

1 **1. Title page**

2 **Effects of Chronic Exercise on Endocannabinoid System in Obese Wistar Rats Induced by High Fat Diet.**

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18 **Short title:** Endocannabinoid system and exercise

19

20 **2. Abstract**

21 **Objective :** The endocannabinoid system is dysregulated during obesity in tissues involved in the control of food intake and energy
22 metabolism. We examined the effect of chronic exercise on the tissue levels of endocannabinoids (eCBs) and on the expression of
23 genes coding for CB1, CB2 receptors (*Cnr1* and *Cnr2* respectively) in the subcutaneous (SAT) and visceral adipose tissues, and in the
24 soleus and extensor digitorum longus (EDL) muscles, in rats fed with standard or high fat diet. **Methods:** Twenty-eight male Wistar
25 rats were placed on high fat diet or standard diet (HFD and Ctl group respectively) during 12 weeks whereafter half of each group was
26 submitted to an exercise-training period of 12 weeks (HFD+training and Ctl+training). Tissue levels of eCBs were measured by LC-
27 MS while expression of genes coding for CB1, CB2 receptors were investigated by qPCR. **Results:** High fat diet induced an increase
28 in anandamide (AEA) levels in Soleus and EDL ($p < 0.02$). In soleus of the HFD group, these results were accompanied by an
29 elevated *Cnr1* mRNA ($p < 0.05$). In EDL, exercise training allowed to reduce significantly this diet-induced AEA increase ($p <$
30 0.005). 2-arachidonoylglycerol (2-AG) levels were decreased and increased by high fat diet in SAT and EDL respectively ($p < 0.04$)
31 but not affected by exercise training. Unlike in HFD+ training, 2-AG level in soleus was also decreased in HFD group comparatively
32 to Ctl group ($p < 0.04$). **Conclusion:** The levels of eCBs and *Cnr1* expression are altered in a tissue-specific manner following high
33 fat diet and chronic exercise reverses some of these alterations.

34 **Key words: Key words:** 2-Arachidonyolglycerol, Anandamide, adipose tissue, CB1 receptor, CB2 receptor, skeletal muscle.

35

36 3. Introduction

37 Targeting the endocannabinoid system (ECS) is an interesting way to prevent weight gain as accumulating evidence highlights the
38 role of endocannabinoids (eCBs) and CB1 receptors in food intake [27] and energy metabolism regulation [37]. The ECS is a
39 complex endogenous signaling system comprising 7-transmembrane domain receptors (cannabinoid CB1 and CB2 receptors), their
40 endogenous lipid-derived ligands (the eCBs) and enzymes for ligand biosynthesis and degradation. The two most studied eCBs are *N*-
41 arachidonylethanolamine (AEA), also known as anandamide, and 2-arachidonoylglycerol (2-AG). AEA is only one of a large family
42 of related bioactives acylethanolamides which includes both *N*-oleylethanolamine (OEA) and *N*-palmitoyl-ethanolamine (PEA) [2]
43 that can share the same enzymes of AEA metabolism [20]. eCBs are not stored in cells but are synthesized on demand from
44 arachidonic acid containing phospholipid precursors in the cell membrane through enzyme activation by multiple pathways possibly
45 in response to elevated levels of intracellular calcium, membrane depolarization and/or receptor stimulation [19]. Enzymes known in
46 eCBs synthesis are N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), protein tyrosine phosphatase N22 (PTPN-22),
47 glycerophosphodiesterase 1 (GDE-1) and α / β -hydrolase 4 (ABHD4) for AEA and diacylglycerol lipase α (DAGL- α),
48 diacylglycerol lipase β (DAGL- β) for 2-AG [12]. AEA is degraded by the fatty acid amide hydrolase (FAAH) while 2-AG
49 catabolism is mediated by monoacylglycerol lipase (MAGL), α / β -hydrolase 6 (ABHD6) and α / β -hydrolase 12 (ABHD12)[12].
50 At the peripheral level, the ECS is an important factor in the modulation of energy metabolism [37]. **Indeed, the entire eCB**
51 **machinery is expressed in peripheral tissues involved in energy balance regulation such as white adipose tissue and skeletal**
52 **muscle [37]. In adipocytes, CB1 activation promotes fatty acid de novo biosynthesis, triglycerides accumulation by activating**
53 **glucose uptake, fatty acid synthase and inhibits lipolysis by decreasing mitochondrial biogenesis [41, 43]. This adipocyte**
54 **endocannabinoid tone is subject to negative feedback control by hormones and peroxisome proliferator-activated-receptors**
55 **PPAR, including leptin [14], insulin [30], PPAR γ [28] and PPAR δ [47]. In the skeletal muscle, CB1 stimulation reduces**
56 **glucose uptake, insulin sensitivity [22, 23], oxidative pathways and mitochondrial biogenesis [6], thus slowing down energy**
57 **expenditure [38]. Taken together, these data suggest that ECS activation induce an energy balance toward energy storage.**
58 **Recent data demonstrate a link between obesity development and ECS deregulation in different tissues involved in**
59 **metabolism regulation [29]. This deregulation is usually characterized by an ECS overactivity as an increase in eCB levels,**
60 **modifications of CB1 expression and alterations in enzymes involved in eCB biosynthesis and degradation were observed**
61 **during obesity [37]. In the visceral fat of diet-induced obese mice, elevated levels of eCBs have been observed [8, 28, 39], but**
62 **reduced levels are instead found in the subcutaneous white adipose tissue of these animals [39]. Regarding the lipogenic action**
63 **of CB1 receptors, the unbalance of the ECS between subcutaneous and visceral white adipose tissues might eventually**
64 **contribute to accumulation in the latter at the expenses of the former, and, hence, to the several metabolic disorders associated**
65 **with visceral obesity [38]. In obese rodent skeletal muscle, AEA and 2-AG might be also increased by alterations in their**
66 **biosynthetic or degrading enzymes possibly contributing to the impairment in glucose uptake and mitochondrial oxydations**
67 **[19]. Thus this overactivity in peripheral tissues involved in energy homeostasis may participate in excessive and/or ectopic fat**
68 **accumulation, insulin sensitivity impairment and related metabolic disorders [14, 29]. Whether ECS deregulation is a**
69 **consequence or a cause of obesity, it represents a primary target for the treatment of abdominal obesity and associated metabolic**
70 **changes [33].**
71 A healthy lifestyle approach could be effective to diminish ECS **overactivity** [13] and may represent a safer alternative than the
72 pharmaceutical approaches. In subcutaneous and visceral adipose tissues from rodents, chronic exercise limits the CB1 gene
73 expression increase induced by high fat diet [47]. Thus, it is possible that chronic physical activity counteracts ECS deregulation in
74 these tissues. However this potential beneficial adaptation of the ECS remains to be confirmed in tissues involved in energy balance
75 control and adaptable to exercise, such as the skeletal muscle [35]. In obese volunteers, exercise improves muscle metabolism by
76 enhancing glucose uptake, insulin sensitivity, oxidative pathways and mitochondrial biogenesis [35]. These elements are also
77 negatively regulated by ECS and might be disturbed by CB1 overactivity during obesity [6, 23, 41]. Thus, it could be hypothesized
78 that chronic exercise will reverse obesity-induced ECS alterations in skeletal muscle.

