoid's effects on cells are complex and still a controversial issue. Several data suggest that cannabinoids protect cells against stressful conditions in receptor-independent [8–11], receptor-dependent [12, 13] or both mechanisms [14, 15] in in vitro and in vivo experimental settings. We have recently demonstrated that the CP55,940 a non-selective CB_1/CB_2 cannabinoid receptor agonist, was able to prolong survival and improve locomotor activity in *Drosophila melanogaster* against paraquat (a mitochondrial complex I redox cycling compound) in a receptor-independent mechanism [16]. Moreover, CP55,940 and JWH-015 cannabinoids have also shown to protect lymphocytes against paraquat-induced apoptosis- a type of cell death- by blocking reactive oxygen species (ROS) [17]. These observations suggest that some cannabinoids are

endowed with antioxidant properties. Therefore, we hypothesize that the protective action of cannabinoids against PQ-induced oxidative stress may take place on the mitochondria.

It is known that brain cortex mitochondria (as many other mitochondrial types) are able to buffer transient changes in intracellular calcium (Ca^{2+}), presenting high capacity to accumulate this cation under normal physiological conditions. But in the presence of a calcium overload, their ability to retain calcium is markedly diminished triggering a sudden increase in the permeability of the inner mitochondrial membrane to solutes with a molecular mass of up to 1,500 kDa and protons, a phenomenon known as the mitochondrial permeability transition (MPT, for a review see ref [18]). The MPT thus provokes the collapse of the mitochondrial transmembrane potential ($\Delta\psi_m$) and swelling of the mitochondrial matrix. This mitochondrial process is one of the central molecular mechanisms involved in cell death process [19]. On the other hand, ROS derived molecules are normally produced by mitochondria during the electron transport in the respiratory chain and they can play physiological roles in intracellular signalling. However, when the production of these molecules is higher or the scavenging processes against them fail, they can be potentially harmful inducing oxidative damage to proteins, nucleic acids, lipids and polysaccharides and MPT. In this context, it is important to search for new and effective compounds with antioxidant abilities that could be used as therapeutic strategies for oxidative stress related disorders [20].

To further characterize the effects of cannabinoids, we evaluated the effect of the synthetic cannabinoids CP55,940 and JWH-015 in isolated rat cortical mitochondria exposed to paraquat. Oxygen consumption, mitochondrial membrane potential and MPT were determined as parameters of mitochondrial function. Additionally, we assessed the ability of cannabinoids to scavenge anion superoxide radicals and hydrogen peroxide in mitochondria-free superoxide anion generation system and in mitochondria exposed to PQ. Understanding the mechanistic action of cannabinoids on mitochondria may provide insights into more effective therapeutic approaches against oxidative stress stimuli.

Materials and Methods

Reagents

The cannabinoid CP55,940 (cat #C1112) and JWH-015 (cat#J4252) and other reagents, if not otherwise specified, were purchased from Sigma–Aldrich.

Isolation of Rat Cortical Mitochondrial Fraction

Brains were extracted and the cortical region was dissected, suspended in MSTE (0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA and 10 mM Tris–HCl, pH 7.2), homogenized 1:5 (w/v) in the presence of complete protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.4 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin) and centrifuged at 600 g and 8,000 g for 10 min. The resulting pellet was washed and resuspended in the same buffer, containing both synaptic and non-synaptic mitochondria, corresponding to cortical mitochondria from neurons and glia. Protein content was assayed by using Folin phenol reagent and bovine serum albumin was used as standard. All isolation procedures were performed at 0–4°C [21].

Preparation of Sub-Mitochondrial Particles

Mitochondria from cortical regions (2 young rats, 3 months) were pooled for the preparation of sub-mitochondrial particles (SMP) by a modification of the method described in ref. [22]. Given that these inside out SMP lack the matrix enzymes capable of artificially induced tight coupling, this procedure of SMP purification warrants an optimal experimental system for studying superoxide generation (see section Determination of Superoxide Anion production). Therefore, fresh mitochondrial fraction (0.5 ml) was diluted with 1 ml buffer 140 mM KCl, 20 mM Tris pH 7.4, and sonicated for 20 s in ice water bath 4°C at 55% of maximal output in a Branson sonifier, followed by 1.5 min interval. After 10 cycles the suspension was centrifugate at 10,000 rpm for 10 min. The pellet was resuspended in the same buffer and centrifuged again at 10,000 rpm for 10 min. Finally, the pellet was resuspended in 0.5 ml of the same buffer. The protein concentration of this fraction was 4-9 mg protein/ml.

