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In vivo effects of CB_1 receptor ligands on lipid peroxidation and antioxidant defense systems in the brain of healthy and ethanol-treated rats

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Abstract
In vivo experiments were conducted to study the effects of N-(piperidin-l-yl)-5-(4-chlorophenyl)-1-(2,4-cochlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A; a potent and selective CB_1-receptor antagonist) and arachidonyl-2-chloroethylamide (ACEA; a selective CB_1-receptor agonist) on spontaneous lipid peroxidation, glutathione (GSH) level and activities of antioxidant enzymes in rat tissues. Single doses of SR141716A (3 mg/kg, *ip*) and ACEA (10 mg/kg, *ip*) had no effect on all indices, studied in the brain, except for a decrease in GSH level by 10 mg/kg of SR141716A. The effects of repeated administration of the CB_1-receptor ligands (3 mg/kg, *ip*, once daily for 2 days) on the above indices in the brain and liver of control and ethanol-treated animals were also studied. Two weeks after ethanol exposure, the rats lost weight (by 41%), which correlated with their decreased water and food consumption (by 52% and 33%, respectively). The time of ethanol action was not sufficient to change the biochemical parameters in the brain, except for the lipid peroxidation. However, a decrease in GSH level and superoxide dismutase activity, as well as an increase in lipid peroxidation and glucose-6-phosphate dehydrogenase activity were registered in the liver. The repeated administration of CB_1 receptor ligands restored some of ethanol-induced changes. The present results suggested lack of pro-oxidant activity and potential antioxidant ability of the studied CB_1 receptor ligands, which might contribute to their beneficial effects.

Key words:
SR141716A, lipid peroxidation, GSH-level, antioxidant enzymes

Abbreviations: ACEA – arachidonyl-2-chloroethylamide, GSH-Per – glutathione peroxidase, GSSG-Red – glutathione reductase, LP – lipid peroxidation, ROS – reactive oxygen species, SOD – superoxide dismutase, SR141716A – N-(piperidin-l-yl)-5-(4-chlorophenyl)-1-(2,4-cochlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide, TBARs – thiobarbituric acid-reactive substances, Win 55212-2 – R-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl) methyl] pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate]

Introduction

More than 70 naturally occurring cannabinoids are known and some of them are traditionally used for therapy of a variety of disorders. This determines the great interest in cannabinoid drugs and an abundance of data about their effects can be found in many reviews and experimental studies [10, 28–31, 34].

It is believed that the pathogenesis of different diseases (inflammation, ischemia/reperfusion injury, diabetes, ulcers, etc.) is associated with free-radical processes and peroxidation of important cell structures. Oxidative stress in the brain is a potential causal factor in aging and age-related neurodegenerative disorders, Alzheimer's, Huntington's and Parkinson's diseases [6, 14, 26, 27]. According to Ramirez et al. [33], cannabinoids succeed in preventing the neurodegenerative process, occurring in this disease; in addition to their neuroprotective action, some of them possess an antioxidant activity [13, 22].

Reactive oxygen species (ROS) play an important role in ethanol-induced intoxication, too [4, 11, 15, 38]. There are many studies on the effects of CB_1 receptor ligands (agonists and antagonists) in alcohol-treated animals [8, 36, 37], but the results are not associated with the ethanol-induced oxidative stress. For example, Colombo et al. [8] established a stimulatory effect of R-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl) methyl] pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate] (WIN 55,212-2; a non-selective cannabinoid agonist) on ethanol intake, which was completely prevented by administration of 0.3 mg/kg N-(piperidin-l-yl)-5-(4-chlorophenyl)-1-(2,4-cochlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A); they concluded that these results added a further support to the hypothesis suggesting that the cannabinoid CB_1 receptor is a part of the neural substrate, regulating ethanol intake. The studies of Lallemand et al. [18–20] with SR141716A confirmed the involvement of CB_1 cannabinoid receptors in ethanol-induced behavioral effects and revealed that CB_1 receptors might be implicated in ethanol absorption/distribution, particularly after administration of high ethanol doses. The blockade of CB_1 receptors with SR141716A (0.03–3 mg/kg) reduced both ethanol self-administration and conditioned reinstatement of alcohol-seeking behavior in rats [7]. SR141716A-induced CB_1 receptor blockade may be potentially useful in alcoholism treatment [35], but data whether SR141716A affects the parameters of ethanol-induced oxidative stress *in vivo* have not been found, yet.