79 This study aimed at identifying the changes in the tissue concentrations of AEA and 2-AG as well as of two AEA congeners, OEA
80 and PEA, together with corresponding alterations in the expression of genes encoding *Cnr1* and *Cnr2* for eCB receptors (CB1, CB2)
81 and enzymes involved in the anabolic (ABHD4, GDE-1, NAPE-PLD and PTNP-22, for AEA, OEA and PEA; DAGL- α and DAGL-
82 β , for 2-AG) and catabolic (FAAH, for AEA, OEA and PEA; ABHD6, ABHD12 and MAGL, for 2-AG) pathways of the eCBs, after
83 regular endurance training in the subcutaneous (SAT) and visceral (VAT) adipose tissues, and in the **slow-type** soleus and **fast-type**
84 extensor digitorum longus (EDL) muscles of Wistar rats fed with a standard or a high fat diet. Finally, we analyzed the expression of
85 the transient receptor potential vanilloid type-1 (TRPV1) channel, which is activated by eCBs as well as by AEA congeners, and is
86 considered a ionotropic receptor for eCBs [49].
87

88 4. Methods

89 Animals and experimental procedures

90 Twenty-eight male Wistar rats (3 weeks old) were housed in groups of three per cage. After one week of acclimatization, rats were
91 divided in 2 groups and fed with a standard diet or a high fat diet in order to induce obesity during 24 weeks. After 12 weeks, half of
92 the rats were submitted to 12 weeks of exercise training (Ctl+training, n = 7 and HFD+training, n = 7). The second half of the rats
93 remained untrained for 12 weeks (Ctl and HFD groups for rats on standard (n=7) and high fat (n = 7) diets respectively). Before and
94 after the training period, Ctl+training and HFD+training performed a MAV (Maximal Aerobic Velocity) test on the treadmill. Five
95 days before sacrifice, all rats were subjected to an oral glucose tolerance test (OGTT). At the end of the training period, all rats were
96 anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). Soleus (slow-type postural muscle) and EDL (fast-
97 type muscle) muscles, epididymal VAT, inguinal SAT and blood were collected.
98 All procedures described were approved by both the Agricultural and Forest Ministry and the National Education Ministry
99 (Veterinary service of health and animal protection).

100

101 Diet-induced obesity

102 Rats were fed *ad libitum* with two different types of diet during the 24 weeks of the experimentation:

- 103 - A high fat diet (Purified Diet 231 HF, Safe, Augy. France) with an energy equivalent of 5.05 kcal.g⁻¹. It contained
104 26.9% of proteins, 39.7% of lipids and 10.1% of carbohydrates.
- 105 - A standard diet with an energy equivalent of 2.90 Kcal.g⁻¹. It contained 16% of proteins, 3 % of lipids, 60% of
106 carbohydrates and 21% of other components (fiber, mineral, humidity).

107 Fatty acid compositions of the two diets were provided by the manufacturer (Safe, Augy. France) and are described in table 1. Food
108 and caloric intake by each rat and their weight gain were estimated 2 times per week during the experimentation.

109

110 Maximal aerobic velocity test

111 Animals in the HFD+training and Ctl+training were familiarized with treadmill running (L810, Bioseb. France) during 10 min for 5
112 days at a velocity of 20 cm.s⁻¹ and a 0° slope. Electric shocks were used sparingly to motivate the rats to run. After the familiarization
113 period, Ctl+training and HFD+training groups performed a graded exercise test to voluntary exhaustion. The test started at 20 cm.s⁻¹
114 for 5 min, followed by speed increment of 3 cm.s⁻¹ every 3 minutes until the animal could no longer keep up with the treadmill speed.
115 Exhaustion was reached when animal sat longer than 10 s on electric shock grid. MAV was defined as the velocity of the last 3 min
116 stage completed. The same protocol was repeated 1 week before rats sacrifice to determine the change in MAV with exercise training
117 (two days before the OGTT).

118

119 Exercise training program

120 The day after the baseline MAV test, Ctl+training and HFD+training groups started the 12-week exercise training period that
121 consisted of treadmill running for 1hour/day, 5 days/week at an intensity set between 70 – 80 % of the MAV. The intensity was
122 increased by 1 cm.s⁻¹ every week to take into account the adaptations to exercise training. Animal exercised at the same hour of the
123 day at the end of the room dark cycle (7:30 am). Control groups were in the same room during the training session and handled in the
124 same way to induce a similar stress level. Three days before the sacrifice, exercise training was stopped to avoid the acute effect of
125 exercise, fatigue or stress.

126

127 Oral Glucose Tolerance Test

128 Five days before sacrifice, the animals were fasted overnight. Basal blood glucose level, defined as T0, was determined using an
129 automatic glucometer (Accu-Chek Performa; Roche Diagnostics) before oral administration (4 ml.kg⁻¹ of body weight) of a D-glucose
130 solution (50%). Tail vein blood glucose was then measured at 30, 60, 90, 120 min after the administration. Total Area under the curve
131 (AUC) was calculated using the trapezoidal method [36].

132

133 **Samples collection**

134 The day before the end of the experiment, rats were fasted in order to obtain the same nutritional state for each. For all groups, soleus,
135 EDL, SAT from the inguinal region and VAT from the epididymal fat were quickly removed from animals anesthetized with
136 pentobarbital sodium (60 mg.kg⁻¹ of body weight, intraperitoneal injection). Samples were weighed, immediately frozen in liquid
137 nitrogen and stored at -80°C until analyses.

138 Blood samples were collected by cardiac puncture and then directly drawn into pre-cooled 5-mL EDTA (Ethylenediaminetetraacetic
139 acid) tubes. The later were immediately centrifuged (less than 5 min after sampling) and plasma was removed and frozen (- 80 °C)
140 until analysis.

141

142 **Plasma analyses**

143 The fasting glucose concentration in plasma was measured using a commercially available colorimetric assay kit (Cayman Chemical
144 Company, USA). Fasting plasma insulin was determined using a commercially available rat insulin enzyme immunoassay kit (SPI-
145 BIO, France).

146

147 **Measurements of tissue eCBs**

148 The extraction, purification, and quantification of eCBs from tissues have been performed as previously described [20]. Briefly, the
149 tissues were dounce-homogenized and extracted with chloroform/methanol/Tris-HCl 50 mmol.l⁻¹ pH 7.5 (2:1:1, vol/vol) containing
150 internal standards ([2H]8 AEA; [2H]5 2-AG, [2H]5 PEA and [2H]4 OEA 5 pmol each). The lipid-containing organic phase was dried
151 down, weighed, and pre-purified by open-bed chromatography on silica gel. Fractions were obtained by eluting the column with 99:1,
152 90:10 and 50:50 (v/v) chloroform/methanol. The 90:10 fraction was used for AEA, 2-AG, PEA and OEA quantification by liquid
153 chromatography – atmospheric pressure chemical ionization – mass spectrometry by using a Shimadzu high-performance liquid
154 chromatography apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2020) quadruple mass spectrometry via a Shimadzu
155 atmospheric pressure chemical ionization interface as previously described [20]. The amounts of analyses in tissues quantified by
156 isotope dilution with the above mentioned deuterated standards, were expressed as pmol.g⁻¹ of wet tissue weight.