Determination of Superoxide Anion Production

Superoxide production measurements were carried out in brain SMP using a spectrophotometric assay based on superoxide-dependent oxidation of adrenaline to adrenochrome at 37°C ($\varepsilon_{480\ nm} = 4.0\ mM^{-1}\ cm^{-1}$). The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), SMP (0.25 mg protein/ml), 0.1 μM catalase and 1 mM epinephrine. NADH (50 μM) was used as substrate. In order to generate a detectable level of superoxide anion, SMP were incubated in the presence or absence of different paraquat (PQ) concentrations. Superoxide dismutase (SOD) was used at 0.1–0.3 μM final concentration to give assay specificity [22]. The cannabinoid effect was studied by preincubation of SMP with 1 μM CP55,940 and JWH-015 for 2 min before PQ

addition. The superoxide anion production was also determined in the presence of PQ and cannabinoids alone. The PQ and cannabinoids solutions were performed in DMSO.

Detection of H_2O_2 Production

H_2O_2 generation was determined in intact brain cortex mitochondria by the scopoletin-HRP method, following the decrease in fluorescence intensity at 365-450 nm (λexc-λem) at 37°C [22]. The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), 0.8 μM HRP, 1 μM scopoletin, 6 mM malate and glutamate and 0.3 μM SOD. Calibration was made using H_2O_2 (0.05–0.35 μM) as standard to express the fluorescence changes as nmol H_2O_2/min mg protein [23]. Cannabinoid effect on hydrogen peroxide production was evaluated by fresh mitochondria preincubation with 1 μM CP55,940 and JWH-015 for 2 min in the presence or absence of 250 μM PQ.

Mitochondrial Respiration

Oxygen consumption of the total isolated cortical mitochondria was measured with a high-resolution respirometer (Oroboros Oxygraph, Paar KG, Graz, Austria). Mitochondrial protein (0.5 –1 mg/ml) was incubated in a reaction medium consisting of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl, 5 mM malate plus glutamate, 5 mM KH_2PO_4, 4 mM $MgCl_2$ (pH 7.4), and 0.2% bovine serum albumin, at 30°C. Cannabinoid effect on respiratory rate in state 3 and 4 was evaluated by mitochondria pre-incubation with 1 μM. The mitochondrial fraction obtained from the brain cortex tissue showed a respiratory control ratio (RCR) of 5.7 ± 0.1 ($n = 4$) in presence of malate plus glutamate as substrates [24, 25], indicating that mitochondrial isolation procedure did not affect mitochondrial physiology. CP55,940 or with JWH-015 for 2 min before PQ addition.

Determination of Mitochondrial Membrane Potential

For estimation of the mitochondrial membrane potential, isolated rat brain cortex mitochondria (0.025 mg/ml) were incubated at 37°C for 20 min in MSH buffer supplemented with 5 mM malate, 5 mM glutamate, and 1 mM phosphate (State 3 respiratory rate) in the presence of 30 nM of the potentiometric probe $DiOC_6$ (3) [26, 27]. The changes in fluorescence were determined by cytometry measurement of FL-1 green fluorescence for $DiOC_6$(3). Samples were protected from light until placed in the cytometer. Fresh mitochondria were prepared for each experiment and were used within 4 h after isolation. Auto-florescence of the mitochondrial preparation, without probe, was measured, and the protonophore and depolarizing agent (0.5 μM) FCCP was used as a positive control. A common marker, indicating the relative fluorescence intensity of the mitochondrial population analyzed, was used to quantify the resulting changes in membrane potential in each experiment. The $\Delta\psi_m$ was determined on fresh mitochondrial samples with or without 250 μM PQ. Cannabinoid effect on $\Delta\psi_m$ was evaluated by mitochondria pre-incubation with 1 μM CP55,940 and JWH-015 for 2 min before PQ addition and followed by the probe loading.

Scavenging Activity of Cannabinoids Against Superoxide Anion Generation in a Mitochondria-Free System

The superoxide-dependent adrenaline auto-oxidation to adrenochrome system was used to assess the in vitro reactivity of the cannabinoid against superoxide anion. For the assay, different concentrations of JWH-015 and CP55,940 (0.25–1 μM) were incubated in 50 mM Glycine buffer, pH 10.2, 5 min before of 0.16 μM adrenaline addition and performed as previously described [28]. Kinetics of the superoxide dependent adrenaline auto-oxidation to adrenochrome was followed by a spectrophotometer at 480 nm at 25°C. The results were expressed as nmol of adrenochrome/min ml ($\varepsilon = 4\ mM^{-1}\ cm^{-1}$). 5 Units CuZn SOD (0.3 μM) was used to give assay specificity.

