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Cannabinoid-Induced Changes in the Activity of Electron Transport Chain Complexes of Brain Mitochondria

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Abstract The aim of this study was to investigate changes in the activity of individual mitochondrial respiratory chain complexes (I, II/III, IV) and citrate synthase induced by pharmacologically different cannabinoids. In vitro effects of selected cannabinoids on mitochondrial enzymes were measured in crude mitochondrial fraction isolated from pig brain. Both cannabinoid receptor agonists, Δ^9-tetrahydrocannabinol, anandamide, and R-(+)-WIN55,212-2, and antagonist/inverse agonists of cannabinoid receptors, AM251, and cannabidiol were examined in pig brain mitochondria. Different effects of these cannabinoids on mitochondrial respiratory chain complexes and citrate synthase were found. Citrate synthase activity was decreased only by Δ^9-tetrahydrocannabinol and AM251. Significant increase in the complex I activity was induced by anandamide. At micromolar concentration, all the tested cannabinoids inhibited the activity of electron transport chain complexes II/III and IV. Stimulatory effect of anandamide on activity of complex I may participate on distinct physiological effects of endocannabinoids compared to phytocannabinoids or synthetic cannabinoids. Common inhibitory effect of cannabinoids on activity of complex II/III and IV confirmed a non-receptor-mediated mechanism of cannabinoid action on individual components of system of oxidative phosphorylation.

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Introduction

Cannabinoids can be categorized into (i) phytocannabinoids, e.g., Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD); (ii) endocannabinoids, e.g., anandamide (AEA) and 2-arachidonoylglycerol (2-AG); and (iii) synthetic cannabinoids, e.g., R-(+)-WIN55,212 (WIN) and HU-210. Synthetic cannabinoids include potent cannabinoid receptor agonists and antagonists/inverse agonists. E.g., AM251, analogue of rimonabant (SR141716A), is an example of antagonist/inverse agonist of the type 1 cannabinoid receptor (CB_1) (Gatley et al. 1997).

The biochemical targets of cannabinoids include both CB_1 and CB_2 receptors and downstream targets, which are regulated by chronic drug administration and receptor-independent effects. By retrograde signaling, endocannabinoids play a vital role in the modulation of synaptic plasticity in the CNS. Recently, it was confirmed that some effects of endocannabinoids on mitochondrial physiology are independent of their target cannabinoid receptors by reducing calcium sensitivity and perturbing membrane properties of these organelles (Catanzaro et al. 2009). CBD could prevent apoptotic signaling via restoration of Ca^{2+} homeostasis (Ryan et al. 2009). Further, CBD affected various functions of mitochondria in BV-2 microglial cells, leading to biphasic increase in intracellular calcium levels. CBD interaction with voltage-dependent anion-selective channel protein 1 (VDAC1) conductance may be responsible for anticancer and immunosuppressive properties, but it

cannot exclude the possibility that increased concentration of CBD in mitochondria could increase ROS formation and mitochondrial permeability (Rimmerman et al. 2013).

Numerous studies have suggested mitochondria as target of cannabinoids (Mahoney and Harris 1972; Bartova and Birmingham 1976; Sarafian et al. 2003, 2006; Stork and Renshaw 2005; Athanasiou et al. 2007). It was reported very early that THC is a highly effective inhibitor of complex I activity in rat brain and heart mitochondria in vitro (Bartova and Birmingham 1976). Recently, it was shown that the CB_1 receptors are present in outer mitochondrial membranes, where their activations led to decreased cyclic AMP concentration, protein kinase A activity, complex I activity, and mitochondrial respiration; i.e., activation of mitochondrial CB_1 receptors regulates energy metabolism (Benard et al. 2012). In vitro study regarding the effects of CB_1 receptor agonists (THC, AEA, HU210) on mitochondrial functions, decreased oxygen consumption, and biphasic changes in complex I and/or complex II/III activities in rat heart mitochondria have been described (Athanasiou et al. 2007). Another study exploring the reduced effects of cannabinoids on energy metabolism revealed a decline in ATP production and decrease in mitochondrial membrane potential in the pulmonary transformed cell line. This study presumed that the inhibitory effects of cannabinoids on energy metabolism might be caused by impaired function of NADH supply or inhibition of the citric acid cycle (Sarafian et al. 2003).