157

158 **RNA purification and quantitative real-time PCR (qPCR)**

159 Total RNA was isolated from native tissues by use of the TRI-Reagent (Sigma-Aldrich, Milan, Italy), reacted with DNase-I (1 U/ml;
160 Sigma-Aldrich) for 15 min at room temperature, and followed by spectrophotometric quantification. Final preparation of RNA was
161 considered DNA- and protein-free if the ratio between readings at 260/280 nm was ≥ 1.7 . Isolated mRNA was reverse transcribed by
162 use of SuperScript III Reverse Transcriptase [Life Technologies, Monza (MI), Italy]. The quantitative real-time PCR was carried out
163 in CFX384 real-time PCR detection system [Bio-Rad, Segrate (MI), Italy] with specific primers [20] by the use of SYBR Green
164 master mix kit [Bio-Rad, Segrate (MI)] (See table 2 for primers sequences).

165 Samples were amplified simultaneously in quadruplicate in one-assay run with a non-template control blank for each primer pair to
166 control for contamination or primer-dimers formation, and the ct (cycle threshold) value for each experimental group was determined.
167 The housekeeping gene (the hypoxanthine-guanine phosphoribosyltransferase, hprt) was used as an internal control to normalize the
168 ct values, using the $2^{-\Delta Ct}$ formula. Differences in mRNA content between groups were as expressed as $2^{-\Delta\Delta Ct}$.

169

170 **Statistical analyses**

171 Data are shown as means \pm SD, except where otherwise indicated. Normal Gaussian distribution of the data was verified by the
172 Shapiro-wilk test. Two or 3-way ANOVAs were used to evaluate the evolution of weight during the first 12 weeks of the experiment
173 (Time x Diet), the evolutions of weight (Time x Diet x Exercise) and MAV (Time x Diet) during the exercise-training period and
174 mean caloric intake during each 12-week period (the first 12 weeks, Time x Diet and the further 12 weeks training period, Time x
175 Diet x Exercise). Multiple comparisons were made with the Newman-Keul post-hoc test. A two-way ANOVA was used to evaluate
176 the effects of diet, exercise training and the diet x exercise interaction on metabolic parameters (fasting glucose, insulin, AUC during

177 OGTT), and on tissue eCBs and congeners levels. Multiple comparisons were made with the Bonferroni post-hoc test if significant
178 main effects or interaction were observed with ANOVA. Concerning gene expression data, ctl group was compared with other groups
179 by use of the Mann-Whitney U test. Statistical significance was set at $p < 0.05$ for all analyses. All calculations were made with
180 Statistica 6.0 (Statsoft, Tulsa, USA).

181

182 5. Results

183 Effect of diet and/or exercise on body weight, food intake, maximal aerobic velocity, basal glucose and insulin levels, and 184 glucose tolerance (Table 3)

185 Body mass increased with time and this all the more in case of high fat diet throughout the 12 and 24 weeks of study. During the
186 second period (12 to 24 weeks), exercise training slowed down the time-induced body mass gain. Mean caloric intake per day and per
187 rat were increased in rats on high fat diet but the increase induced by time slowed down with this diet.

188 MAV was measured only in exercise trained groups to avoid a familiarization in Ctl and HFD groups that could affect the results.
189 Two-way ANOVA for MAV revealed that MAV was reduced by high fat diet **but that** exercise training increased MAV in a
190 comparable extent **in both diets**.

191 Plasma insulin levels were neither affected by diet nor by exercise training. Fasting plasma glucose concentrations were increased
192 by high fat diet but this increase was strongly reduced by exercise training. Consequently, at the end of the experimentation, the
193 HFD group was exposed to much higher plasma glucose concentrations compared to all the other groups, included the
194 HFD+training group. Glucose AUC during the OGTT was also significantly increased by high fat diet but without significant
195 protecting effect of exercise training.

196 Effect of diet and/or exercise on AEA, 2-AG, PEA, OEA levels (Table 4) and the expression of genes coding for eCB 197 receptors and enzymes implicated in the synthesis or degradation of eCBs in adipose (Figure 1) and skeletal muscle (Figure 198 2) tissues.

199 Adipose tissue

200 In SAT, high fat diet decreased 2-AG whereas exercise training or combination of both had no effect on this eCB nor on AEA and
201 its congeners levels. Despite this diet effect on 2-AG, the mRNA levels of the enzymes for 2-AG biosynthesis or degradation
202 were not affected significantly in HFD group (Fig.1c). While chronic exercise did not change the expression of genes coding for
203 enzymes involved in AEA and 2-AG metabolism in lean rats, we found a significant increase in the expression of genes coding for
204 GDE-1, FAAH, DAGL- α , ABHD12 and MAGL when exercise training was combined with high fat diet in SAT (Fig.1b and 1c).
205 Concerning the expression of genes coding for cannabinoid receptors in SAT, high fat diet in lean rats reduced significantly *Cnr1*
206 mRNA levels (Fig.1a). However, when chronic exercise was added to high fat diet, this diet effect was completely reversed with
207 *Cnr1* mRNA levels strongly enhanced in HFD+training group (Fig.1a). *Trpv1* mRNA levels were not affected by diet, but were
208 increased by exercise training in both lean and high fat diet rats compared to the ctl group (Fig.1a).

209 In VAT, as seen in table 4 and figure 1, high fat diet or exercise training or the combination of both did not induce significant
210 changes in eCBs or AEA congeners levels but affected gene expression of eCBs biosynthetic or degradation enzymes. Twenty-
211 four weeks of high fat diet increased significantly *Gde-1* and *Ptpn-22* mRNA levels in sedentary rats (Fig. 1e). This increase was
212 not observed anymore for *Gde-1* mRNA levels in the HFD + training group, but was always present for *Ptpn-22* in the
213 ctrl+training and HFD+training groups (Fig. 1e). Expression of FAAH, the major AEA degrading enzyme (Fig. 1e), was
214 significantly higher in HFD, ctrl+training and HFD+training groups as compared to ctl. High fat diet increased significantly
215 DAGL- α and ABHD-12 mRNA in sedentary rats (Fig.1f). This increase in *Abhd12* gene expression was also observed in
216 ctrl+training group but not in the HFD+training group (Fig.1f). Exercise combined with high fat diet had no effect on enzyme gene
217 expression involved in 2-AG metabolism even though MAGL mRNA was slightly but non-significantly increased (Fig.1f). While
218 *cnr1* gene expression was significantly increased in all groups comparatively to ctl group in VAT, *cnr2* mRNA level was
219 significantly decreased in all experimental conditions (Fig.1d). *Trpv1* mRNA levels was only increased significantly in HFD group
220 (Fig. 1d).

221

222 *Skeletal muscles*

223 In the soleus, 24 weeks of high fat diet induced significant higher AEA and OEA concentrations whereas exercise training had no
224 significant effect. Contrary to AEA and its congeners, soleus 2-AG concentrations were affected when diet was paired with
225 exercise training. Between group comparison indicated that HFD group presented significant lower 2-AG concentration than Ctl
226 group and this 2-AG decrease was no longer observed in the HFD+ training group.

