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RESEARCH ARTICLE

Protein level alteration of endocannabinoid system components after chronic, oral self-administration of three atypical antipsychotics in rat

László-István Bába[1], Melinda Kolcsár[1*], Zsolt Gáll[1], László Csaba Bencze[2], Adrian Man[3], Imre Zoltán Kun[4]

1. Department of Pharmacology and Clinical Pharmacy, George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Targu Mures, Romania
2. Biocatalysis and Biotransformation Research Center, Faculty of Chemistry and Chemical Engineering, Babeș-Bolyai University, Cluj-Napoca, Romania
3. Department of Microbiology, George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Targu Mures, Romania
4. Doctoral School, George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Targu Mures, Romania

Objective: Atypical antipsychotics (AAPs) often cause metabolic adverse effects (mAE) such as weight gain and dyslipidemia. The mechanisms underlying AAP induced mAE are not fully elucidated. The endocannabinoid system (ECS) is a key system in the regulation of energy metabolism that may be involved in AAPs induced mAE. In this experiment, we studied the expression of three major components of ECS: cannabinoid receptor 1 (CB1), fatty acid amidohydrolase (FAAH) and monoacyl glycerol lipase (MAGL) after chronic administration in rat of three AAPs: olanzapine (Ola), aripiprazole (Ari) and cariprazine (Car). **Methods**: Drugs were self-administered orally, in two doses by female, adult Wistar white rats (n=6 per treatment group) for six weeks. After the treatment period, the animals were sacrificed and visceral (perirenal) white fat pads were collected. The fat tissue samples were homogenized and the expression level of CB1, FAAH and MAGL were compared by western-blot analysis. **Results**: An increase of CB1 expression was noticed after the treatment with 1.5 mg/kg/day Ola, although not statistically significant. All three drugs augmented the FAAH expression, the effect being significant after the treatment with 0.25 mg/kg/day Car. The expression of MAGL was not influenced significantly by the three AAPs, nevertheless, an increasing tendency can be remarked in the case of Ari and Car. **Conclusions**: Promoting the CB1 expression in adipose tissue could contribute to weight increasing and other mAE effects of Ola. The tendency of Ari and Car to enhance the breakdown enzymes expression might have some role in more favorable mAE of these drugs.

Keywords: endocannabinoid system, atypical antipsychotic, adipose tissue, western blot

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Introduction

Third generation antipsychotics or *„dopamine stabilizers"*, are a distinct subgroup of atypical antipsychotics (AAPs), that are used in severe mental diseases including schizophrenia, major depression and bipolar disorder [1]. AAPs are also used off-label for several other conditions [1]. AAPs have distinct adverse effects that differ greatly from those of the first-generation antipsychotics, being mainly of metabolic nature [2]. Their potential of inducing metabolic adverse effects (mAE) varies greatly. Some drugs are considered metabolically safe (aripiprazole – Ari, paliperidone, ziprasidone, lurasidone), while others carry a high risk for mAE (olanzapine – Ola, clozapine, zotepine) [2,3]. Cariprazine is a new AAP that is a partial agonist on both $D_{3/2}$ receptors with almost ten times higher affinity for D_3, but there is limited experience regarding its adverse effects [4]. The importance of the fact that Car has almost ten times higher affinity for D_3 is not clear yet, but it seems likely, that it is involved in the pro-cognitive, antidepressant effect and in the effectiveness against the negative symptoms of schizophrenia [5,6]. According to *Nasrallah*, the receptor subtypes mainly involved in AAP-induced weight gain are H_1, and 5-$HT_{2C/1A}$ [7]. In this regard, Car has a beneficial binding profile (similar to that of Ari), exerting only a slight affinity to the H_1-receptors, and displaying high binding affinity only for 5-HT_{1A} subtype of the serotonergic receptors [4]. This is in accordance with a recent study regarding the adverse effects of Car, reporting negligible mAE [6].

The drugs studied in this experiment (Ola, Ari and Car) were chosen having in mind that Ola is considered an AAP with very pronounced mAE while Ari is more or less devoid of such effects. This allowed us to compare a new drug (Car) with substances that are considered references in terms of mAE (Ola and Ari).

An endogenous signaling system that is involved in energy homeostasis is the endocannabinoid (EC) system (ECS) [8]. ECS is comprised of several transmitters/modulators, receptors that mediate the effect of these, and the enzymes that are involved in the synthesis and degradation of the mediators. The two endocannabinoid mediators that are largely accepted are: 2-arachidonoyl glycerol (2-AG) and arachidonoyl-ethanolamine (anandamide) [9,10]. The two major receptors in the ECS are cannabinoid receptor 1 and 2 (CB1 and CB2) [9,11]. The enzymes involved in the termination of the EC signaling are fatty acid amidohydrolase (FAAH) and monoacyl glycerol lipase (MAGL)

* Correspondence to: Melinda Kolcsár
E-mail: melinda.kolcsar@umfst.ro

(hydrolases of anandamide and 2-AG, respectively) [11]. They have a molecular weight of ~63 (FAAH) and ~33.5 kDa (MAGL) respectively [12,13]. The most important role of the ECS is to govern the metabolic processes in such a manner, that favors energy preservation [14]. This involves multiple mechanisms, including central (stimulation of energy intake and inhibition of energy expenditure) and peripheral ones.