Analysis of Swelling in Isolated Brain Cortex Mitochondria

Brain cortex mitochondrial swelling was monitored as a decrease in absorbance at 540 nm ($\Delta A_{540\ nm}$/min.mg protein), after a single bolus of 100 μM Ca^{2+} (100 μM $CaCl_2$ equivalents to 1.0 μmol Ca^{2+}/mg mitochondrial protein). Rat brain cortex mitochondria corresponding to 0.1 mg protein were suspended in MSH buffer supplemented with 5 mM malate, 5 mM glutamate, 1 mM phosphate and 2 mM $MgCl_2$, pH 7.4, at 30°C. In these conditions mitochondria swell, their refractive index changes and they scatter less light [29–31]. Cyclosporine A (CsA) at 1 μM added 3 min before Ca^{2+} addition was used as an inhibitor of mitochondrial permeability transition pore. Several laboratories have found that loading mitochondria with 1.0 μmol Ca^{2+}/mg protein is able to induce maximal MPT in liver mitochondria [32]. Incubation of brain cortex mitochondria with the same Ca^{2+} amount/mg protein induces only partial swelling that can be maximal after adding the 20-residue channel-forming peptide alamethicin. Thus, for unspecific permeabilization, 20 μg/ml alamethicin was used [33–35].

Quantification of mitochondrial swelling was performed with the data from three different experiments.

Statistics

Values in figures are mean values ± SEM. At least four independent experiments for each experimental condition were performed. Results were compared using an unpaired Student *t*-test.

Results

Cannabinoids Suppress Superoxide Anion Radical in a Free-Mitochondria Superoxide Radical Generation System

To demonstrate specific scavenger activity of cannabinoids against superoxide radicals, a mitochondria-free superoxide radical generation system was performed. As depicted in Fig. 1, both cannabinoids showed a dose dependent inhibitory effect against superoxide anion, yet with different potencies. Although the amount of adrenochrome production in the presence of either 0.25 μM JWH-015 or CP55,940 was similar to the system without cannabinoids (22 nmol/ml min adrenochrome), indicating no ability of cannabinoids to scavenge superoxide anion at this concentration, at 0.5 and 1 μM JWH-015 presented 17 and 42% less adrenochrome production, respectively, and 0.5 and 1 μM CP55,940 adrenochrome production decreased 30.2 and 35%, respectively, being significantly different as compared with the system alone.

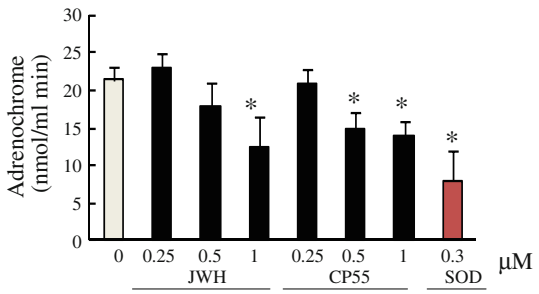

Fig. 1 Scavenging activity of the cannabinoids CP55,940 and JWH-015 against mitochondria-free superoxide anion generate system. Glycine buffer (50 mM, pH 10.2) was pre-incubated for 5 min with JWH-015 and CP55,940 (0.25–1 μM, *black bars*) or without cannabinoids (negative control, *white bar*) or with 5 Units CuZn SOD (0.3 μM) alone, used to give assay specificity (positive control, *red bar*). After this time, 0.16 μM adrenaline was added. Kinetics of the superoxide dependent adrenaline auto-oxidation to adrenochrome was followed by a spectrophotometer at 480 nm at 25°C. The results were expressed as nmol of adrenochrome/min ml ($\varepsilon = 4$ mM^{-1} cm^{-1}). *Bars* represent the slopes (nmol adrenochrome/ml min) obtained from kinetic studies of superoxide-dependent adrenaline auto-oxidation to adrenochrome. Data are mean ± SEM performed in triplicate ($n = 3$). *Statistically different from the adrenaline auto-oxidation alone. ($P < 0.001$). For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article

Cannabinoids Suppress Superoxide Anion Radical Detection on Submitochondrial Particles (SMP) from Rat Brain Cortex Exposed to Paraquat (PQ)

Since mitochondria are an important source of reactive oxygen species within most mammalian cells, we assessed the basal amount of superoxide anion radical ($O_2^{\bullet -}$) generation by brain SMP. Endogenous superoxide anion radical generation increased linearly up to 16 ± 2 (nmol $O_2^{\bullet -}$)/min with increasing concentration of SMP in the range of 0.025-0.1 mg protein. As expected, pre-incubation of mitochondrial particles with 0.1–0.3 μM SOD diminished markedly mitochondrial $O_2^{\bullet -}$ production (data not shown), thus a final concentration of 0.025 mg protein SMP was used in these experiments.

In order to assess the effect of cannabinoids on $O_2^{\bullet -}$ mitochondrial production under PQ exposition, aliquots of brain SMP were initially exposed to increasing PQ concentrations, up to 1,500 μM. As shown in Fig. 2a, the production of ($O_2^{\bullet -}$) induced by paraquat rose in a concentration dependent fashion up to a maximum of 16 ± 1 nmol ($O_2^{\bullet -}$)/min mg protein. Since 250 μM PQ is the minimal concentration at which almost 50% anion superoxide radicals were generated and provoked a clear effect on mitochondrial depolarization (see below and Fig. 4), this concentration was used for further experiments. Aliquots of SMP were then pre-incubated with 1 μM CP55,940 [36] and JWH-015 for 2 min before exposure to 250 μM PQ. As shown in Fig. 2b, the amount of $O_2^{\bullet -}$ detected was markedly decreased with both cannabinoids compared with those levels obtained with PQ alone. Interestingly, JWH-015 was three times more effective than CP55,940 ($P < 0.01$). In fact, JWH-015 was able to reduce about 50% the basal level of $O_2^{\bullet -}$ detected.