227 The increase of AEA levels induced by high fat diet in the soleus was accompanied by the modification of the gene expression of
228 enzymes involved in AEA metabolism in HFD group (Fig. 2b). We observed higher levels of *Abhd4* mRNA and lower levels of
229 *Ptpn-22* and *Faah* mRNA. The altered mRNA levels of these genes, especially *Abhd4* and *Faah* could explain the increase in AEA
230 and OEA levels induced by high fat diet. However, they were also observed following exercise program in lean and obese rats, in
231 which exercise training had no significant effect on AEA and OEA levels. Concerning enzymes potentially involved in 2-AG
232 metabolism (Fig. 2c), the mRNA of *Abhd6* was overexpressed in HFD, Ctl+training and HFD+training groups, whereas *Abhd12*
233 mRNA was less expressed in HFD and Ctl+training group. *Magl* mRNA expression was also affected by exercise and was lower
234 in Ctl+training and HFD+training groups. *Cnr1* gene expression in soleus was significantly increased with HFD compared to Ctl
235 group, and this difference was no longer observed with exercise training (Fig. 2a).

236 In the EDL, AEA and 2-AG concentrations were increased with high fat diet. Whereas exercise had not effect on 2-AG level, it
237 induced a significant decrease in AEA. Moreover, it allowed to reduce the AEA increase induced by high fat diet. Thereby post
238 hoc test revealed that HFD group presented a significant higher AEA concentration than all other groups. The increase in AEA
239 levels in HFD group was again accompanied by a significant increase in *Abhd4* mRNA levels and a decrease in *Faah* mRNA
240 levels, as shown in figure 2e. In this tissue, however, only the increase in *Abhd4* mRNA level was still observed with exercise in
241 Ctl+training and HFD+training groups, whereas the decrease in *Faah* levels was not, thus possibly explaining the reversal of the
242 elevation of AEA levels by chronic exercise. Gene expression of *Ptpn22* (Fig. 2e) was significantly increased with exercise only in
243 lean rats. Neither high fat diet nor exercise training nor the combination of both affected gene expression of enzymes involved in
244 2-AG metabolism (Fig. 2d).

245 As shown in figure 2a, the mRNA expression of genes coding for CB1, CB2 and TRPV1 in EDL were not altered by high fat diet,
246 exercise nor the combination of both.

247

248 6. Discussion

249 The aim of this study was to examine the effect of chronic exercise on the tissue levels of eCBs and two AEA congeners and on
250 the expression of genes coding for CB1, CB2 receptors and the enzymes responsible for synthesis and degradation of eCBs in
251 adipose tissues and skeletal muscles, in Wistar rats fed with standard or high fat diet. Our results can be summarized by stating
252 that high fat diet or exercise training induced different changes in eCB levels and/or eCB receptor expressions in all tissues
253 analyzed. Noteworthy, the changes induced by high fat diet were tissue-specific and some of these changes were reversed by
254 exercise training.

255 As expected, rats fed with a high fat diet exhibited abnormalities that precede diabetes as indicated by higher fasting glucose levels
256 and glucose intolerance during an OGTT. Basal insulin levels remained even so **unchanged** with the high fat diet regimen, which
257 may reflect a relative deficiency of insulin secretion. However, this finding must be taken with caution as metabolic perturbations
258 induced by HFD depend on the dietary regimen and the animal model used [17]. We confirmed that endurance exercise improves
259 MAV and metabolic syndrome risk factors such as basal glycemia and weight gain in rat model of diet induced obesity (DIO)
260 [42]. Nevertheless, although glucose intolerance during the OGTT seemed to be slightly improved in the HFD +training group, the
261 difference with the HFD group did not reach statistical significance.

262 *Effect of High Fat Diet on the ECS in the adipose tissue and skeletal muscle.*

263 Elevated levels of eCBs and *Cnr1* gene expression have been observed in the visceral fat of DIO rodents [39, 47] but reduced eCBs
264 levels are instead found in the SAT of these animals [39]. This is in line with our present results of decreased 2-AG levels and *Cnr1*
265 mRNA levels in the SAT, and of increased *Cnr1* mRNA levels in the VAT, of rats fed with high fat diet. The 2-AG decrease in SAT
266 induced by high fat diet is difficult to explain regarding mRNA expression of its biosynthetic and/or degrading enzymes in the 2
267 groups on high fat diet. While no changes appeared in HFD group, biosynthetic (*dagl- α*) and degrading enzyme (*abhd12*, *magl*)
268 mRNA levels were overexpressed at the same time in the HFD+training group. Thus, this lack of consistency between the two HFD
269 groups suggests that more than mRNA expression of enzymes, enzymatic activities and eCB precursors may be more involved in the
270 2-AG decrease induced by high fat diet. **Whatever, these results confirm the existence of an unbalanced eCB tone between these**
271 **two adipose tissue depots in favor of VAT. Regarding the inhibitory effect of insulin on 2-AG and the insulin resistance that**
272 **occurs during obesity in visceral more than subcutaneous adipocytes, these 2-AG unbalance might be the result of the loss of**
273 **the possible insulin inhibition of eCBs levels in VAT [39]. Thus this lack of CB1 stimulation by the 2-AG decrease in SAT**
274 **may impair adipocyte glucose uptake [43] and then participating to the hyperglycemia observed in rat submitted to high fat**
275 **diet. Moreover the unbalanced eCB tone might impair lipogenesis in SAT and its capacity to buffer energy surplus leading to**
276 **fat accumulation in favor of VAT and other tissues not suited for lipid storage and hence to the metabolic disorders therewith**
277 **associated [45].**

278
279 In agreement with previous results [11, 39], we have confirmed that both SAT and VAT express mRNA coding for the CB2 receptor.
280 **Activation of the CB2 receptor in adipose tissue might promote tissue inflammation by increasing macrophage infiltration and**
281 **the related inflammatory response and contribute to the development of insulin resistance [11]. Even if CB2 receptor are**
282 **expressed in adipocytes [39], the presence of this receptor in the adipose tissue is mainly attributed to the stromal vascular**
283 **fraction [11]. Deveaux et al.[11] have already reported an increase in *Cnr2* expression in adipose tissue of mice under high fat**
284 **diet.** Interestingly, this *Cnr2* expression increase was the result of macrophage infiltration rather than a raise in adipocyte *Cnr2*
285 expression. In our study, while one may expect an increase in *Cnr2* mRNA in the epididymal adipose tissue in the HFD group,
286 accompanying the probable obesity-associated macrophage infiltration as already observed by Deveaux et al. [11] in mice, we instead
287 observed a decrease. We did not measure macrophage infiltration or adipose tissue inflammation, but the possible lack of
288 inflammation in our study could not justify the decrease in *Cnr2* mRNA levels, as no change in expression would be expected in this

289 case. Therefore, it is possible that the observed decrease is due to high fat diet-induced non-inflammatory regulatory events in these
290 cells. Further investigations are required to explain this phenomenon.

291 **Importantly, we also observed changes in the expression of *Trpv1* mRNA in the VAT during high fat diet. TRPV1 is known to**
292 **be binded by AEA and to be the molecular integrator of inflammatory mediators [40].** Recent studies underlined also the
293 significant role in the regulation of the glucose homeostasis [49]. Although TRPV1 “knockout” mice are protected against high fat
294 diet-induced obesity [49], TRPV1 activation has been suggested to reduce adipocyte differentiation and obesity [48], to induce a
295 brown phenotype during adipocytes differentiation [3], and to suppress accumulation of VAT in mice fed with a high fat diet [21].
296 Activation of TRPV1 in VAT might thus counteract the effect of unbalanced eCB tone in favor of VAT during high fat diet-induced
297 obesity and prevent visceral fat accumulation.