The existence of CB1 in adipocytes was shown in 2003 [14]. Since then, a whole functional system in both white and brown adipocytes has been described [15,16]. Having in mind the pivotal role of ECS in energy metabolism, it`s role in AAP driven mAE has been long hypothesized. In animal experimental settings, the in-depth mechanistic study of ECS in AAP induced mAE was undertaken in the last decade. So far it has been demonstrated, that the administration of cannabinoid antagonists/inverse agonists block the metabolic effects of Ola both at central and peripheral (white adipose tissue [AT], liver) levels [17]. Results were also published about the energy metabolism influenced by Ola [18]. More than that, lipolysis and glucose transport in adipocytes isolated from chronic Ola treated rats is compromised, but not that of ziprasidone (an AAP with low incidence of treatment-emerged mAE) treated animals [19].

We previously observed the influence of the mentioned three AAPs on the body weight of animals. Being in accordance with the human clinical profile of the mentioned drugs, and also with the available animal experimental data, we found a weight gain lowering effect after Ari and Car administration and a steady increase of body weight after chronic Ola administration [20]. In the present study we intended to elucidate, if the ECS, at the level of AT is influenced by the chronic administration of the three AAPs, potentially contributing to the effect on body weight, and adipocyte maturation seen in our mentioned previous experiment [20]. Furthermore, we compared a novel AAP, Car with better-known drugs concerning to AT-specific alterations of the ECS. Therefore, we quantified the expression of three major components of the ECS (CB1, FAAH and MAGL) from the AT after chronic self-administration of the mentioned drugs, each in two different doses in rat.

Methods

Animals, drug administration and study design

The mentioned drugs were administered in two different doses (Ola: 0.5 and 2 mg/kg tid, Ari: 0.5 and 1 mg/kg tid, Car: 0.1 and 0.25 mg/kg once daily), to female adult, Wistar white rats. The doses were chosen having in mind earlier studies, in a way that they mimicked an antipsychotic treatment in the human clinical setting [21–23]. These doses were corresponding to average human therapeutical ones, considering the „Human equivalent dose" (HED), calculated by using the formula recommended by Reagan-Shaw and coworkers, except the case of 6 mg/kg Ola [24]. This, translated to HED, exceeds typical doses of Ola (i.e. 5-15 mg/day), but was chosen based on earlier experiments, being a dose that robustly causes weight gain [21].

The drugs were used by self-administration, according to a method described by Weston-Green et al. [21]. Briefly, an ~0.3 g pellet was made of 73% powdered standard laboratory chow, 18% flour, 9% sucrose, in which the drug was homogenously dispersed. This pellet was offered to the animals for consumption, by an automated system, three times daily (in the case of Car, only one pellet contained the active ingredient, since this substance has sufficiently long half-life). Standard laboratory chow was simultaneously available to the animals, throughout the whole experiment. After a few days of accommodation, the animals learned to consume these pellets. Car was obtained from Shanghai Resuperpharm Tech ltd., for Ola and Ari, dosage forms available for human use were freshly powdered and the corresponding quantity was taken to prepare the drug-containing pellets (Ola – Olanzapin, oral tablets, 20 mg - Actavis; Ari – Aryzalera, coated tablets, 10 mg - Krka). The animals were left seven days for accommodation and trained to consume the dry-dough cookie pellets. One group served as control (Ctr) and received vehicle-only pellets.

The animals were housed individually, at room temperature, with a 12/12-hours light-dark cycle. After the accommodation period, the animals were treated for six weeks with the three atypical antipsychotics in the mentioned two doses ($n=6$ for each group). The cages were observed daily to ensure that all animals consumed the pellets. All of the rats consumed the drug-containing pellets throughout the experiment, except one animal from the Ola 6 mg/kg group, that discontinued the consumption of the pellets after four weeks of treatment. We tried to recondition the animal to consume the pellets for a few days, but did not succeed, thus the animal was sacrificed. Therefore, only five values were included in this treatment group. Body weight was weekly measured for dose correction. After the drug administration has ended, the animals were sacrificed by cardiac puncture under ketamine-xylazine anesthesia.

The animals had *ad-libitum* access to standard laboratory chow and tap water. This way, the animal model mimicked the human clinical setting and avoided the stress related to immobilization and forced administration (gavage or injection). The experimental procedures undertaken were in accordance with the current EU guidelines (directive nr.: 2010/63/UE, European Parliament and European Council for the protection of animals used for scientific purposes). The study also received agreement from the ethical commission of the G. E. Palade University of Medicine, Pharmacy, Science and Technology of Targu Mures (decision no. 276/10.08.2017).