The aim of this study was to investigate *in vivo* effects of SR141716A (a selective CB_1 receptor antagonist) and arachidonyl-2-chloroethylamide (ACEA; a selective CB_1 agonist) on lipid peroxidation, total glutathione (GSH) level and antioxidant enzyme activities in the brain of control and ethanol-treated rats. The present study is an extension of our previous *in vitro* study, where an inhibition of Fe^{2+}-induced lipid peroxidation in the rat brain and a decrease in OH-provoked deoxyribose degradation were found in the presence of both CB_1 ligands (unpublished data).

Materials and Methods

Animals and treatment

Male Wistar rats, weighing 180–200 g were housed at 22–25°C, under an alternating 12-h light/dark cycle. Prior to the experiments, the animals were fasted for 24 h with *ad libitum* access to water.

The control animals were treated with single doses of SR 141716A (3 mg/kg or 10 mg/kg, *ip*) or ACEA (10 mg/kg, *ip*) and 60 min later were decapitated, under light ether anesthesia. The both cannabinoids (3 mg/kg, *ip*) were also administered once daily for 2 days in controls and ethanol-treated rats and the animals were killed 60 min after the second dose.

Ethanol-treated rats received 10% ethanol (v/v) instead of water, for a period of two weeks.

Materials

All reagents used were of analytical grade. ACEA (in ethanol) and SR141716A were from Tocris (England); 2-thiobarbituric acid and riboflavine – from Sigma-Aldrich (Germany); NADPH and GSH – from Boehringer (Mannheim, Germany). SR141716A was solved in DMSO and all other solutions, used in the experiments, were prepared with over-glass redistilled water.

Preparations

Rat brain was washed with cooled 0.15 M KCl, homogenized in 15 ml cooled 0.15 M KCl-10 mM potassium phosphate buffer (pH = 7.4) and centrifuged at 3,000 rpm for 10 min for obtaining a post-nuclear fraction.

Rat liver was also used in the present experiments; the liver, perfused with cooled 0.15 M KCl, was used for obtaining a 10% homogenate in 0.15 M KCl and was centrifuged for 10 min at 600 × g (a "post-nuclear" homogenate).

Procedures

Protein content was measured by the method of Lowry et al. [21]. *Lipid peroxidation* (LP) in the absence and in the presence of an inducer (5×10^{-5}M Fe^{2+}) was determined by the amount of the thiobarbituric acid-reactive substances (TBARs), formed in fresh preparations for 60 min at 37 C [16]. The absorbance was read at 532 nm against appropriate blanks; the absorbance at 600 nm was considered to be a non-specific baseline and was, therefore, subtracted from A_{532}. *Total glutathione* (GSH) level was measured according to Tietze [39]. *Cu, Zn-superoxide dismutase* (SOD) activity was determined according to Beauchamp and Fridovich [1]; one unit of SOD activity was the amount of the enzyme, producing 50% inhibition of nitro-blue tetrazolium-reduction. *Glutathione peroxidase* (GSH-Per) activity was measured by the method of Gunzler et al. [12]. *Glutathione reductase* (GSSG-Red) activity was measured by the method of Pinto and Bartley [32]. *Glucose-6-phosphate dehydrogenase* (Glu-6-P-DH) activity was determined according to Cartier et al. [3].

Statistical analysis

The results were statistically analyzed by one-way ANOVA with Dunnett post-test; $p < 0.05$ was accepted as the minimum level of statistical significance of the established differences.

The experiments have been performed according to the "Principles of laboratory animal care" (NIH publication No. 85–23, revised 1985), and the rules of the Ethics Committee of the Institute of Physiology, Bulgarian Academy of Sciences (registration FWA 00003059 by the US Department of Health and Human Services).