Cannabinoids Scavenge H_2O_2 on Intact Mitochondria upon PQ Treatment

H_2O_2 is the product of the $O_2^{\bullet -}$ dismutation catalyzed by either SOD enzymatic-or non-enzymatic reactions. Thus, to evaluate whether H_2O_2 produced by PQ was removed by cannabinoids, intact brain cortex mitochondria were incubated with 250 μM PQ in the absence or presence of 1 μM agonists. As shown in Fig. 3, while H_2O_2 generation increased almost threefold upon PQ treatment ($P < 0.01$) compared to untreated mitochondria, CP55,940 and JWH-015 significantly reduced mitochondrial H_2O_2 production by 44 and 50%, respectively, when co-incubated with PQ.

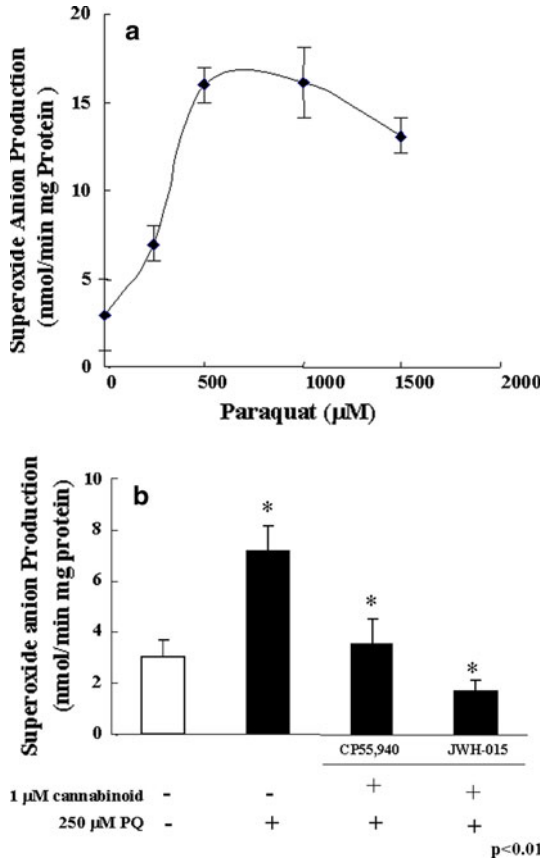

Fig. 2 Effect of cannabinoids on superoxide anion production ($O_2^{\bullet -}$) in rat brain mitochondria upon PQ exposure. **a** Rat brain submitochondria particles (SMP) were untreated or treated with increasing concentrations of PQ and detection of ($O_2^{\bullet -}$) was performed as described in *Materials and Methods.* **b** Effect of (1 μM) cannabinoids on ($O_2^{\bullet -}$) production in rat brain SMP under paraquat exposition. Values represent the mean ± SEM of 4 different mitochondrial samples. * $P < 0.01$ as compared with control value

Strikingly, no H_2O_2 was detectable when mitochondria were incubated with CP55,940 and JWH-015 alone.

CP55,940 and JWH-015 do not Restore Mitochondrial Respiratory Function

Brain cortex mitochondrial respiratory activity was measured in state 4 (resting state) and in state 3 (maximal respiration rate), in basal conditions and after incubation with 250 μM PQ. The effect of pre-incubation of brain cortex mitochondria with cannabinoids CP55,940 and JWH-015 before PQ addition was also evaluated. As shown in Table 1, incubation of brain cortex mitochondria with PQ increased mitochondrial state 4 respiratory rate by 94%, as compared with untreated mitochondria. Incubation of mitochondria with 1 μM CP55,940 or JWH-015 alone did not change mitochondrial state 4 respiratory rate. Pre-incubation with these two cannabinoids before PQ addition only slightly decreased state 4 respiratory rate, although