298
299 Skeletal muscle plays a crucial role in energy homeostasis regulation by being the primary site of glucose disposal and fatty acid
300 oxidation [19]. This role could be altered by ECS dysregulation in obesity and/or hyperglycemia, as suggested by the literature [6, 15,
301 24, 29]. Our results confirmed the study of Iannotti et al.[20] in obese Zucker fa/fa rats. We indeed observed that 24 weeks of high fat
302 diet increased AEA levels in both the soleus and EDL. **As CB1 stimulation reduces glucose uptake, insulin sensitivity [22, 23],**
303 **oxidative pathways and mitochondrial biogenesis [6], these EDL and soleus AEA increases may participate to the glucose**
304 **intolerance observed in rats under high fat diet.** AEA increases are in line with the lower expression of the gene coding for FAAH,
305 the main AEA-degrading enzyme [37] and with the higher mRNA levels of *Abhd4*, coding for an AEA-biosynthetic enzyme
306 (ABHD4) in HFD group. Although *Ptnp22* mRNA levels (coding for PTNP22 another potential AEA-biosynthetic enzyme) were
307 instead decreased in the soleus, this may not influence significantly AEA levels, as PTNP22 involvement in the biosynthesis of the
308 eCB has so far been shown only *in vitro* [25].

309
310 Contrary to AEA, the levels of 2-AG in the soleus were decreased in the HFD group compared to other groups. Previous studies [20,
311 29] have focused on skeletal muscle 2-AG level in rodent obesity models and found conflicting results (i.e. no change or increase). In
312 the present study, the observed decrease of 2-AG concentrations in soleus was probably due to the strong increase of the expression of
313 the gene coding for ABHD6 (*Abhd6*), a 2-AG degrading enzyme. These results confirm the existence of a lower rate of production of
314 2-AG in the skeletal muscle, suggested by Crespillo et al. [9], who reported a decrease and an increase in expression of genes coding
315 for DAGL α and MAGL, respectively. However, a similar effect on 2-AG levels is not always observed in rodents with DIO [20, 29]
316 and we found a significant increase in EDL with high fat diet. These discrepancies among studies may be explained by the influence
317 of the diet, the animal species and the presence of metabolic disturbances on eCB levels [29, 37]. Also muscle composition (i.e. slow
318 vs. fast-twitch muscle fibers) might be another factor affecting ECS dysregulation during obesity and/or hyperglycemia. Indeed, in the
319 present study, the levels of eCBs and congeners, as well as the expression of genes related to eCB enzymatic machinery, were
320 differentially regulated in the soleus and EDL, which are a slow and a fast-twitch muscle, respectively [31]. This difference in ECS
321 response to diet between slow and fast twitch muscles was also observed at the level of eCB receptor expression. As in previous
322 studies conducted in DIO rodents [20, 32], CB1 mRNA expression was increased in the soleus, confirming the existence of ECS
323 alteration, which reduces insulin signaling, glucose uptake and oxygen consumption in this tissue [6, 23, 26]. However, a different
324 situation was found in the EDL, where we observed no change in CB1 (or CB2) mRNA expression. Likewise, in the abdominal
325 muscle, which has a mixed fiber composition, Crespillo et al. [9] detected a trend towards a decrease in CB1 mRNA, and a significant
326 decrease in CB2 mRNA expression, after a 10-week high fat diet period. **Thus muscle ECS response to high fat diet seems to**
327 **depend on muscle fiber composition and may induce specific metabolic responses. Indeed insulin sensitivity alteration induced**
328 **by CB1 activation [22, 23], and the overexpression in CB1 mRNA in soleus may explain in part the larger decrease in insulin**
329 **sensitivity generally observed in slow twitch fibers during chronic hyperglycemia [18].** More investigations are needed to
330 confirm that the ECS is involved in metabolic specific muscle responses to high fat diet.

331 Taken together these ECS tone unbalance between adipose tissues and ECS muscle overactivity might participate in weight
332 gain and in the glucose intolerance observed in rats submitted to high fat diet. Regarding the importance of 1) insulin muscle
333 sensitivity during glucose challenge [10], 2) muscle fiber composition on insulin resistance [18] and 3) glucose uptake and
334 insulin sensitivity reduction induced by CB1 activation [22, 23], we can speculate that ECS alteration in muscle mainly
335 composed by slow twitch fibers could be more deleterious for insulin sensitivity than in fast twitch muscle.

336 *Effect of Exercise on ECS in adipose tissues and skeletal muscles of lean and obese rats*

337 Chronic exercise alone had no effect on eCB tone in the SAT of lean rats, whereas it upregulated the mRNA expression of some eCB
338 biosynthetic and degrading enzymes and prevented the decrease of *Cnr1* mRNA level induced by the high fat diet. Yan et al. [47]
339 have already shown that chronic exercise alone does not affect *Cnr1* expression in the SAT of lean rats. However, they observed also
340 a normalization of *Cnr1* mRNA levels when they combined regular exercise to high fat diet in comparison to diet alone. Exercise
341 training tended also to prevent the 2-AG decrease induced by high fat diet in SAT but non-significantly ($p=0.06$) **and thus, the**
342 **deleterious unbalanced ECS tone in favor of visceral fat accumulation. This effect might be induced by the leptin level**
343 **decrease observed with exercise in obese people [34] that might reduce leptin inhibitory effect on 2-AG [14]. This is in line**
344 **with previous study [5] that observed 2-AG level normalization in SAT after weight loss and fat mass decrease in obese people.**
345 **Indeed fat mass loss induced also a leptin secretion decrease in adipose tissue [4] that might contribute to 2-AG level**
346 **normalization. We did not measure fat mass, and even if the body weights of the HFD and HFD+training groups were not**
347 **significantly different we can speculate that training period has decreased fat mass and increased lean mass.**

348 Concerning the VAT, chronic exercise did not reverse the upregulation of *Cnr1* and the downregulation of *Cnr2* mRNA induced by
349 the high fat diet. However, Yan et al. [47] observed a decrease in *Cnr1* gene expression when they added chronic exercise to HFD.
350 This discrepancy with our study could be explained by differences in the two protocols used. In our study, rats were exposed to
351 exercise training after a high fat diet period of 12 weeks, whereas Yan et al. [47] submitted their rats to exercise from the beginning of
352 the high fat regimen. Thus, it is likely that exercise might prevent the changes in eCB receptor expression induced by high fat diet in
353 the VAT, but not reverse them if they are already present.

354 Training reversed *Trpv1* mRNA upregulation observed in VAT in HFD group. Considering the TRPV1 protective effect against ECS
355 overactivation in the VAT during the high fat diet-induced obesity, it is not surprising to observe a normalization of *Trpv1* mRNA
356 level as exercise might also reverse the negative effects of the high fat diet on this adipose depot. We also observed changes in the
357 expression of *Trpv1*, which was upregulated by training in both lean and high fat diet rats in the SAT. TRPV1 might become
358 upregulated by exercise in the SAT in order to enhance energy expenditure [21] in this adipose depot, regardless of the diet.