Results

SR141716A, at a single dose (3 mg/kg, *ip*, had no effect on spontaneous and Fe^{2+}-induced LP in the brain of control animals (Tab. 1). After injection of SR141716A at a higher dose (10 mg/kg, *ip*), a slight decrease in brain GSH level, but not in the activities of GSH-dependent enzymes (GSH-Per, GSSG-Red and related with them Glu-6-P-DH) and SOD, was found (Tab. 2). At the dose used (10 mg/kg, *ip*), the

Tab. 1. *In vivo* effects of SR141716A and ACEA on lipid peroxidation in the rat brain of control animals

Test	Control	SR 141716A		ACEA
		3 mg/kg	10 mg/kg	10 mg/kg
Spontaneous LP	4.5 ± 0.35	5.2 ± 0.12	5.0 ± 0.37	4.7 ± 0.31
Fe^{2+}-induced LP	10.8 ± 0.48	11.5 ± 0.16	12.0 ± 0.61	10.4 ± 0.19

The animals were treated with single doses of SR 141716A or ACEA and killed 60 min later. The brain LP was measured after 60 min incubation at 37 C in the absence and in the presence of 5×10^{-5}M Fe^{2+}. The amount of TBARs, formed was expressed in nmoles MDA/mg of protein, using a molar extinction coefficient $1.56 \times 10^{5}M^{-1}cm^{-1}$. Values represent the mean ± SEM of 6 animals

Tab. 2. *In vivo* effects of SR141716A and ACEA on GSH level and antioxidant enzyme activities in the rat brain

Test	Control	SR 141716A		ACEA
		3 mg/kg	10 mg/kg	10 mg/kg
Brain homogenate				
GSH level	3346 ± 182	3373 ± 152	2622 ± 187*	3019 ± 146
GSH-Per	29.0 ± 1.96	30.0 ± 0.11	33.1 ± 1.82	27.7 ± 0.67
GSH-Red	9.8 ± 0.45	11.8 ± 0.67	11.5 ± 1.65	9.9 ± 0.46
Glu-6-P-DH	15.8 ± 1.08	17.6 ± 0.85	14.3 ± 0.47	16.1 ± 0.74
Cu, Zn-SOD	11.4 ± 0.93	11.1 ± 0.58	12.8 ± 0.38	11.7 ± 0.32

The animals were treated with single doses of SR 141716A or ACEA and killed 60 min later. The total GSH-level was expressed in ng/mg protein; the Glu-6-P-DH, GSH-Per and GSSG-Red activities in nmoles NADP(H)/min/mg of protein (molar extinction coefficient of $6.22 \times 10^{6}M^{-1}cm^{-1}$) and the SOD activity in U/mg protein. Values represent the mean ± SEM of 5 animals. Statistically significant differences *vs.* controls at $p < 0.05$

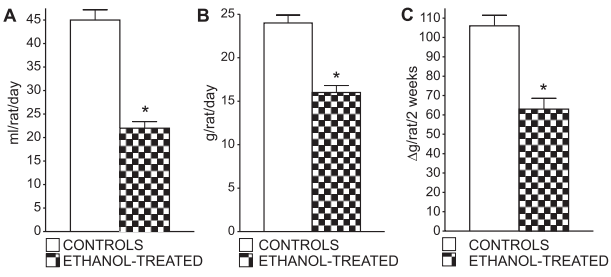

Fig.1. Changes in water consumption (**A**), food consumption (**B**) and body weight gain (**C**) in ethanol-treated rats. Ethanol-treated rats received 10% ethanol for a period of two weeks. Values represent the mean ± SEM of 6 animals. Statistically significant differences *vs.* controls at $p < 0.05$