Tissue collection and protein extraction

After the sacrification, visceral (perirenal) adipose fat pads were rapidly dissected and kept at 20 ^{0}C until further processing. Tissue protein extraction method was optimized

in order to maximize the amount of proteins extracted. We found that homogenization with tissue bead mill (IKA ultra-turrax, IKA-Werke GmbH & Co., Germany) and NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, in 50 mM Tris-Cl, adjusted to pH=8.0; 300 µL/ 100 mg tissue sample) resulted good extraction yield from the AT samples. Protease-Inhibitor Mix G (SERVA Electrophoresis GmbH, Germany, cat. no.: 39101.01) was added to the lysis buffer to protect the proteins from enzymatic degradation. After homogenization, the tissue homogenate was centrifuged three times (10.000 g, 4 ^{0}C, 10 min), and extracts were saved for Western blotting (WB) at -20 ^{0}C.

Western blotting

Prior to the electrophoresis, the protein concentration was determined spectrophotometrically (BioSpectrometer, Eppendorf GmbH, Austria). Protein probes were denaturized using 6x Laemmli buffer (10% w/v SDS, 20% glycerol, 120 mM Tris-Cl, 50 mM dithiothreitol, 0.2% bromophenol blue in dH_2O) at 98 ^{0}C for 5 minutes. Then, protein extracts were loaded (volumes corresponding to 10 µg of total protein) in 10% polyacrylamide gel and separated by electrophoresis (3-6 samples per treatment group). In order to reduce the inter-membrane variability, induced by the different transfer efficiency, the proteins were transferred to PVDF membrane using commercially available transfer packs (Trans-Blot® Turbo™ Transfer Pack – Bio-Rad, USA) with a semidry blotting system (Trans-Blot® Turbo™ - Bio-Rad laboratories, USA). Blotting efficiency was optimized in order to maximize the amount of protein of interest transferred, separately for each protein (2.5 A, 25 V for 7 min - CB1; 1.0 A, 25 V for 30 min - FAAH and 2.5 A, 25 V, 5 min - MAGL). All procedures were undertaken under the same experimental conditions as recommended by Eaton and coworkers to maximize reproducibility and minimize inter-lane and inter-membrane inconsistency [25].

After the transfer, membranes were washed with phosphate-buffered saline (PBS) and blocked overnight at 4 ^{0}C (for CB1) or one hour at room temperature (for FAAH, MAGL) with 3% bovine serum albumin (BSA, Carl Roth GmbH & Co, Germany cat. no.: 8076.3) in PBS. The following antibody dilutions were used: 1:1000 for anti-CB1 (Abcam plc, Cambridge, UK, cat. no.: ab23703), and anti-FAAH (St. John`s lab, London, UK. cat. no.: STJ23602), and 1:500 for anti-MAGL antibody (Biorbyt, Cambridge, UK, cat. no.: orb337279), in antibody diluent (1% BSA in PBS). After incubation with primary antibodies, the membranes were washed three times, for 5 min., with membrane wash buffer (1% BSA, 0.2% Tween 20 in PBS). HRP conjugated goat anti-rabbit secondary antibody (Abcam plc, Cambridge, UK, cat. no.: ab205718) was used in 1:5000 dilution with incubation undertaken at room temperature for one hour, on a rocking plate. After secondary antibody incubation, the membranes were washed three times with wash buffer and incubated with ECL WB substrate (Pierce ECL WB substrate, ThermoFisher, USA) for 30 min in a ChemiDoc MP Imaging System (Bio-Rad, USA). All protein samples were run at least in duplicate, and the values were averaged before statistical analysis for each animal.

Gel loading and loading control

Since the WB is a complex experimental procedure, where many steps invoke possible errors that might influence the results, the current practice in molecular biology is to normalize the obtained signals. Traditionally, the so called `house-keeping proteins` (HKP) were used to normalize the signal. These are ubiquitously expressed proteins, that have a relative constant level among a number of cell types and tissues. However, some experts have challenged the validity of house-keeping proteins in WB experiments [26]. First, the assumption that the house keeping proteins are present in equal levels in different tissues is fundamentally wrong [25]. Second, HKP levels can be influenced by different interventions and disease states. Not to mention, that there are substantial differences in expression between various parts of the same tissue [25].

Considering the mentioned flaws of the HKP normalization, we decided to use total protein stain (TPS) for normalization of the probes. This method normalizes the specific signal to the total protein content of the lane, evidenced by a protein stain. The method described by Welinder and Ekblad was used for TPS [27]. Briefly, membranes were air-dried after immunodetection and stained with 0.1% Coomassie R-350 Blue solution (methanol/water, 1:1) for 5 min, then, they were destained for 7 min (acetic acid/ethanol/water, 1:5:4). Finally, the membranes were dried and a colorimetric picture was taken. The total lane volume was measured using ImageLab (Bio-Rad Laboratories, Inc. USA, ver. 6.1). The obtained band volumes were normalized to the corresponding lane volumes (Figure 1).