359 Importantly, twelve weeks of regular exercise reversed some eCB dysregulation induced by high fat diet in the skeletal muscle. In the
360 soleus, the 2-AG decrease induced by high fat diet was not longer observed in HFD+training group probably due to a decrease in
361 *magl* expression, the gene of the key enzyme in the hydrolysis of this endocannabinoid. *Cnr1* mRNA upregulation in the HFD group
362 was prevented by exercise training in the soleus muscle. This is in agreement with data by Wiklund et al. [46] who reported a decrease
363 in CB1 protein expression in the skeletal muscle of mice fed with a high fat diet after an aerobic exercise period. Thus, this decreased
364 in *Cnr1* mRNA expression in the soleus might participate to an improvement in glucose metabolism previously observed during
365 chronic antagonism of CB1 by increasing muscle glucose uptake [22, 26]. However, it is worth noting that similarly to the
366 Ctl+training group, AEA levels tended to remain elevated in the HFD+training, suggesting an additive and positive effect of chronic
367 exercise and high fat diet on AEA. Moreover the gene expression of several eCB biosynthetic (PTPN-22) and degrading (FAAH,
368 ABHD12, MAGL) enzymes in Ctl+training and HFD+training was altered in the same manner as in the HFD group, in agreement
369 with a higher AEA turnover in the skeletal muscle. This potential similarity between HFD and exercise is surprising as it suggests that
370 chronic exercise, by increasing muscle AEA levels, could **reduce glucose uptake, insulin sensitivity [22, 23], oxidative pathways**
371 **and mitochondrial biogenesis [6] and at least negatively influence muscle metabolism.** However, some studies [6, 15] have
372 already demonstrated that AEA may also exert beneficial effects on markers of muscle glucose metabolism, mitochondrial biogenesis

373 and oxidative mitochondrial capacity independently of cannabinoid receptors [35]. Nevertheless these beneficial adaptations are the
374 consequence of an acute administration of a high dose of AEA, and not of a chronic treatment with the eCB, such as during increased
375 ECS tone in obesity.

376 Concerning the EDL, a fast twitch muscle, exercise training was not accompanied by a decrease in *faah* expression in obese or lean
377 rats as rather observed during high fat diet alone. These different adaptations of the main AEA degrading enzyme to exercise with or
378 without high fat diet vs. diet alone might explain the decrease in AEA level observed with training in this skeletal muscle. Contrary to
379 the soleus, exercise decreased AEA level in EDL **that might suggest a better improvement in glucose metabolism in fast twitch**
380 **muscle regarding the AEA effect on skeletal muscle glucose transport activity and muscle insulin sensitivity. This is in line**
381 **with previous studies that have demonstrated that the exercise-induced increase in skeletal muscle insulin sensitivity was**
382 **larger in fast twitch muscle [7, 16].**

383 **Taken together, normalizations of the expression of the gene coding for the CB1 receptor in the soleus and of the AEA levels**
384 **in the EDL might participate in improvement in the basal glycemia observed in the HFD+training group. Indeed, previous**
385 **studies have already demonstrated an improvement in skeletal muscle glucose metabolism when reducing eCB tone with a**
386 **CB1 antagonist [22, 23, 26]. However ECS response to exercise in lean and obese rats seems to be different according the**
387 **skeletal muscle type. More investigations are needed to understand the meaning of different eCB remodeling induced by**
388 **exercise in slow and fast twitch muscles.**

389 Our study has several limitations. First, we did not assess protein **level** of receptors or enzymes and **their functionalities**. Second,
390 MAV was measured to show exercise training efficiency. Nevertheless the use of skeletal muscle, adipose or plasma tissue
391 remodeling markers would have been more relevant to highlight the effect of training or diet and their associations with ECS changes.
392 Third, recent findings showed that eCBs and their receptors are sensitive to diet composition and more particularly to polyunsaturated
393 fatty acids (PUFA)[44]. Dietary PUFA induced a remodeling in the phospholipid composition of cell membranes and changed
394 substrate availability for the biosynthesis of eCBs [44]. Alvheim et al. [1] have already observed an increase in arachidonic acid (the
395 substrate for the eCBs) and thus in AEA and 2-AG levels in liver of mice fed with a diet enriched in linoleic acid. In our study, high
396 fat diet used was also mainly composed by this fatty acid, which could have increased arachidonic acid availability and thus
397 influenced the tissue eCB levels [1]. Thus eCB changes presented in our study may be the result of both metabolic disorders
398 associated with obesity and the diet-induced increase in eCB precursors.

399 *Conclusion*

400 In summary, we have confirmed here that weight gain and perturbations of glucose metabolism are accompanied by changes in the
401 ECS in major tissues involved in metabolism regulation which are adipose tissue and skeletal muscle. These alterations are tissues-
402 specific and, notably, for the muscle we observed here for the first time a difference in HFD-induced ECS dysregulation between
403 glycolytic and oxidative muscles. Interestingly, adding chronic exercise to HFD reverses some of these alterations in the different
404 tissues. Nevertheless, depending on the tissue, exercise *per se* could mimic the effect of HFD on the levels of eCBs and/or their
405 receptors and/or their anabolic and catabolic enzymes. This seemingly paradoxical response is observed in the VAT and, in part, in
406 the oxidative soleus muscle, and highlights the need to investigate the role of the ECS also in the beneficial metabolic adaptations
407 induced by chronic exercise.

408

409

410

411 **7. List of abbreviations**

412 2-AG: 2-arachidonoylglycerol

413 ABHD4 : α / β -hydrolase 4

414 *Abhd4* : α / β -hydrolase 4 gene

415 ABHD6 : α / β -hydrolase 6

416 *Abhd6* : α / β -hydrolase 6 gene

417 ABHD12 : α / β -hydrolase 12

418 *Abhd12* : α / β -hydrolase 12 gene

419 AEA: anandamide

420 CB1: Cannabinoid receptor 1

421 CB2: Cannabinoid receptor 2

422 *Cnr1*: Cannabinoid receptor 1 gene

423 *Cnr2*: Cannabinoid receptor 2 gene

424 Ctl: Control group

425 Ctl+training: Exercise training + standard diet group

426 DAGL- α : Diacylglycerol lipase α

427 *Dagl- α* : Diacylglycerol lipase α gene

428 DAGL- β : Diacylglycerol lipase β

429 *Dagl- β* : Diacylglycerol lipase β gene

430 DIO: diet induced obesity

431 eCBs: Endocannabinoids

432 ECS: Endocannabinoid system

433 EDL: Extensor digitorum longus

434 EDTA: Ethylenediaminetetraacetic acid

435 FAAH : Fatty acid amide hydrolase

436 *Faah* : Fatty acid amide hydrolase gene

437 GDE-1: Glycerophosphodiesterase 1

438 *Gde-1*: Glycerophosphodiesterase 1 gene

439 HFD: High fat diet group

440 HFD+training: Exercise training + high fat diet group

441 MAGL: Monoacylglycerol lipase

442 *Magl*: Monoacylglycerol lipase gene

443 MAV : Maximal aerobic velocity

444 NAPE-PLD: N-acylphosphatidylethanolamine-phospholipase D

445 *Nape-pld*: N-acylphosphatidylethanolamine-phospholipase D gene

446 OEA: N-oleoylethanolamine
447 OGTT : Oral glucose tolerance test
448 PEA: *N*-palmitoyl-ethanolamine
449 **PPAR: Peroxisome proliferator-activated-receptor**
450 PTPN-22: Protein tyrosine phosphatase N22
451 *Ptpn-22*: Protein tyrosine phosphatase N22 gene
452 PUFA: polyunsaturated fatty acids
453 SAT: Subcutaneous adipose tissue
454 RNA: Ribonucleic acid
455 mRNA : Messenger RNA
456 TRPV1: Transient receptor potential cation channel subfamily V member 1
457 ***Trpv1*: Transient receptor potential cation channel subfamily V member 1 gene**
458 VAT: Visceral adipose tissue
459