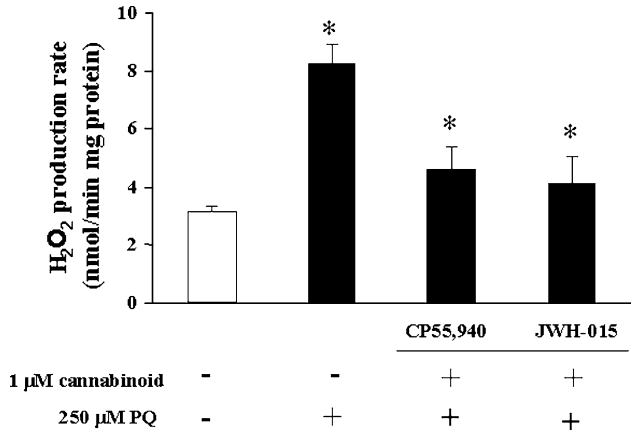

Fig. 3 Effect of cannabinoids on hydrogen peroxide (H_2O_2) generation in rat brain mitochondria upon PQ exposure. Intact rat brain mitochondria were untreated or treated with 250 μM PQ in the absence or presence of 1 μM cannabinoids and detection of H_2O_2 was performed as described in *Materials and Methods.* Notice that no H_2O_2 was detected when SMP were incubated with agonists alone. Values represent the mean ± SEM of 4 different mitochondrial samples. * $P < 0.01$ as compared with control value

Table 1 Effect of CP55,940 and JWH-015 on brain cortex mitochondrial respiration upon paraquat (PQ) treatment

Condition	Oxygen consumption (ng-at O/min.mg prot)
Control (state 4)	12.9 ± 0.9
+ ADP (state 3)	74.1 ± 0.1
PQ (state 4)	25 ± 2*
+ ADP (state 3)	62 ± 2
CP55,940 (state 4)	12 ± 2
+ ADP (state 3)	57 ± 7
CP55,940 + PQ (state 4)	20 ± 2
+ ADP (state 3)	51 ± 2
JWH-015 (state 4)	11 ± 1
+ ADP (state 3)	49 ± 7
JWH-015 + PQ (state 4)	19 ± 2
+ ADP (state 3)	53 ± 7

Brain mitochondria were untreated or treated with 250 μM PQ in the presence or absence of (1 μM) cannabinoids as described in *Materials and Methods*. Values represent the mean ± SEM of 4 different mitochondrial samples

* $P < 0.05$ as compared with control value

differences were not significant. No significant changes were observed in state 3 respiratory rate or in any of the assayed conditions.

CP55,940 and JWH-015 Prevent PQ-Induced Mitochondrial Membrane Depolarization

We further evaluated the agonist's effect on $\Delta\psi_m$ upon PQ treatment. As illustrated in Fig. 4a, mitochondria treated

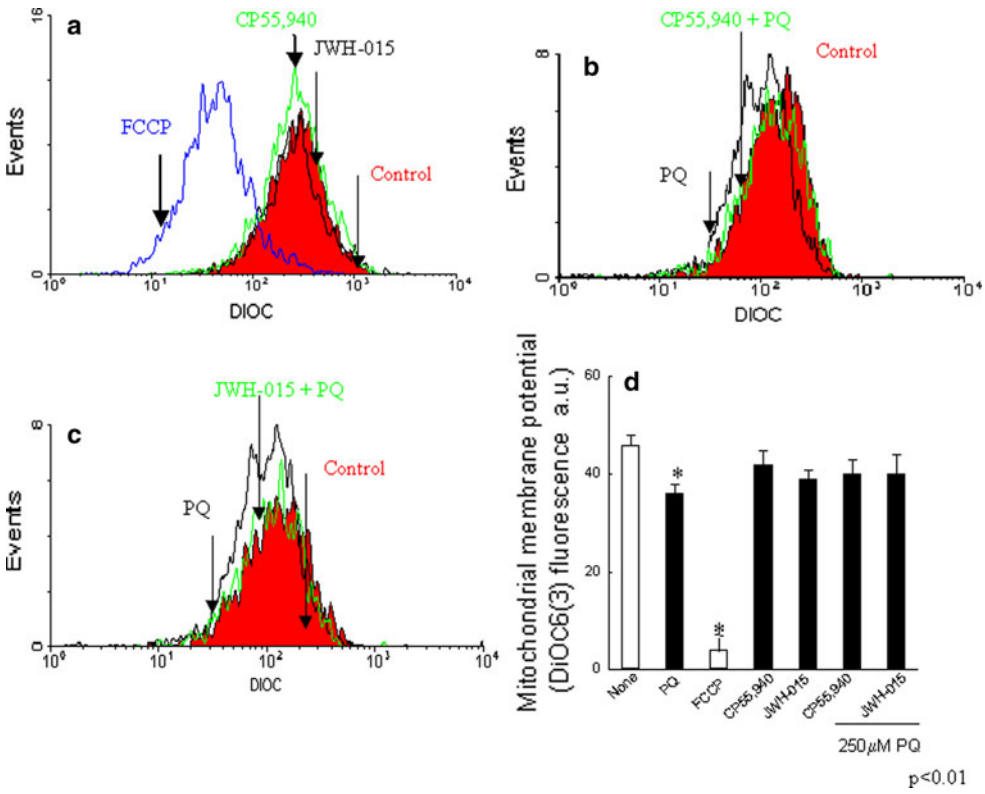

Fig. 4 Effect of cannabinoids on mitochondrial transmembrane potential ($\Delta\Psi_m$) upon PQ exposure. Intact rat brain mitochondria in State 3 were **a** untreated (control) or treated with (1 μM) cannabinoids alone or **b**, **c** with cannabinoids and 250 μM PQ. FCCP, a protonophore, was used as positive mitochondria depolarizing agent. Assessment of $\Delta\Psi_m$ was performed as described in *Materials and Methods*. **d** *Bars* scheme of the quantification of $D_iOC_6(3)$ fluorescence (a.u.) of the different treatment (mean values ± SEM); (* $P < 0.01$). Each histogram represents a typical experiment out of three