In order to demonstrate the robustness of TPS as a normalization method, a calibration curve was prepared using BSA as reference protein within the range of 0-15 µg/lane, showing good linearity R^2>0.96 (Figure 2).

Since there were more samples than would fit on a single gel, the treatment and control group samples were balanced by randomly choosing samples to load in a gel.

Statistical analysis

The values normalized to the total protein stain were divided by the average of normalized values of control animals. This way, the relative changes in expression levels were compared to the control group. For CB1 and FAAH one-way ANOVA, while for MAGL expression, a Kruskal-Wallis test was run with the corresponding post-tests (Dunnett`s multiple comparisons for CB1 and FAAH and Dunn`s multiple comparisons for MAGL). Significance level was set α=0.05 for all statistical tests. Values declared in the results section and figures are mean ± standard error of mean.

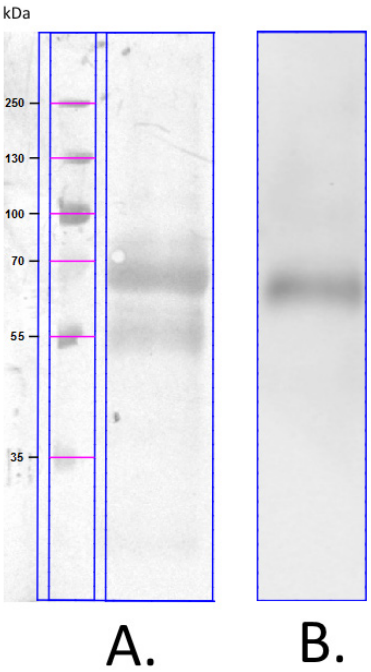

Fig. 1. Normalization of the WB probes using total protein stain technique. A: the total lane volume representing the total amount of protein in a sample, stained with Coomassie. On the left, the molecular weight marker can be seen. B: Specific signal of the protein of interest (in this case FAAH) with a clear band between 55 and 70 kDa weight.

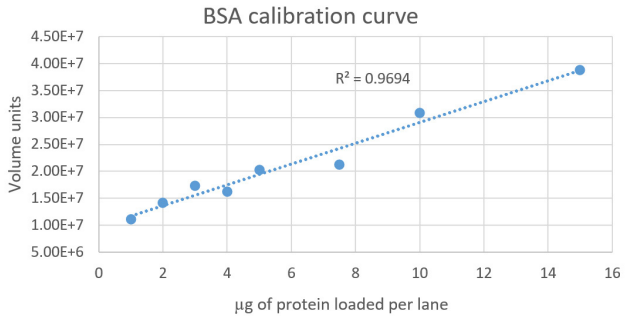

Fig. 2. The calibration curve for total protein stain. The curve was made with a dilution series from 1 to 15 µg of BSA per lane, showing good linearity (R2=0.9694) within the concentration range of 1-15 µg.

Results

1. Expression level of CB1

CB1 expression was not influenced significantly by none of the drugs applied, dispersion of values was considerable, but an increasing tendency in the Ola 1.5 mg/kg, and – in a lesser extent – Ari 3 mg/kg and also in both doses of Car treated groups can be observed (Ctr.: 1.00±0.23; Ola 1.5 mg/kg: 3.09±0.835; Ari 3 mg/kg: 1.86±0.68; Car 0.1 mg/kg: 1.50±0.73; Car 0.25 mg/kg: 1.95±0.62) (Figure 3).

2. Expression level of FAAH

All the treatment groups displayed an increasing tendency in FAAH expression, significant difference can be seen in the case of Car 0.1 mg/kg (Ctr.: 1.00±0.05; Ola 1.5 mg/kg: 3.01±0.59; Ola 6 mg/kg: 3.36±0.88; Ari 1.5 mg/kg: 4.01±0.95; Ari 3 mg/kg: 3.45±0.81; Car 0.1 mg/kg: 4.49±0.67; Car 0.25 mg/kg: 2.79±0.57) (Figure 4).

3. Expression level of MAGL

The expression of MAGL was not influenced significantly by the drugs administered and a high dispersion (similar to that seen in the case of CB1) was observed. Nevertheless, an increasing tendency can be seen in Ari 1.5 mg/kg (2.87±1.02), Car 0.25 mg/kg (2.84±1.95) groups, and – in a lesser extent – after Ola 1.5 mg/kg (1.52±0.52) (Figure 5).