We have reported earlier inhibitory effects of cannabinoids on both activity of mitochondrial enzyme, monoamine oxidase (Fisar 2010), and mitochondrial respiratory rate (Fisar et al. 2014). In continuation of our previous studies, we investigate here the effects of cannabinoids on individual enzyme complexes and citrate synthase (CS) using both antagonists/inverse agonists of CB_1 receptors, AM251 and cannabidiol (CBD), and the cannabinoid receptor agonists, Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD), anandamide (AEA), and WIN 55,212-2 (WIN).

The hypothesis that some effects of cannabinoids can be related to cannabinoid-induced changes in activity of mitochondrial respiratory chain complexes has been tested.

Materials and Methods

Isolation of Brain Mitochondria

The crude mitochondrial fraction was isolated from pig brain cortex as described previously (Fisar et al. 2010). Briefly, brain cortex was separated without cerebellum, brain stem, and midbrain. The grey matter was homogenized in ice-cold-buffered sucrose (0.32 mol/l sucrose, 4 mmol/l HEPES; pH 7.4); it was centrifuged at 1000*g* for 10 min to remove unbroken cells, nuclei, and cell debris. The supernatant was carefully decanted; the pellet was resuspended in buffered sucrose and centrifuged again under the same conditions. Supernatants were collected and centrifuged at 10,000*g* for 15 min. The final pellet containing crude mitochondrial fraction was washed twice with buffered sucrose (10,000*g*, 15 min), resuspended to a protein concentration of 20–40 mg/ml, and stored at −70 °C until the assays. Protein concentration was determined by the method of Lowry (Lowry et al. 1951), with bovine serum albumin as the standard.

Measurement of Drug Effect on Enzyme Activity

Crude mitochondrial fraction was resuspended with hypotonic buffer (25 mmol/l potassium phosphate, 5 mmol/l $MgCl_2$, pH 7.2) and freeze-thawing and three times ultrasonicated to achieve the maximum of enzyme activities. Samples were incubated with selected cannabinoids for 30 min at 30 °C. Enzyme activity was determined at relatively high drug concentrations (50 μmol/l) for all the cannabinoids, upon prior measurement of the full inhibitory curve for the effect of THC on complex II/III activity. Samples were measured in 3 ml of total reaction volume at 30 °C; the final protein concentration was 150 μg/ml. Activities of respiratory chain complexes and CS were measured spectrophotometrically using Uvicon XL spectrophotometer (SECOMAM, Alès, France). All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) except for AEA and Tocrisolve from Tocris Bioscience (Tocris House, Bristol, UK). Enzyme assays used in our study are stated below.

Complex I (NADH Dehydrogenase (Ubiquinone), EC 1.6.5.3)

Previously published method was used (Folbergrova et al. 2010; Hroudova and Fisar 2010) with a slight modification. Complex I activity was measured as the rotenone-sensitive rate of NADH oxidation at 340 nm. The reaction medium was composed of 25 mmol/l potassium hydrogen phosphate (pH 7.2), 5 mmol/l $MgCl_2$, 2.5 mg/ml bovine serum albumin (BSA), 2 mmol/l KCN, and 0.3 mmol/l NADH. The reaction was started by the addition of coenzyme Q_1 (in final concentration 33 μmol/l) and measured for 10 min. Afterwards, rotenone was added in final concentration 50 μmol/l and the inhibited rate was measured for further 2 min.

Complex II/III (Succinate Cytochrome *c* Oxidoreductase, EC 1.8.3.1)

Mitochondrial complex II/III activity was measured spectrophotometrically as antimycin A sensitive rate of cytochrome *c* reduction at 550 nm using succinate as a substrate (Trounce

et al. 1996). Reaction mixture consisted of 50 mmol/l potassium hydrogen phosphate (pH 7.4), 0.625 mmol/l EDTA, 2 mol/l KCN, 0.03 mmol/l cytochrome *c*, 20 mmol/l succinate, 1 % BSA, and 2 μg/ml rotenone.