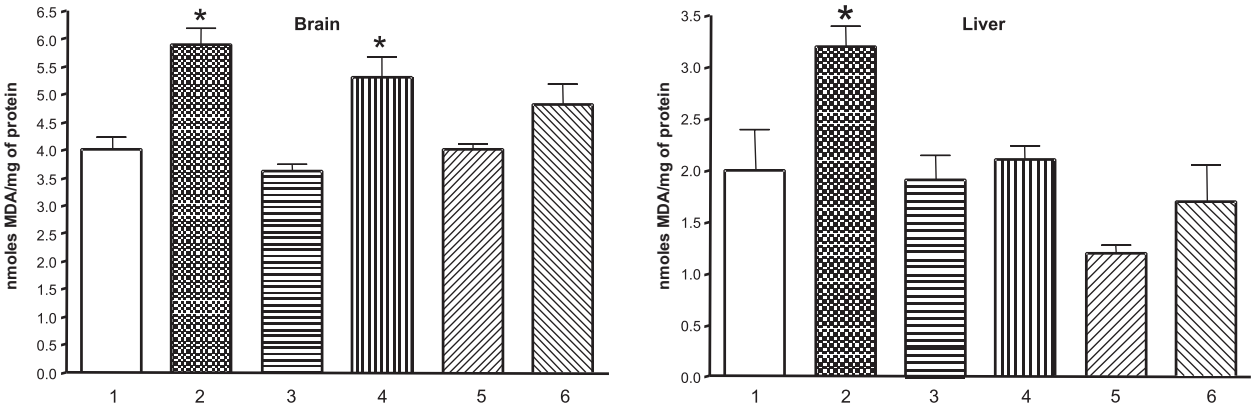

Fig. 2. *In vivo* effects of CB_1 receptor ligands on spontaneous lipid peroxidation in the brain and liver of control and ethanol-treated rats *Experimental groups*: controls (1) and treated with 10% ethanol for two weeks (2); part of the animals in above groups were treated with SR 141716A or ACEA (3 mg/kg, once daily for 2 days): ethanol + SR 141716A (3); ethanol + ACEA (4); SR 141716A (5); ACEA (6) and all animals were killed 60 min after the second dose of CB_1 ligands. The LP was measured in the brain and liver homogenates after 60 min incubation at 37°C. The amount of TBARs, formed was expressed in nmoles MDA/mg of protein, using a molar extinction coefficient $1.56 \times 10^5 M^{-1} cm^{-1}$. Values represent the mean ± SEM of 6 animals in each experimental group. Statistically significant differences *vs.* controls at $p < 0.05$

CB_1-agonist ACEA had no effect on all the studied biochemical parameters (Tab. 1, 2).

The effects of repeated administration of SR141716A and ACEA (3 mg/kg, *ip*, once daily for 2 days) on LP, GSH level and antioxidant enzyme activities in tissues of control and ethanol-treated animals were also studied. We choose the brain in the present experiments, because the brain is one of the main tissues where CB_1 receptors are present and the chronic alcohol consumption leads to oxygen radical formation and lipid peroxidation in the rat brain [24]. Besides, the liver was chosen as a peripheral target of the metabolic actions of endocannabinoids [25], because reactive oxygen species (ROS) are involved in the mechanism of alcohol-induced damage of this major ethanol-metabolizing organ [41].

As seen in Figure 1 (A, B, C), the ethanol-treated animals lost weight (with 41%); the body weight changes correlated with a decrease in water (by 52%) and food (by 33%) consumption. The both CB_1 ligands had not an additional effect on these parameters both in controls and ethanol-treated animals. In comparison with controls, a higher level of the spontaneous LP was measured in the brain and liver of ethanol-treated animals (Fig. 2). This effect was abolished in both tissues after SR141716A administration

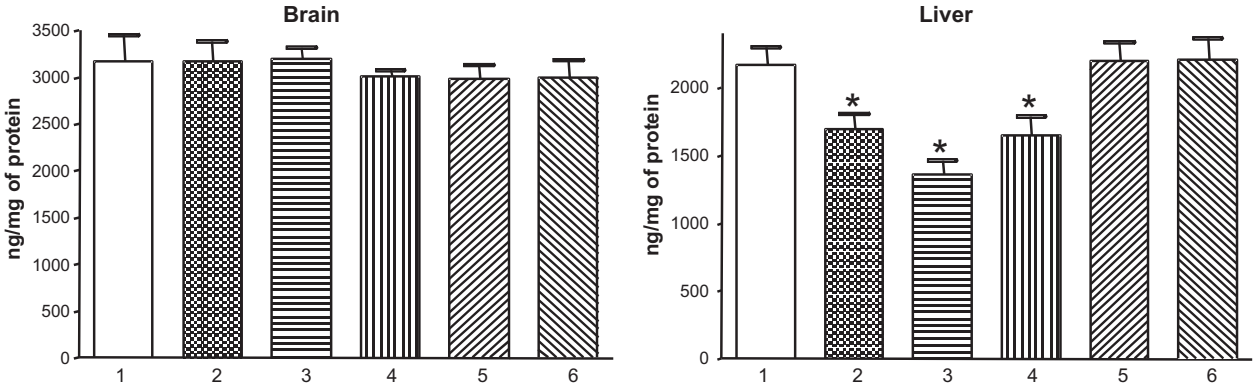

Fig. 3. Effects of SR141617A and ACEA on total GSH-level in the brain and liver of control and ethanol-treated rats. *Experimental groups*: controls (1) and treated with 10% ethanol for two weeks (2); part of the animals in above groups were treated with SR 141716A or ACEA (3 mg/kg, once daily for 2 days): ethanol + SR 141716A (3); ethanol + ACEA (4); SR 141716A (5); ACEA (6) and all animals were killed 60 min after the second dose of CB_1 ligands. The total GSH-level was expressed in ng/mg protein. Values represent the mean ± SEM of 5 animals. * Statistically significant differences *vs.* controls at $p < 0.05$