Discussion

In the present study, we compared the effects of Ola, Ari and Car on the expression of three key components of ECS: CB1, FAAH and MAGL. An increasing tendency of CB1 expression in the case of the group treated with 1.5 mg/kg Ola per day can be observed. The result was not significant, and the dispersion of data was considerable, but the importance of this finding has to be addressed. In the clinical setting, Ola is a drug with pronounced mAE. In rodent models of AAP induced mAE these observations are well mirrored [21,28]. As demonstrated by Weston-Green and colleagues in a rat experimental model for Ola administration (doses ranging from 1.5 to 6.0 mg/kg per day for 14 days), the ECS at central levels is contributing to the weight-gain seen with this drug [29].

Regarding the peripheral, ECS-related mechanisms that control adipose tissue function, it has been demonstrated, that under in-vitro circumstances the expression of CB1 receptor increases both at RNA and protein levels during adipogenesis [14,30]. More than that, the activation of CB1 is associated with proliferation, while the blockade of CB1 with the inhibition of proliferation of preadipocytes [14,30]. Engeli et al. described a similar increase in the expression level of CB1 and FAAH at protein and mRNA levels during adipogenesis in-vitro. They also observed a reduction of mRNA levels in obese subjects compared to lean, suggesting the existence of a feedback mechanism [31]. The activation of CB1 not only controls the adipogenesis, but also the lipogenesis, stimulating the glucose uptake, and the transcription of key lipogenic transcription factors and enzymes [14]. It is worth to mention, that CB1 negatively regulates the trans-differentiation (development of beige phenotype in the white adipocyte), with lowering the expression of key genes in thermogenesis and mitochondrial biogenesis (UCP-1, PGC-1α) [9]. Moreover, the blockade of CB1 receptor under in-vivo conditions counteracts the effects of high-fat diet on adiposity [32]. These results underscore the role of CB1 in the control of fat tissue mass.

Exploring the involvement of peripheral ECS in the mAE of AAPs, Lazzari et al. demonstrated that administration of rimonabant (a CB1 inverse agonist) prevents the white adipose tissue accumulation in rat triggered by chronic Ola administration [17]. At genomic level, the alteration of a cluster of genes involved in adipocyte function (adipocyte maturation, inflammation, and adipokines) was also proven to be influenced by Ola treatment, for some of these even at protein levels, but little is known about the effect of AAPs

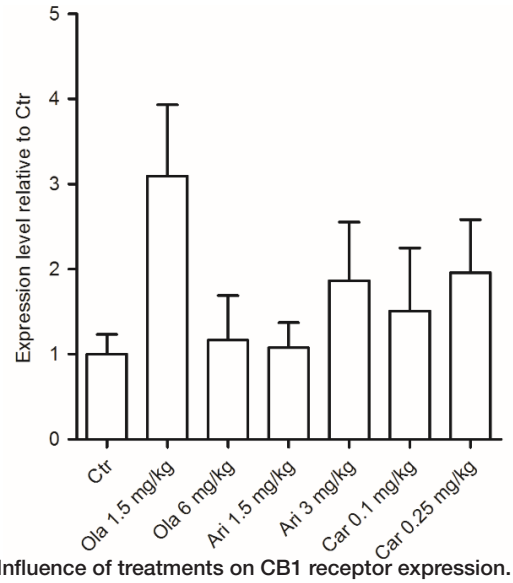

Fig. 3. Influence of treatments on CB1 receptor expression. A difference close to the significance threshold can be observed in the case of Ola 1.5 mg/kg group. None of the other treatment groups displayed considerable effect on the expression level of CB1.

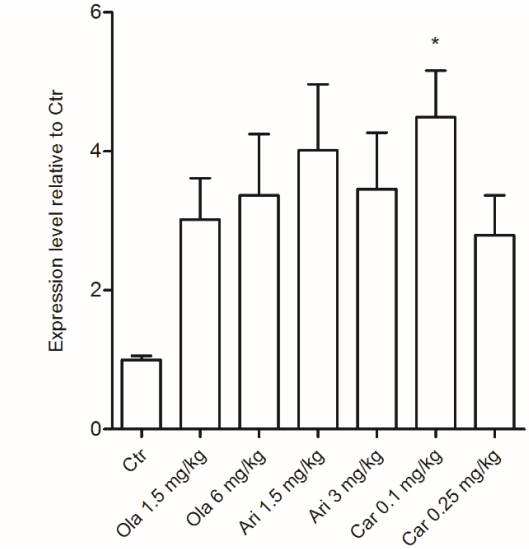

Fig. 4. Influence of treatments on adipose tissue FAAH expression. All the three AAPs presented an increasing tendency on FAAH expression, with significant difference noticed in the case of Car 0.1 mg/kg (noted with *, compared to Ctr).