Complex IV (Cytochrome-*c* Oxidase, EC 1.9.3.1)

Cytochrome-*c* oxidase activity was measured as a decrease of absorbance during oxidation of reduced cytochrome *c* at 550 nm. The reaction mixture consisted of 20 mmol/l potassium hydrogen phosphate (pH 7.0); reaction was started with reduced cytochrome *c* (final concentration 25 μmol/l) and was monitored for 1 min (Rustin et al. 1994).

Citrate Synthase (CS, EC 2.3.3.1)

CS activity was measured as a color change of 5,5′-dithiobis-(2-nitrobenzoic) acid (DNTB). Incubation medium was composed of 100 mmol/l Tris/HCl (pH 8.1), 0.1 % Triton X-100, 0.2 mmol/l DTNB, and 0.3 mmol/l acetyl-CoA. The reaction was initiated by the addition of 0.5 mmol/l oxaloacetate and absorbance was measured at 412 nm for 3 min (Srere 1969).

Data Analysis and Statistics

Enzyme activities were evaluated as a slope of time dependence of absorbance of samples using LabPower Junior software (SECOMAM). Each independent measurement had a control, i.e., sample containing all components except for the drug. Relative changes of enzyme activities evoked by the drugs were determined assuming that the activity of the control sample is equal to 100 %. All data presented were expressed as the mean±standard deviation. Results were analyzed by STATISTICA (data analysis software system, version 12.0, StatSoft, Inc., Tulsa, OK, USA). The *t* test for single means was used to calculate test statistics in order to compare the enzyme activities in samples with and without the drug. Analysis of variance (ANOVA) and post hoc Scheffé test were used to analyze the cannabinoid-induced differences between several group means (CS, complex I, complex II/III, and complex IV).

Results

The effects of cannabinoids on activity of CS and electron transport chain (ETC) complexes were assessed and compared to control samples from pig brain mitochondria. We observed that the most affected complexes were complexes II/III and IV owing to the tested cannabinoids; CS activity was minimally changed (Fig. 1). Small but statistically significant decrease of CS activity was caused only by THC (p=0.0034) and AM251 (p=0.018), when compared to controls. Statistically significant decrease of complex I activity was found for CBD (p=0.0044); AEA induced increase of complex I activity (p=0.0031). Activity of complex II/III was strongly inhibited by AEA (p=0.00031), THC (p=0.00012), WIN (p=0.0020), and CBD (p=0.000074). Complex IV activity was significantly inhibited by all the tested cannabinoids, AEA (p=0.0075), THC (p=0.000009), CBD (p=0.024), AM251 (p=0.015), and WIN (p=0.0063).

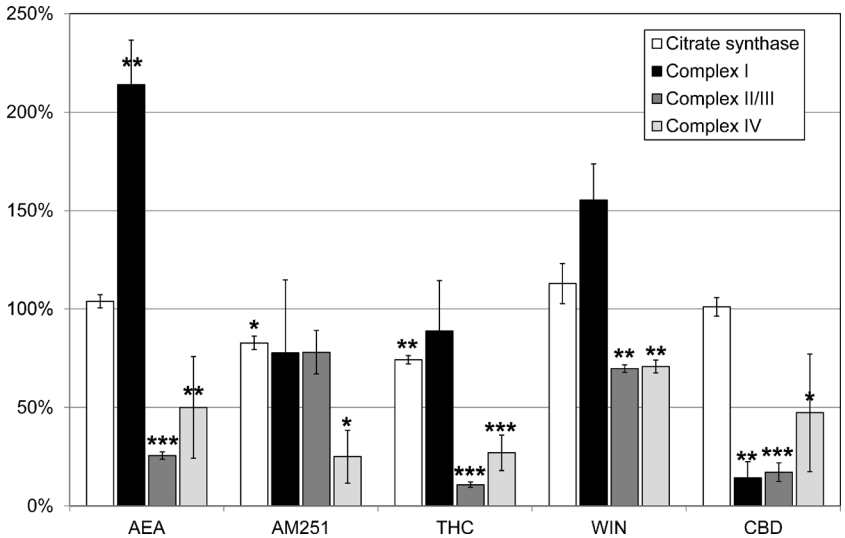

Fig. 1 Effects of cannabinoids on activities of citrate synthase and individual respiratory chain complexes I, II/III, and IV. The pig brain mitochondria were incubated with tested drugs for 30 min at 30 °C; relative cannabinoid-induced changes in enzyme activities were determined by comparison with activity of the control sample (sample without drug added). Values are means±standard deviation of three to six independent measurements; significantly different mean values are marked by an *asterisk* when compared to reference value 100 % of controls using *t* test for single means (*p<0.05; **p<0.01; ***p<0.001)