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465

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583 12. Figures legends

584 **Fig 1.** Expression level analysis of the genes related to endocannabinoid metabolism and function in control (Ctl, n=4), high fat diet
585 (HFD, n=4), control with chronic exercise (Ctl+training, n=4) and high fat diet with chronic exercise (HFD+training, n=4) groups.
586 mRNA expression levels of genes encoding for (a) endocannabinoid receptors (*cnr1*, *cnr2*, *trpv1*), (b) enzymes involved in AEA and
587 AEA congeners biosynthesis (*abhd4*, *gde-1*, *nape-pld*, *ptpn22*) and catabolism (*faah*), (c) enzymes involved in 2-AG biosynthesis
588 (*dagla*, *daglβ*) and catabolism (*abhd6*, *abhd12*, *magl*) were measured in subcutaneous adipose tissue. mRNA expression levels of
589 genes encoding for (d) endocannabinoid receptors, (e) enzymes involved in AEA and AEA congeners biosynthesis and catabolism, (f)
590 enzymes involved in 2-AG biosynthesis and catabolism were measured in visceral adipose tissue. The results obtained by qPCR are
591 reported using the $2^{-\Delta\Delta Ct}$ formula using *hprt* as housekeeping gene. Each column is the mean \pm S.E. of at least four independent
592 determinations performed each in quadruplicate. * Significantly different from ctl group: $p < 0.05$.

593 **Fig 2.**

594 Expression level analysis of the genes related to endocannabinoid metabolism and function in control (Ctl, n=4), high fat diet (HFD,
595 n=4), control with chronic exercise (Ctl+training, n=4) and high fat diet with chronic exercise (HFD+training, n=4) groups. mRNA
596 expression levels of genes encoding for (a) endocannabinoid receptors (*cnr1*, *cnr2*, *trpv1*), (b) enzymes involved in AEA and AEA
597 congeners biosynthesis (*abhd4*, *gde-1*, *nape-pld*, *ptpn22*) and catabolism (*faah*), (c) enzymes involved in 2-AG biosynthesis (*dagla*,
598 *daglβ*) and catabolism (*abhd6*, *abhd12*, *magl*) were measured in soleus. mRNA expression levels of genes encoding for (d)
599 endocannabinoid receptors, (e) enzymes involved in AEA and AEA congeners biosynthesis and catabolism, (f) enzymes involved in
600 2-AG biosynthesis and catabolism were measured in extensor digitorum longus muscles. The results obtained by qPCR are reported
601 using the $2^{-\Delta\Delta Ct}$ formula using *hprt* as housekeeping gene. Each column is the mean \pm S.E. of at least four independent
602 determinations performed each in quadruplicate. * Significantly different from ctl group: $p < 0.05$.

603

Table 1. Fatty acid composition of the standard (Ctl) and the high fat diet (HFD).

	Ctl	HFD
Total Fat (g/kg)	27,50	395,47
Total Saturated Fat (g/kg)	6,34	140,36
C10:0	-	0,32
C12:0	0,03	0,32
C14:0	0,17	4,19
C15:0	0,03	0,32
C16:0	5,30	86,93
C17:0	0,03	1,29
C18:0	0,58	45,97
C20:0	0,10	0,94
C22:0	0,06	0,07
C24:0	0,06	-
Total Monounsaturated Fat (g/kg)	5,61	171,18
C16:1	0,19	8,14
C17:1	0,03	0,65
C18:1	5,09	158,96
C19:1	-	0,00
C20:1	0,30	2,80
C22:1	-	0,65
Total Polyunsaturated Fat (g/kg)	15,57	86,54
C18:2	13,70	78,14
C18:3	1,13	3,24
C18:4	-	2,58
C20:2	0,03	1,29
C20:3	-	0,65
C20:4	0,06	-
C20:5	0,14	-
C22:1	0,19	-
C22:4	-	0,32
C22:5	0,06	0,32
C22:6	0,22	-
C24:1	0,06	-
Total ω3 fatty acids (g/kg)	1,57	3,91
Total ω6 fatty acids (g/kg)	13,70	75,35

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Table 2: Primers sequences used in qPCR analysis

Gene	FORWARD Sequence (5'->3')	REVERSE Sequence (5'->3')	Enter Accession Number	Product length (bp)
<i>abhd12</i>	CAGGCGTGCGGTCGAAACCA	TCAAGCTGCAGTCGGCGTCC	NM_001024314.1	189
<i>abhd4</i>	TCTGGCGTCAAGCGGAGGGA	ACGCCACCCCCAAAGCCATG	NM_001108866.1	299
<i>abhd6</i>	AGCGTCTGCTCCCATCCCCA	TGGCTTGCCAGTGGCGTGAA	NM_001007680.1	255
<i>cnr-1</i>	CTGAGGGTTCCCTCCCGGCA	TGCTGGGACCAACGGGGAGT	NM_012784.4	285
<i>cnr-2</i>	GCGGCTAGACGTGAGGTTGGC	TCCTTCAGGACCAAGAGTCTCAGCCT	NM_020543.4	335
<i>dagla</i>	GGCCGCACCTTCGTCAAGCT	ATCCAGCACCGCATTGCGCT	NM_001005886.1	380
<i>daglβ</i>	AGACCCGGGTGCAATGCTGC	GCCCTGGTGTGTGGGTCACG	NM_001107120.1	212
<i>faah</i>	GGCAGAGCCACAGGGGCTATCA	TGGGGCTACAGTGCACAGCG	NM_024132.3	349
<i>gde-1</i>	GCAGCCCCTTCAACGCCTGT	GATGGCCGCCAGCGTGTTCT	NM_019580.4	172
<i>magl</i>	CGGAACAAGTCGGAGGTTGA	TGTCCTGACTCGGGGATGAT	NM_138502.2	220
<i>nape-pld</i>	AGGCTGGCCTACGAATCACGT	ATGGTACACGGGGGACGGCG	NM_199381.1	150
<i>ptpn-22</i>	TGGTCGTGGGAGAGCCGCTT	GGGCCACTTTTTGCGCCTGC	NM_001106460.1	263
<i>trpv1</i>	AGACATCAGCGCCCGGGACT	CCAGCTTCAGCGTGGGGTGG	NM_031982.1	151

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Table 3. Effect of diet and / or exercise on body weight, food intake, maximal aerobic velocity (MAV), basal glucose and insulin levels, oral glucose tolerance test (OGTT) in control (Ctl, n=7), high fat diet (HFD, n=7), control with chronic exercise (Ctl+training, n=7) and high fat diet with chronic exercise (HFD+training, n=7) groups.