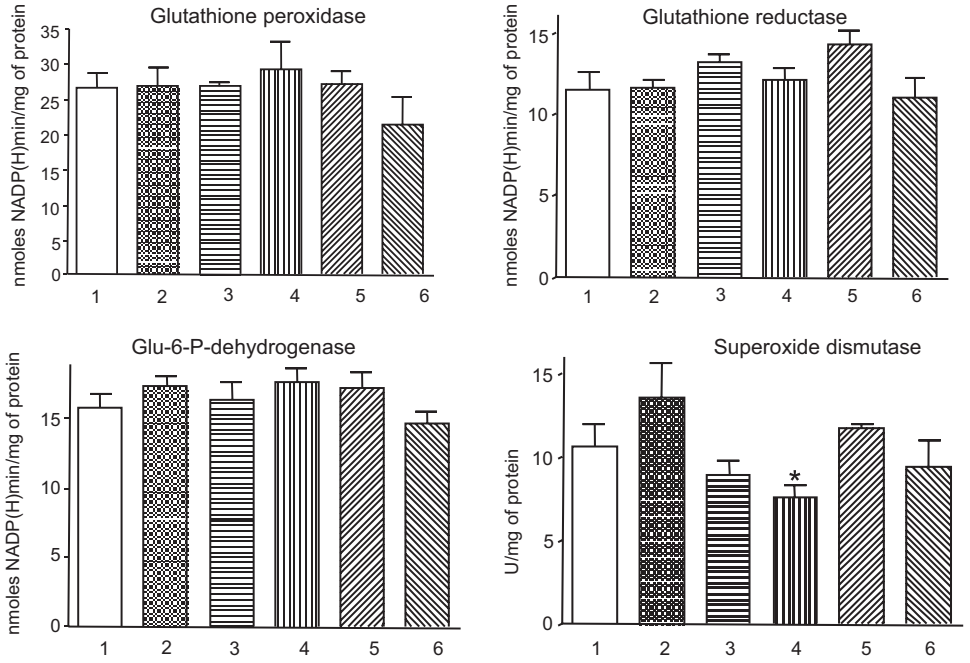

Fig. 4. Effects of SR141617A and ACEA on antioxidant enzyme activities in the brain of control and ethanol-treated rats. *Experimental groups*: controls (1) and treated with 10% ethanol for two weeks (2); part of the animals in above groups were treated with SR 141716A or ACEA (3 mg/kg, once daily for 2 days): ethanol + SR 141716A (3); ethanol + ACEA (4); SR 141716A (5); ACEA (6) and all animals were killed 60 min after the second dose of CB_1 ligands. GSH-Per, GSSG-Red and Glu-6-P-DH activities were expressed in nmoles NADP(H)/min/mg of protein (molar extinction coefficient of 6.22 $10^6 M^{-1} cm^{-1}$); SOD activity was expressed in U/mg of protein. Values represent the mean ± SEM of 5 animals. * Statistically significant differences *vs.* ethanol-treated animals at $p < 0.05$