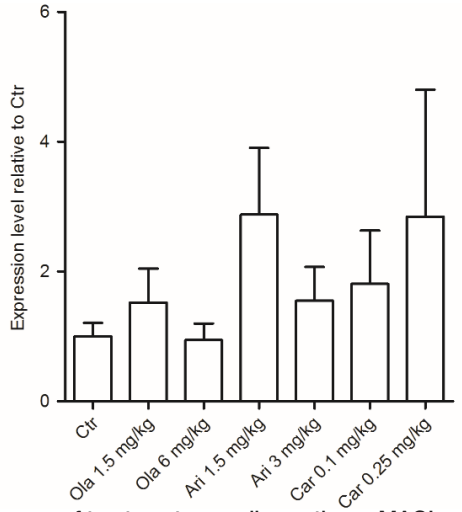

Fig. 5. Influence of treatments on adipose tissue MAGL expression. Ari, Car and – in a lesser extent – Ola 1.5 mg/kg presented an increasing tendency on MAGL expression.

on the expression of ECS components in the AT [19,21]. Our result adds to the existing knowledge in this field. To the best of our knowledge, this is the first experimental report of FAAH, MAGL and CB1 expression in visceral adipose tissue at protein level in rat, after chronic Ola, Ari and Car treatment. Having in mind the above-mentioned role of CB1 receptor in adipose tissue biology, the increasing tendency in its expression by Ola could potentially stimulate the adipo- and lipogenesis, offering to some extent, an explanation for the well-documented expansion of visceral adiposity caused by this drug [17,18,21,33,34]. The importance of the fact that CB1 expression was increased only in the 1.5 mg/kg Ola treated group remains to be elucidated. The effect in case of Ari and Car administration is negligible on CB1. Notably, Skrede et al. found expansion of the mesenteric adipose tissue, and no increase in total visceral adipose tissue after ad-libitum fed rats under chronic Ari treatment [34]. It must be remarked, that in the same experiment, animals treated with Ola did not gain weight when kept under a pair-fed paradigm (were given the amount of chow that vehicle group consumed in the previous day). This further points out the importance of the central component (hyperphagia) of weight gain caused by Ola [34]. The fact that Ari and Car did not influence the expression of CB1 might contribute to the better metabolic profile of the two drugs, because they could not induce a pathological expansion of the visceral adipose tissue, possibly driven by ECS activation.

The biological role of FAAH is to hydrolyze anandamide, but other signaling molecules are also degraded by this enzyme (fatty acid amides like palmitoylethanolamide, oleoylethanolamide) [11]. The result that all three AAPs stimulated the expression of FAAH could result in an accelerated turnover of EC mediators (mainly that of anandamide) that could potentially reduce the lipid accumulation and thus contribute to the mild mAE of Ari and Car. In the case of Ola, the FAAH expression increased in a similar way to Ari and Car. The importance of this finding is not known yet, and it seems plausible, that under in-vivo conditions, the increase in FAAH expression is eclipsed by other mechanisms, that finally result in an expansion of adipose tissue with this drug. A slight difference can be observed between the two doses of Ari and Car in terms of FAAH expression, that could reflect a dose-dependent effect of these drugs. This could bear clinical importance. A potential consequence of this phenomenon might be, that there is a dose range, at which the drugs stimulate more intensely the expression of this enzyme, resulting accelerated turnover of the EC mediators.

The expression of MAGL was not influenced markedly by the three AAPs studied, however, an increasing tendency can be observed in case of 1.5 mg/kg Ari and 0.25 mg/kg Car treatment. The increase of expression of this enzyme again could mean an accelerated hydrolysis of its primary substrate, namely 2-AG [11]. Lowering the endocannabinoid tone is considered a strategy, that is beneficial in

a plethora of metabolic derangements, including obesity, type-2 diabetes, and dyslipidemias [35,36]. Since rimonabant, the first brain penetrating CB1 inverse agonist was withdrawn from the global drug market because of serious adverse effects, the peripherally restricted antagonists/inverse agonists were in the limelight. Several of these show promising results in various animal models of metabolic disease [37–39]. Nevertheless, accelerating the turnover of a mediator which is a high affinity agonist at both CB1 and CB2 receptors is lowering the endocannabinoid tone in the periphery, and could be a beneficial feature of a drug. The observation that Ari and Car administration resulted in a dose-dependent influence on the expression of MAGL could mean that in some doses, other mechanisms may also be present.

Limitations

The limitations of this study have to be mentioned. First, besides being a key enzyme in ECS, MAGL has other biological roles also. Since 2-AG is an important intermediate in lipid metabolism and also in the arachidonic acid signaling, MAGL participates in the functioning of these systems, too [11]. As a consequence, AAP administration could have resulted in alteration of these systems, and not that of ECS.

Second, besides the two enzymes involved in the degradation of ECs, several others have been described (Cyclooxygenase-2 [COX-2], N-acyl ethanolamine amino hydrolase for anandamide; alpha/beta domain-containing hydrolases 6/12, and under certain circumstances FAAH and COX-2 for 2-AG). Since these are involved in the termination of EC signal, they could modulate the peripheral endocannabinoid tone [11]. Again, the AAP administration could have influenced these enzymes rather than the ECS. Based on the present experiment, we cannot rule out neither of these two possibilities.