	Ctl	HFD	Ctl+training	HFD+training	Main effects by ANOVA
Weight (g)					
<i>Baseline</i>	95.4 ± 5.4	90.1 ± 3.7	93.6 ± 6.0	92.9 ± 3.4	Time, p < 0.0001 Diet, p < 0.0001
<i>After diet period</i>	361.4 ± 37.4	437.3 ± 34.7†	366.7 ± 22.8	436.6 ± 27.1†	Time x Diet, p < 0.0001 Time, p < 0.0001 Diet, p < 0.0001 Ex, NS
<i>After diet and exercise period</i>	428.3 ± 46	532.6 ± 42.0†	421.0 ± 29.1	494.6 ± 24.4*	Diet x Ex, NS Time x Diet, p < 0.005 Time x Ex, p < 0.0001 Time x Ex x Diet, p < 0.05
Mean caloric intake (Kcal.day⁻¹)					
<i>After diet period</i>	55.9 ± 4.2	69.4 ± 3.5	57.21 ± 3.5	69.2 ± 4.1	Time, p < 0.0001 Diet, p < 0.0001 Ex, NS
<i>After diet and exercise period</i>	68.3 ± 11.9	75.9 ± 1.7	70.3 ± 3.9	75.6 ± 2.1	Diet x Ex, NS Time x Diet, p < 0.005 Time x Ex, NS Time x Ex x Diet, NS
MAV (cm.s⁻¹)					
<i>After diet period</i>			45.9 ± 5.2	41.6 ± 3.9	Time, p < 0.0001 Diet, p < 0.05
<i>After diet and exercise period</i>			61.9 ± 6.0	55.8 ± 5.0	Time x Diet, NS
Plasma insulin (ng.ml⁻¹)	2.8 ± 0.9	2.9 ± 0.5	2.2 ± 0.8	2.5 ± 0.3	Diet, NS Ex, NS Diet x Ex, NS
Plasma glucose (mg.dL⁻¹)	83 ± 3	103 ± 12§	86 ± 5	92 ± 5	Diet, p < 0.0001 Ex, NS Diet x Ex, p < 0.05
OGTT (AUC)	247.3 ± 12.4	306.7 ± 20.4	247.7 ± 16.5	286.1 ± 9.8	Diet, p < 0.0001 Ex, NS Diet x Ex, NS

Data are means ± SD;

The main effects from two- and three- way ANOVAs are as follows: Time, time effect; Diet, diet effect; Ex, exercise training effect; x, Interaction between variables, with repetitions on the Time effect

* Significantly different from Ctl + training, p < 0.05; † Significantly different from Ctl and Ctl + training groups, p < 0.05; § Significantly different from all groups, p < 0.05

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Table 4. Subcutaneous and visceral adipose tissues, and soleus and extensor digitorum longus (EDL) muscles concentrations of endocannabinoids and anandamide congeners in control (Ctl, n=7), high fat diet (HFD, n=7), control with chronic exercise (Ctl+training, n=7) and high fat diet with chronic exercise (HFD+training, n=7) groups.

	Ctl	HFD	Ctl + training	HFD + training	Main effects by ANOVA
Subcutaneous adipose tissue					
<i>AEA (pmol.mg⁻¹)</i>	28.58 ± 4.23	33.47 ± 3.22	26.15 ± 4.57	31.27 ± 4.44	Diet, NS Ex, NS Diet x Ex, NS
<i>2-AG (pmol.mg⁻¹)</i>	0.60 ± 0.15	0.32 ± 0.12	0.52 ± 0.19	0.46 ± 0.15	Diet, p < 0.02 Ex, NS Diet x Ex, NS
<i>PEA (pmol.mg⁻¹)</i>	0.43 ± 0.09	0.34 ± 0.17	0.38 ± 0.09	0.38 ± 0.09	Diet, NS Ex, NS Diet x Ex, NS
<i>OEA (pmol.mg⁻¹)</i>	0.35 ± 0.10	0.35 ± 0.07	0.35 ± 0.07	0.34 ± 0.02	Diet, NS Ex, NS Diet x Ex, NS
Visceral adipose tissue					
<i>AEA (pmol.mg⁻¹)</i>	40.23 ± 8.42	35.40 ± 17.37	39.37 ± 16,12	28.55 ± 6.79	Diet, NS Ex, NS Diet x Ex, NS
<i>2-AG (pmol.mg⁻¹)</i>	0.28 ± 0.15	0.27 ± 0.05	0.33 ± 0.13	0.28 ± 0.21	Diet, NS Ex, NS Diet x Ex, NS
<i>PEA (pmol.mg⁻¹)</i>	0.54 ± 0.11	0.80 ± 0.38	0.63 ± 0.22	0.50 ± 0.11	Diet, NS Ex, NS Diet x Ex, NS
<i>OEA (pmol.mg⁻¹)</i>	0.39 ± 0.08	0.41 ± 0.09	0.42 ± 0.10	0.40 ± 0.04	Diet, NS Ex, NS Diet x Ex, NS
Soleus					
<i>AEA (pmol.mg⁻¹)</i>	18.21 ± 2.98	38.60 ± 10.84	30.96 ± 18.29	35.71 ± 15.28	Diet, p < 0.02 Ex, NS Diet x Ex, NS
<i>2-AG (pmol.mg⁻¹)</i>	2.74 ± 0.80	1.74 ± 0.40*	2.21 ± 0.65	2.28 ± 0.60	Diet, NS Ex, NS Diet x Ex, p < 0.04
<i>PEA (pmol.mg⁻¹)</i>	0.86 ± 0.33	0.70 ± 0.23	0.60 ± 0.14	0.61 ± 0.15	Diet, NS Ex, NS Diet x Ex, NS
<i>OEA (pmol.mg⁻¹)</i>	0.21 ± 0.02	0.28 ± 0.05	0.22 ± 0.06	0.26 ± 0.05	Diet, p < 0.006 Ex, NS Diet x Ex, NS
EDL					
<i>AEA (pmol.mg⁻¹)</i>	8.15 ± 1.81	18.45 ± 4.35 [§]	6.73 ± 2.05	11.34 ± 4.15	Diet, p < 0.00002 Ex, p < 0.005 Diet x Ex, p < 0.05
<i>2-AG (pmol.mg⁻¹)</i>	0.68 ± 0.15	0.85 ± 0.21	0.73 ± 0.29	0.91 ± 0.16	Diet, p < 0.04 Ex, NS Diet x Ex, NS
<i>PEA (pmol.mg⁻¹)</i>	0.56 ± 0.24	0.76 ± 0.42	0.54 ± 0.24	0.44 ± 0.18	Diet, NS Ex, NS Diet x Ex, NS
<i>OEA (pmol.mg⁻¹)</i>	0.24 ± 0.10	0.42 ± 0.17	0.27 ± 0.14	0.25 ± 0.06	Diet, NS Ex, NS Diet x Ex, NS

Data are means ± SD;

* Significantly different from Ctl group, p < 0.05; § Significantly different from all the groups, p < 0.05

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