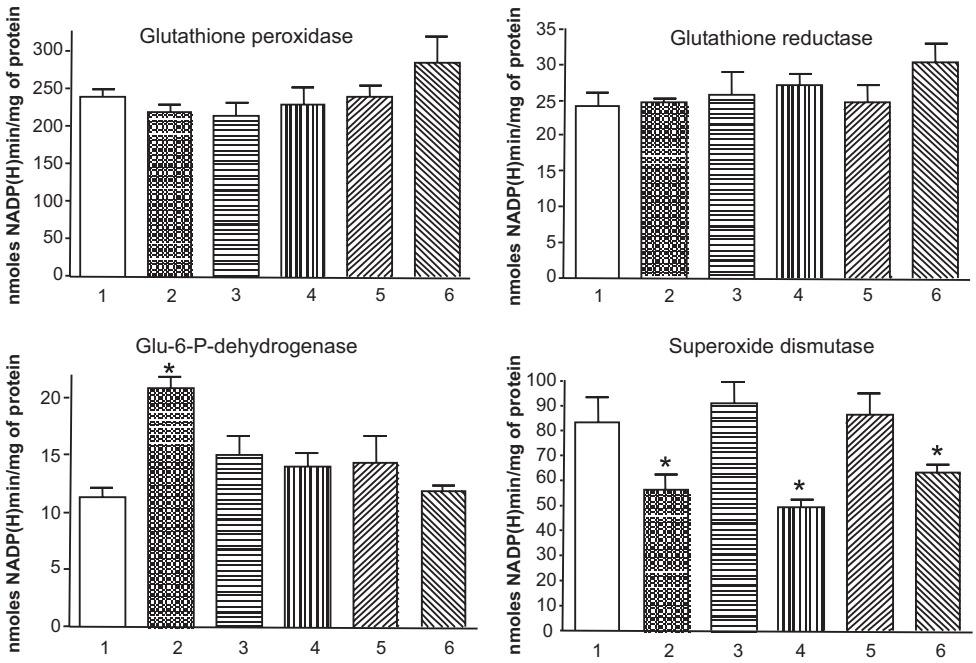

Fig. 5. Effects of SR141617A and ACEA on antioxidant enzyme activities in the liver of ethanol-treated rats. *Experimental groups*: controls (1) and treated with 10% ethanol for two weeks (2); part of the animals in above groups were treated with SR 141716A or ACEA (3 mg/kg, once daily for 2 days): ethanol + SR 141716A (3); ethanol + ACEA (4); SR 141716A (5); ACEA (6) and all animals were killed 60 min after the second dose of CB_1 ligands. GSH-Per, GSSG-Red and Glu-6-P-DH activities were expressed in nmoles NADP(H)/min/mg of protein (molar extinction coefficient of 6.22 $10^6 M^{-1} cm^{-1}$); SOD activity was expressed in U/mg of protein. Values represent the mean ± SEM of 5 animals. Statistically significant differences *vs.* ethanol-treated animals at $p < 0.05$

and only in the liver after ACEA injection. In addition, the CB_1 receptor ligands, tested in control animals did not exert any effects on the spontaneous LP in the brain and liver; only a tendency to a decrease in liver LP was observed after SR141716A.

The 2-week treatment with ethanol did not lead to any changes in GSH level and antioxidant enzyme activities in the rat brain (Fig. 3, 4). However, in the rat liver, total GSH level (Fig. 3) and SOD activity (Fig. 5) were decreased, while Glu-6-P-DH activity was increased (Fig. 5). When ACEA or SR141716A were administered to ethanol-treated animals, additional changes in the above indices were not observed in the brain, except for a slight decrease in SOD activity after ACEA administration (Fig. 4). In the liver of ethanol-treated animals, the cannabinoids decreased the ethanol-induced Glu-6-P-DH activity to its control value (Fig. 5), without changing the already decreased GSH level (Fig. 3). SR141716A fully abolished the ethanol-induced decrease in SOD activity, while ACEA was without effect (Fig. 5). In addition, both CB_1 ligands alone did not change the biochemical indices measured in the brain and liver of control animals (Fig. 3, 4, 5), with the exception of ACEA-induced decrease in liver SOD activity (Fig. 5).

Discussion

Cannabinoids have been reported to protect neural cells from oxidative damage [13, 22, 23], this protective action was attributed to both CB_1 receptor-dependent and CB_1 receptor-independent mechanisms. Some investigators believe that the antioxidant effects of cannabinoids are not CB_1 receptor-dependent [5, 13, 22], while the findings of other authors confirm the role of CB_1 receptors [2, 9, 17]. For example, Carracedo et al. [2] have found that WIN-55,212-2 (a cannabinoid without antioxidant properties) and THC (Δ^9-tetrahydrocannabinol, a cannabinoid with antioxidant properties) prevent the effect of H_2O_2 on cell viability, which was abrogated by co-incubation with SR141716A. The authors suggest that the protective role of cannabinoids in this model relies on the activation of the CB_1 receptors, i.e. these cannabinoids, apart from their antioxidant properties, act *via* the CB_1 receptors. Since the underlying protective mechanisms against oxidative injury are yet incompletely understood, an increase in the number of the studies in this area is observed.

The present study was designed to extend the knowledge of the *in vivo* effects of the SR141716A and ACEA, especially on the ethanol-induced oxidative changes in the rat brain and liver. It was also an extension of our previous *in vitro* study with these CB_1 receptor ligands (unpublished data).