with 1 μM agonist alone maintain a basal $\Delta\psi_m$ similar to that observed in intact untreated mitochondria. A dramatic decrease in $\Delta\psi_m$ was detected in mitochondria upon FCCP treatment (used as positive control for comparative purposes). However, when mitochondria were incubated with 250 μM PQ, a moderate loss of basal $\Delta\psi_m$ by about 22% was observed compared to untreated mitochondria (Fig. 4b–c). Interestingly, both CP55,940 (Fig. 4b) and JWH-015 (Fig. 4c) were able to return ($\Delta\psi_m$) to basal state levels when co-incubated with PQ. Figure 4d shows quantification of D_iOC_6 (3) fluorescence (a. u.) in the different conditions.

CP55,940 and JWH-015 Inhibit Mitochondrial Permeability Transition Pore (MPT) Under Ca^{2+} Exposure

Since increased Ca^{2+} concentrations leads to mitochondrial swelling and loss of mitochondrial membrane potential, we examined whether the cannabinoid CP55,940 and JWH-015 could prevent this mitochondrial phenomena. As depicted in Fig. 5, while (1 μM) cannabinoids alone showed no effect on MPT, addition of (100 μM) Ca^{2+} induced a rapid swelling of mitochondria, as evaluated by light scattering decrease (absorbance) at 540 nm. Remarkably, both CP55,940 and JWH-015 co-incubated with Ca^{2+} were significantly able to block mitochondrial swelling or MPT. In fact, CP55,940 (Fig. 5a) and JWH-015

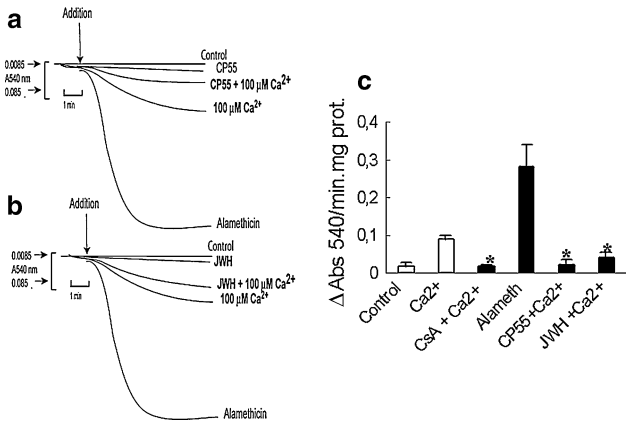

Fig. 5 Effect of CP55,940 and JWH-015 on mitochondrial swelling. Brain cortex mitochondrial swelling was analyzed by the decrease in absorbance at 540-nm/min.mg protein. 25 μg of mitochondrial protein suspended in 1 ml of MSH buffer, supplemented with 5 mM malate, 5 mM glutamate, 1 mM phosphate and 1 mM $MgCl_2$, was exposed to single addition of 100 μM Ca^{2+} and 20 μg/ml of alamethicin. Incubation with (1 μM) CP55,940 (**a**)/JWH-015 (**b**) alone or co-incubated with 100 μM Ca^{2+} are described. *Bar* graphs describe mitochondrial swelling quantification (**c**). Data correspond to mean values ± SEM of three different experiments of mitochondrial swelling. *Statistically different from corresponding swelling calcium values ($P < 0.05$)

(Fig. 5b) showed 76 and 56% protection against calcium overload-induced MPT (percentages were calculated using a maximal mitochondrial swelling of 100% when incubated with Ca^{2+}). Of note, quantification of mitochondrial

swelling showed that both cannabinoids were significantly able to diminish the Ca^{2+}-induced mitochondrial swelling effect to a similar extent as CsA (Fig. 5c). Since alamethicin, a peptide antibiotic that can act as a monovalent cation ionophore, induces unspecific mitochondrial permeabilization, this molecular event was included for illustrative purposes (Fig. 5a–c).