Dose-response curve of inhibition was performed for THC (Fig. 2), measured for the most effected complex II/III. IC_{50} was found 40.9±3.7 μmol/l, Hillslope 2.25±0.75. Thus, drug concentration 50 μmol/l was chosen for measurement of effects of THC and other cannabinoids on mitochondrial enzyme activity.

ANOVA was used to compare effects of several cannabinoids on mean activity of CS and ETC complexes I, II/III, and IV. Complex I activity was increased after incubation with AEA (with statistical significance) and WIN compared to decreased complex I activity after incubation with THC and CBD. Complex II/III activity after incubation with THC, CBD, and AEA was found significantly decreased compared to the effects of both WIN and AM251. The similar inhibitory effect of all tested cannabinoids was found on complex IV activity; i.e., we did not find any noteworthy difference between complex IV activity after incubation with AEA, THC, CBD, WIN, or AM251. Consequently, the analysis confirmed that only complex IV activity was inhibited equally by all the tested cannabinoids.

Discussion

We measured the effect of pharmacologically different cannabinoids on activity of citrate synthase and individual mitochondrial respiratory chain complexes. The relative high concentrations of THC needed for complex II/III inhibition (Fig. 2) indicate non-receptor mechanism of action of cannabinoids on activity of mitochondrial respiratory complexes. We suppose that changes in lipid-protein interaction and/or assembly of respiratory complexes may be affected by lipophilic cannabinoids. Higher doses of cannabinoids were necessary to see the respiration effects compared to recent studies

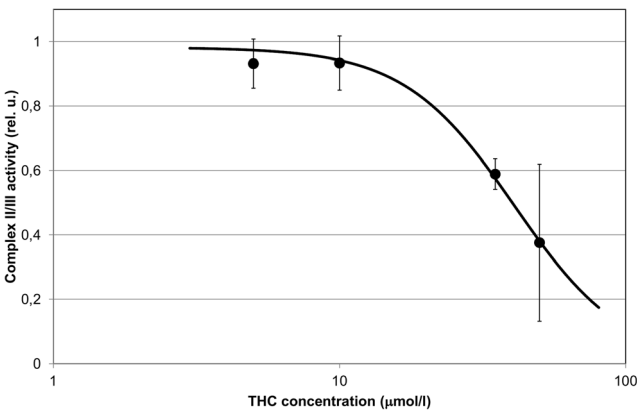

Fig. 2 Inhibition of basal complex II/III activity by THC in a brain crude mitochondrial fraction. Concentration-response curve is displayed as plot of the initial activity of complex II/III against THC concentration. The samples were incubated with drugs at 30 °C for 30 min as described in "Material and Methods." The control samples were measured simultaneously. Values are mean±standard deviation of three to six independent measurements

(Benard et al. 2012; Hebert-Chatelain et al. 2014) that can be related to the procedure of sample preparation. Purity of mitochondrial fraction and concentration of endocannabinoids may modify effect of exogenously added cannabinoids.

CS estimates mitochondrial content and normalizes enzymatic activities of ETC complexes. Studies examining the effects of tested cannabinoids on CS activity are missing. Slightly decreased CS activity after incubation with synthetic cannabinoid CP-55940 was noticed in mitochondria obtained from rat brain and liver after 11 days of treatment with CP-55940 (Costa et al. 1996). We found decreased CS activity after incubation with THC or AM 251, but not after incubation with AEA, WIN, or CBD. We suppose that relatively little effects of cannabinoids on CS compared to effects on activities of respiratory complexes (Fig. 1) could be caused due to a strong accumulation of cannabinoids in the lipid part of membranes.