This *in vivo* study showed that 3 mg/kg of SR141716A did not change the spontaneous and Fe-induced LP, the GSH level and the activities of antioxidant enzymes tested in the brain of control animals. The effects of 10 mg/kg SR141716A and 10 mg/kg ACEA were similar, only a decrease in GSH level by 10 mg/kg SR141716A was found that might be due to some non-specific toxic effect of this very high dose. On the second day of the treatment with SR141716A or ACEA (3 mg/kg/day), no changes in the above parameters were also found in the brain and liver of control rats, except for a decrease in hepatic SOD activity by ACEA. Taken together, these results support our *in vitro* data for a lack of pro-oxidant action of the tested CB_1 ligands.

Lallemand et al. [19], evaluating the effects of SR141716A administration (1, 3 and 10 mg/kg/day) on ethanol preference during a chronic ethanol exposure (alcohol 10% v/v) or at the cessation of this procedure, have found that a 30-day concomitant administration of SR141716A with ethanol increases, while SR141716A administration after induction of chronic alcoholism drastically diminishes the preference for ethanol.

In the present study, CB_1 ligands were administered 2 weeks after treatment with 10% ethanol. The latter alone decreased body weight, food and water consumptions. CB_1 ligands (3 mg/kg/day, for two days) alone had no effect on these parameters, both in controls and ethanol-treated animals. According to Trillou et al. [40], a 5-week treatment with 10 mg/kg/day SR141716A led to reduction in food intake and body weight, but these effects were less pronounced at 3 mg/kg/day SR141716A. Probably, the low dose of SR141716A used in the present study and the short time of its exposure (3 mg/kg, once daily for 2 days), is the reason for the lack of effects of SR141716A on the above parameters in the ethanol-untreated animals; hence, the beneficial effects of SR141716A, observed in the ethanol-treated group could not be ascribed to SR141716A-induced decrease in ethanol intake.

Ethanol treatment led to an increase in TBARs amount in both brain and liver, but changes in GSH level and in some of the antioxidant enzyme activities were found only in the liver. Therefore, studies on antioxidant defense systems in animal tissues at a longer time of ethanol-exposure are envisaged in the future.

In the brain of ethanol-treated animals, SR141716A, but not ACEA, restored LP to its control value. In the liver of ethanol-treated animals, both tested canabinnoids restored LP. SR141716A restored the activities of Glu-6-P-DH and SOD, whereas ACEA restored the ethanol-induced changes in Glu-6-P-DH activity only. The lack of ACEA effect on ethanol-induced changes in SOD activity might probably be due to its inhibitory effect on this enzyme.

In our previous *in vitro* study, it was found that SR141716A and ACEA decreased OH-provoked deoxyribose degradation (a test for OH-scavenging activity of different substances). The inhibitory effect of SR141716A was stronger than that of ACEA, suggesting a better antioxidant activity of SR141716A (unpublished data). All these findings suggest that the protective role of the cannabinoids, studied in the present model of ethanol exposure, does not rely on activation of the CB_1 receptors, thus, it might be assumed that these compounds, especially SR141716A, act in CB_1-independent manner. According to Chen and Buck [5] the antioxidative cytoprotection by cannabinoids, observed in non-neuronal cells lacking cannabinoid receptors, is consistent with a receptor-independent mechanism. In the present study, the main changes in antioxidant status after ethanol-treatment were found in the liver, where the CB_1 receptor ligands restored some of them.

In conclusion, the present *in vivo* results showed that SR141716A and ACEA had no effect on the indices responsible for cell defense against ROS in the brain and liver of control (ethanol-untreated) animals. These results, together with our previous *in vitro* data, proved the lack of pro-oxidant activity of these cannabinoids. The protective effects of the studied CB_1 receptor ligands, observed in the brain and liver of ethanol-treated animals, suggest an antioxidant activity, but these data are not a sufficient evidence for the putative efficacy of these cannabinoids, especially of SR141716A, as antioxidant agents in alcohol abuse. In order to fully elucidate the action of the above CB_1 receptor ligands, further studies of their effects on antioxidant indices in tissues, subjected to oxidative injury, e.g. cerebral ischemia and age-related neurodegenerative disorders (Alzheimer's or Parkinson's diseases), would be of interest.

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