Discussion

In the present work, we report for the first time that cannabinoid agonists CP55,940 and JWH-015 not only attenuate mitochondrial damage against paraquat-induced oxidative stress by scavenging superoxide anion radical and hydrogen peroxide, but also block Ca^{2+}-induced mitochondrial permeability transition, thereby maintaining mitochondrial membrane potential. Specifically, cannabinoids were able to suppress superoxide anion radical detection in a mitochondria-free superoxide radical generation system, as evaluated by superoxide-dependent adrenaline auto-oxidation to adrenochrome assay and on SMP from rat brain cortex exposed to 250 μM PQ, as demonstrated by a spectrophotometric assay. Although the interaction between cannabinoids and $O_2^{\bullet -}$ appears clearly to occur in both systems, the chemical reactions involved are not known at the moment. Interestingly, JWH-015 co-incubated with PQ reduced to roughly one-fourth $O_2^{\bullet -}$ production when compared to PQ alone. Moreover, JWH-015 was two times more effective in removing $O_2^{\bullet -}$ than CP55,940. These features may rely on difference in the chemical structure of the cannabinoids, i.e., while CP55,940 (a synthetic phenolic non-classical cannabinoid) contains three hydroxyl groups, JWH (a synthetic aminoalkylindol cannabinoid) possess no phenolic groups (Fig. 6). In contrast to the accepted concept that a phenolic lead structure is required for antioxidant activity in cannabinoid compounds [10], our results support the notion that some non-phenolic cannabinoids (e.g. JWH-015) may exert similar or better chemical antioxidant activity than those compounds with phenolic structure. These observations suggest that structural modification of the prototype JWH-015 and structure–activity relationship studies might uncover compounds with enhanced antioxidant activity and biological effectiveness [3]. Taken together, our findings suggest that cannabinoids directly act as a very efficient $O_2^{\bullet -}$ sink which not only prevent H_2O_2 generation but also might prevent $O_2^{\bullet -}$ interacting with other intra-mitochondrial molecules (e.g.

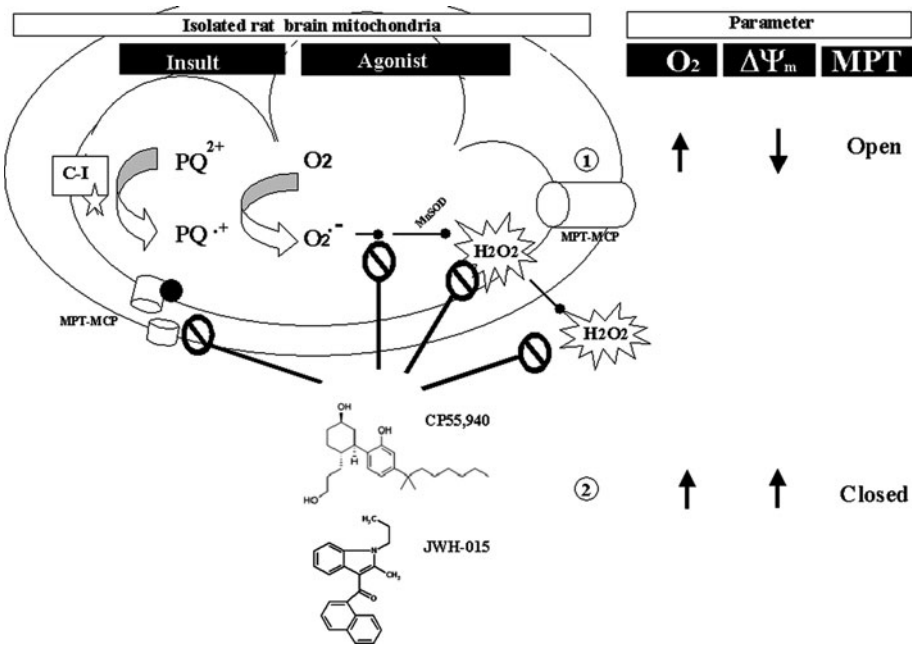

Fig. 6 Scheme of proposed cannabinoid mechanism of action against Paraquat-induced mitochondrial oxidative stress. High mitochondrial membrane potential ($\Delta\Psi_m$) in intact rat brain mitochondria drives PQ compound into the mitochondrial matrix. Once inside, (1) PQ is reduced to the monocation radical $PQ^{+\cdot}$ at complex I in the respiratory chain by electrons donated from NADH. $PQ^{+\cdot}$reacts rapidly with O_2 to produce superoxide ($O_2^{\bullet -}$) [37], thereby consuming high amount of oxygen. In turn, the ($O_2^{\bullet -}$) is enzymatically dismutated by MnSOD into H_2O_2 [38, 39]. Then, H_2O_2 induces mitochondrial permeability transition pore (MPT) and decreases $\Delta\Psi_m$. Interestingly, when cannabinoids are present (2), they can remove both $O_2^{\bullet -}$ and H_2O_2 thereby blocking further ROS signaling [17]. Most interestingly, cannabinoids inhibit MPT probably through interactions with the cyclosporine A-binding cyclophilin-D protein (*black circle*). As a result, cannabinoids maintain the MPT- multiprotein complex (MPC) in a close-stated, high ($\Delta\Psi_m$) but O_2 comsumption is still high. Taken in conjunction these actions, cannabinoids thus protect mitochondria from further damage

MnSOD, aconitase, lipids, nitric oxide radicals) critical for normal mitochondrial functioning. Moreover, on the understanding that neither CB1 nor CB2 receptors are expressed in sub-mitochondrial particles [4], our data suggest that the antioxidant effect of the cannabinoid agonist against PQ-induced ROS generation is mediated through receptor-independent mechanism.