Third, a major flaw of the experimental procedures is that the western-blot is not the most appropriate technique for large sample number, quantitative protein expression studies. Despite of various measures undertaken, the obtained values display a noticeable dispersion (especially in the case of CB1 and MAGL), that limit the significance of the results. For future studies, the use of mass spectrometry techniques (such as matrix-assisted laser desorbtion/ionization time-of-flight mass spectrometry) could result in improved accuracy with lower dispersion of values. For similar reasons, the use of greater number of animals per treatment group could improve the results.

Conclusion

Our results suggest that Ari and Car influenced to some extent the peripheral ECS at the level of adipose tissue: they have no considerable effect on CB1. Although considerably heterogenous, our results showed a slight effect on FAAH and MAGL enzyme expression. This could increase the degradation of EC mediators in the AT. These observations were not significant, excepting the effect of Car in the dose of 0.1 mg/kg per day on FAAH. The increase of CB1 receptor expression in adipose tissue by Ola could contribute to weight-increasing and other mAE of this drug.

Authors' contribuitions

LIB - conception and design of the study, acquisition and interpretation of data, drafting the article, final approval of the version to be published; MK - interpretation of data, revising the article critically and final approval of the paper; ZG - analysis and interpretation of data, revising the paper critically for important intellectual content and final approval of the version to be published; LCB - conception of study, analysis of data, revising the article critically and final approval of the version to be published; AM - conception of study, analysis of data, revising the article critically and final approval of the version to be published; IZK - conception of the study, revising the article critically and final approval of the paper.

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References

1 Stahl SM. Atypical antipsychotics. Stahl's Essent. Psychopharmacol. Neurosci. Basis Pract. Appl., . 4`thCambridge: Cambridge University Press, 2013:141–169.
2 Solmi M, Murru A, Pacchiarotti I, et al. Safety, tolerability, and risks associated with first- and second-generation antipsychotics: a state-of-the-art clinical review. Ther Clin Risk Manag. 2017;13:757–777.
3 Kun IZ, Szántó Z, Kun I, et al. Konvencionális és atípusos antipszichotikumok okozta metabolikus szindróma. Orvostudományi Értesítő. 2017;90:7–18.
4 Citrome L. The ABC's of dopamine receptor partial agonists - Aripiprazole, brexpiprazole and cariprazine: The 15-min challenge to sort these agents out. Int J Clin Pract. 2015;69:1211–1220.
5 Zimnisky R, Chang G, Gyertyán I, et al. Cariprazine, a dopamine D3-receptor-preferring partial agonist, blocks phencyclidine-induced impairments of working memory, attention set-shifting, and recognition memory in the mouse. Psychopharmacology (Berl). 2013;226:91–100.
6 Corponi F, Fabbri C, Bitter I, et al. Novel antipsychotics specificity profile: A clinically oriented review of lurasidone, brexpiprazole, cariprazine and lumateperone. Eur Neuropsychopharmacol. 2019;29:971–985.
7 Nasrallah HA. Atypical antipsychotic-induced metabolic side effects: insights from receptor-binding profiles. Mol Psychiatry. 2008;13:27–35.
8 Horn H, Böhme B, Dietrich L, et al. Endocannabinoids in body weight control. Pharmaceuticals. 2018;11:1–48.
9 Bába L-I, Kolcsár M, Hack B, et al. Az endokannabinoid rendszer: receptoroktól a terápiáig (The endocannabinoid system: from the receptors to therapy). Orvostudományi Értesítő. 2019;92:1–14.
10 Pertwee RG, Howlett a C, Abood ME, et al. International Union of Basic and Clinical Pharmacology. LXXIX . Cannabinoid Receptors and Their Ligands : Beyond CB 1 and CB 2. Pharmacol Rev. 2010;62:588–631.
11 Lu HC, MacKie K. An introduction to the endogenous cannabinoid system. Biol Psychiatry. 2016;79:516–525.
12 Faah - Fatty-acid amide hydrolase 1 - Rattus norvegicus (Rat) - Faah gene & protein. Available at https://www.uniprot.org/uniprot/P97612 Accessed August 21, 2020.
13 Mgll - Monoglyceride lipase - Rattus norvegicus (Rat) - Mgll gene &

protein. Available at https://www.uniprot.org/uniprot/Q8R431 Accessed August 21, 2020.

14 Matias I, Belluomo I, Cota D. The Fat Side of the Endocannabinoid System: Role of Endocannabinoids in the Adipocyte. Cannabis Cannabinoid Res. 2016;1:176–185.

15 Gasperi V, Fezza F, Pasquariello N, et al. Endocannabinoids in adipocytes during differentiation and their role in glucose uptake. Cell Mol Life Sci. 2007;64:219–229.

16 Perwitz N, Fasshauer M, Klein J. Cannabinoid receptor signaling directly inhibits thermogenesis and alters expression of adiponectin and visfatin. Horm Metab Res. 2006;38:356–358.