Athanasiou (Athanasiou et al. 2007) documented that both THC and AEA caused decrease in mitochondrial complex I and complex II/III activity at higher drug concentrations in rat heart mitochondria; complex I activity declined near to zero at 50 μmol/l THC and to 50 % at 50 μmol/l AEA. Our results did not rule out inhibitory effect of THC; however, we observed AEA-induced increase in complex I activity (Fig. 1). We suppose that differences in samples might be responsible for the discrepancy because the effect of drugs on activity of ETC complexes may significantly vary depending on the source of isolated mitochondria (heart, brain, kidney, liver, etc.) (Daley et al. 2005).

Complex I activity was significantly increased after incubation with AEA compared to activity of the control sample (sample without drug added) as well as compared to activity after addition of THC, CBD, WIN, and AM251. The increase of complex I activity after incubation with WIN was significant only when compared to the effects of THC, CBD, and AM251. Significant increase of complex I activity only by AEA (Fig. 1) might be related to chemical structure of AEA that is quite different to other tested cannabinoids. AEA can change its molecular conformations (Barnett-Norris et al. 1998) as well as membrane fluidity differently from cannabinoids containing aromatic structure, such as THC (Velenovska and Fisar 2007). AEA and WIN increased complex I activity compared to other tested cannabinoids, and it can have a positive impact on cellular energetics. This result is in accordance with previous finding that AEA enhanced the brain energy metabolism (Costa and Colleoni 2000).

Activity of both the complexes II/III and IV were decreased by all the tested cannabinoids (all of them with statistical significance except to AM251) (Fig. 1), which indicates that the cannabinoids are capable to cause some mitochondrial dysfunctions. We propose that inhibition of complexes II/III and IV is related to cannabinoid-induced changes in lipid-protein interactions and/or protein complex assembly. Inhibitory

effects of cannabinoids on activity of individual respiratory complexes is in agreement with our previous finding that cannabinoids at micromolar concentrations induce either full or partial inhibition of respiratory rate of intact mitochondria (Fisar et al. 2014).

Our findings that THC inhibits complex II/III and complex IV activity and AEA activates complex I activity are in accordance with previous discovery that THC causes significant increase in mitochondrial hydrogen peroxide production, whereas AEA was without significant effect (Athanasiou et al. 2007). We observed the similar shape of curve measured for THC concentrations in pig brain mitochondria (Fig. 2). Other studies observed inhibitory THC-induced effects: THC inhibited oxygen consumption in mitochondria isolated from human sperm (Badawy et al. 2009) and in human oral cancer cells (Whyte et al. 2010).

Interestingly, CBD inhibited complexes I, II/III, and IV and was found as a neuroprotectant; it had mild (16 %) neuroprotective effect against hydrogen peroxide toxicity and 53 % effect against uncoupler FCCP in SH cells (Ryan et al. 2009). Under pathological conditions involving mitochondrial dysfunctions, CBD might be beneficial in preventing apoptosis. We can speculate that they are dual neurotoxic/neuroprotective effects of some cannabinoids. Preconditioning effect of antimitochondrial actions of CBD similar to effects of antidepressants (Abdel-Razaq et al. 2011) can be under consideration.

We found mostly inhibitory effects of THC, CDB, and AM251 on individual ETC complexes; effects of AEA and WIN included both activation and inhibition. Thus, the performed study confirmed different effects of cannabinoids on several components of mitochondrial respiratory chain.

Conclusions

We investigated the activities of mitochondrial respiratory chain complexes (I, II/III, and IV) and CS activity using both an antagonist/inverse agonists of CB_1 receptors, AM251 and CBD, and CB_1 receptor agonists, THC, AEA, and WIN. Complex I activity was significantly increased only by AEA. Complexes II/III and IV were inhibited by all the tested cannabinoids. CBD seemed to be the most potent inhibitor of mitochondrial functions, including the complex I activity. The outcomes of the investigation support the hypothesis that individual ETC complexes seem to be direct mitochondrial targets for cannabinoids and might contribute to affect further mitochondria-mediated processes. These results also indicate that the direct and/or membrane-mediated effects of cannabinoids on respiratory chain complexes can be involved in regulation of energy metabolism of neurons.

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Conflict of Interest The authors declare that they have no conflicts of interest concerning this article.

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