It has been described that paraquat interacts with complex I to generate $O_2^{\bullet -}$ [37], which in turn is converted into H_2O_2 within mitochondria [38, 39]. In this work, we demonstrated that CP55,940 and JWH-015 were equally able to neutralize PQ-induced H_2O_2 generation according to the scopoletin-HRP assay in intact rat brain cortex mitochondria. These data reinforce the notion that cannabinoids are specific antioxidant molecules which might be useful tools to prevent against ROS damage, in specific pathological conditions. The results of the current investigation therefore demonstrate the capacity of cannabinoids as potential ROS-scavenging molecules, thus increasing cell survival and strongly extending the concept that cannabinoids may display antioxidant action in vitro and in vivo independent of receptor cannabinoid signalling.

It is well known that many of the toxic actions of paraquat are mediated by redox-cycling reactions forming superoxide anion, catalyzed by different NADH dependent reductases [40]. Redox cycling reactions are known to consume significant amounts of oxygen, reducing the levels of oxygen available for cellular metabolic processes. Studies by Gray et al. [41] have clearly demonstrated that micromolar concentrations of paraquat (100 μM) increase cellular oxygen consumption. This activity was not inhibited by cyanide or antimycin, indicating that the actions of paraquat were not dependent on mitochondrial respiration. In the present study, the 94% increase in state 4 respiration by paraquat treated mitochondria is consistent with the fact that the redox cycling of paraquat is consuming a great amount of oxygen. Moreover, ADP dependent respiration was not modified by paraquat treatment, as expected. Therefore, the lack of paraquat effect on state 3 respiration can be explained by the high rate of oxygen uptake by the respiratory chain, with which paraquat cannot compete. Similarly, the lack of cannabinoid ability to restore state 4 respiration on PQ-treated mitochondria can be explained by the fact that these cannabinoids did not interfere with the oxygen uptake by PQ enzymatic redox-cycling reaction.

Previous observations showing that CP55,940/JWH-015 were able to maintain mitochondrial physiology against PQ-induced damage in lymphocytes [17] prompted us to evaluate the effect of CP55,940 and JWH-015 on $\Delta\psi_m$ in isolated rat brain mitochondria incubated with PQ. Effectively, our results confirm that both cannabinoids are capable of restoring the PQ-induced mitochondrial depolarization to basal values compared to PQ alone. In contrast to other investigators who showed that anandamide, THC, and HU-210 (a synthetic classical cannabinoid) cause significant decreases in oxygen consumption and $\Delta\Psi_m$ in rat heart mitochondria and in pulmonary transformed cell line A549 [42, 43], our data suggest that some other cannabinoids such as cannabidiol [44], JWH-015 and CP55,940 might positively target mitochondria against oxidative stress. It is concluded that the observed cannabinoid effect is based on its ability to scavenge superoxide anion and hydrogen peroxide, protecting against ROS-induced $\Delta\Psi m$ depolarization and MPT. Taken together our information suggests that CP55,940 and JWH-015 act up-stream from $\Delta\psi_m$ and independently of mitochondrial respiration.

It is known that PQ induces a Ca^{2+}-dependent permeability increase of the inner mitochondrial membrane leading to membrane depolarization, uncoupling and matrix swelling [45]. We found that both CP55,940 and JWH-015 were able to inhibit the Ca^{2+}-induced MPT. Moreover, both cannabinoids were able to inhibit MPT in a similar fashion as cyclosporine A, a drug that desensitizes the MPT by binding to cyclophilin (CyP)-D. Although the MPT is mediated by a multi-protein complex involving a voltage-dependent, cyclosporine A-sensitive and calcium activated inner membrane channel, according to our present observations, it is tempting to speculate that CP55,940 and JWH-015 might mediate MPT through binding to the CsA-target protein, (CyP)-D. However, further investigation is needed to specifically determine whether those cannabinoids interact directly with (CyP)-D or whether they interact with other protein(s) member(s) of the multi-protein complex.

In summary, the present data demonstrated that CP55,940 and JWH-015 can protect mitochondrial dysfunction against PQ-induced oxidative stress by efficiently scavenging $O_2^{\bullet -}$ and H_2O_2. They also showed the ability of these cannabinoids to prevent Ca^{2+}-induced swelling (Fig. 6). Therefore, synthetic cannabinoids represent highly promising compounds that might aid in the development of mitochondrial therapy for oxidative stress induced neurodegeneration and understanding the mechanistic action of cannabinoids on mitochondria may provide insights into more effective therapeutic approaches.

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