17 Lazzari P, Serra V, Marcello S, et al. Metabolic side effects induced by olanzapine treatment are neutralized by CB1 receptor antagonist compounds co-administration in female rats. Eur Neuropsychopharmacol. 2017;27:1–12.

18 Liebig M, Gossel M, Pratt J, et al. Profiling of energy metabolism in olanzapine-induced weight gain in rats and its prevention by the CB1-antagonist AVE1625. Obesity (Silver Spring). 2010;18:1952–1958.

19 Minet-Ringuet J, Even PC, Valet P, et al. Alterations of lipid metabolism and gene expression in rat adipocytes during chronic olanzapine treatment. Mol Psychiatry. 2007;12:562–571.

20 Bába L-I, Gáll Z, Kolcsár M, et al. Effect on Body Weight and Adipose Tissue by Cariprazine : A Head-to-Head Comparison Study to Olanzapine and Aripiprazole in Rats. Sci Pharm. 2020;88:1–14.

21 Weston-Green K, Huang XF, Deng C. Olanzapine treatment and metabolic dysfunction: A dose response study in female Sprague Dawley rats. Behav Brain Res. 2011;217:337–346.

22 De Santis M, Pan B, Lian J, et al. Different effects of Bifeprunox, Aripiprazole, and Haloperidol on body weight gain, food and water intake, and locomotor activity in rats. Pharmacol Biochem Behav. 2014;124:167–173.

23 Watson DJG, King M V., Gyertyán I, et al. The dopamine D3-preferring D2/D3 dopamine receptor partial agonist, cariprazine, reverses behavioural changes in a rat neurodevelopmental model for schizophrenia. Eur Neuropsychopharmacol. 2016;26:208–224.

24 Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB J. 2008;22:659–661.

25 Eaton SL, Roche SL, Llavero Hurtado M, et al. Total Protein Analysis as a Reliable Loading Control for Quantitative Fluorescent Western Blotting. PLoS One. 2013;8:1–9.

26 Moritz CP. Tubulin or Not Tubulin: Heading Toward Total Protein Staining as Loading Control in Western Blots. Proteomics. 2017;17:1–12.

27 Welinder C, Ekblad L. Coomassie staining as loading control in Western blot analysis. J Proteome Res. 2011;10:1416–1419.

28 Skrede S, Martins L, Berge RK, et al. Olanzapine depot formulation in rat: A step forward in modelling antipsychotic-induced metabolic adverse effects. Int J Neuropsychopharmacol. 2014;17:91–104.

29 Weston-Green K, Huang XF, Deng C. Alterations to melanocortinergic, gabaergic and cannabinoid neurotransmission associated with olanzapine-induced weight gain. PLoS One. 2012;7:1–12.

30 Nisoli E. Endocannabinoids and obesity development - The adipose tissue. Drug Discov Today Dis Mech. 2010;7:e199–e204.

31 Engeli S, Böhnke J, Feldpausch M, et al. Activation of the peripheral endocannabinoid system in human obesity. Diabetes. 2005;54:2838–2843.

32 Tam J, Godlewski G, Earley BJ, et al. Role of adiponectin in the metabolic effects of cannabinoid type 1 receptor blockade in mice with diet-induced obesity. Am J Physiol - Endocrinol Metab. 2014;306:E457-468.

33 Hu Y, Young AJ, Ehli E a., et al. Metformin and berberine prevent olanzapine-induced weight gain in rats. PLoS One. 2014;9:1–9.

34 Skrede S, Fernø J, Vázquez MJ, et al. Olanzapine, but not aripiprazole, weight-independently elevates serum triglycerides and activates lipogenic gene expression in female rats. Int J Neuropsychopharmacol. 2012;15:163–179.

35 André A, Gonthier MP. The endocannabinoid system: Its roles in energy balance and potential as a target for obesity treatment. Int J Biochem Cell Biol. 2010;42:1788–1801.

36 Ruiz de Azua I, Lutz B. Multiple endocannabinoid-mediated mechanisms in the regulation of energy homeostasis in brain and peripheral tissues. Cell Mol Life Sci. 2019;76:1341–1363.

37 Kale VP, Gibbs S, Taylor JA, et al. Preclinical toxicity evaluation of JD5037, a peripherally restricted CB1 receptor inverse agonist, in rats and dogs for treatment of nonalcoholic steatohepatitis. Regul Toxicol Pharmacol. 2019;109:1044983.

38 Barutta F, Bellini S, Mastrocola R, et al. Reversal of albuminuria by combined AM6545 and perindopril therapy in experimental diabetic nephropathy. Br J Pharmacol. 2018;175:4371–4385.

39 Tan S, Liu H, Ke B, et al. The peripheral CB1 receptor antagonist JD5037 attenuates liver fibrosis via a CB1 receptor/β-arrestin1/Akt pathway. Br J Pharmacol. 2020;177:2830–2847.