Expression of the cannabinoid system in muscle: effects of a high-fat diet and CB₁ receptor blockade

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The ECS (endocannabinoid system) plays an important role in the onset of obesity and metabolic disorders, implicating central and peripheral mechanisms predominantly via CB₁ (cannabinoid type 1) receptors. CB₁ receptor antagonist/inverse agonist treatment improves cardiometabolic risk factors and insulin resistance. However, the relative contribution of peripheral organs to the net beneficial metabolic effects remains unclear. In the present study, we have identified the presence of the endocannabinoid signalling machinery in skeletal muscle and also investigated the impact of an HFD (high-fat diet) on lipid-metabolism-related genes and endocannabinoid-related proteins. Finally, we tested whether administration of the CB₁ inverse agonist AM251 restored the alterations induced by the HFD. Rats were fed on either an STD (standard/low-fat diet) or an HFD for 10 weeks and then treated with AM251 (3 mg/kg of body weight per day) for 14 days. The accumulated caloric intake was progressively higher in rats fed on the HFD than the STD, resulting in a divergence in body weight gain. AM251 treatment reduced accumulated food/caloric intake and body weight gain, being more marked in rats fed on the HFD. CB₁ (cannabinoid type 2) receptor and PPARα (peroxisome-proliferator-activated receptor α) gene expression was decreased in HFD-fed rats, whereas MAGL (monoglyceride lipase) gene expression was up-regulated. These data suggest an altered endocannabinoid signalling as a result of the HFD. AM251 treatment reduced CB₁ receptor, PPARγ and AdipoR1 (adiponectin receptor 1) gene expression in STD-fed rats, but only partially normalized the CB₂ receptor in HFD-fed rats. Protein levels corroborated gene expression results, but also showed a decrease in DAGL (diacylglycerol lipase) β and DAGLα after AM251 treatment in STD- and HFD-fed rats respectively. In conclusion, the results of the present study indicate a diet-sensitive ECS in skeletal muscle, suggesting that blockade of CB₁ receptors could work towards restoration of the metabolic adaption imposed by diet.

Key words: AM251, cannabinoid type 1 receptor (CB₁ receptor), diet-induced obesity, endocannabinoid system, inverse agonist, oxidative metabolism, skeletal muscle.

INTRODUCTION

The regulation that the ECS (endocannabinoid system) exerts over food intake and energy metabolism involves not only the activation of CB₁ (cannabinoid type 1) receptors present in central circuits in the hypothalamus, but also a complex interplay of peripheral organs such as adipose tissue, liver, pancreas and muscle [1–3]. A network of fatty-acid-derived signalling molecules, including AEA (anandamide), OEA (oleylethanolamide) and 2-AG (2-arachidonoyl-glycerol), exert their effects mainly by binding to CB₁ and CB₂ receptor or to PPAR (peroxisome-proliferator-activated receptor) α and PPARγ, for which there is growing evidence of their role in obesity and diabetes [4]. CB₁ receptors are among the most important receptors expressed in the brain, but also in many peripheral organs involved in energy metabolism [5], such as liver [6–8], adipose tissue [9,10], gut [11], pancreas [12–14] and skeletal muscle [15,16]. CB₁ receptors are predominantly localized on peripheral immune cells and the haemopoietic system [17], but they have also been found in the brain, liver, pancreas, adipose tissue and cardiac muscle [18,19]. It is well demonstrated that the use of selective CB receptor inverse agonist/antagonists, such as SR141716 (rimonabant) and others with both a similar structure and pharmacological properties, including the CB₁ inverse agonist AM251, are able to regulate energy balance and body weight gain. Studies in various animal models of obesity and subsequent human clinical studies have demonstrated that treatment with CB₁ receptor inverse agonists results in weight loss attributable to a reduction in food intake, but also to an increase in whole-body energy expenditure with the involvement of several peripheral organs [11,20,21]. Blockade of central and peripheral CB₁ receptors by rimonabant has been demonstrated to induce important weight loss and improvement in multiple metabolic parameters correcting hyperinsulinaemia, lowering non-esterified ‘free’ fatty acid levels and reversing insulin resistance, all of which counteract the adverse effects of an overstimulated ECS [21,22]. Thus CB₁ receptor stimulation by an overactive ECS will cause an increase in appetite and food intake, together with a series of metabolic changes in peripheral organs that can lead to weight gain and consequently to obesity and related disorders [23,24].

Abbreviations used: 2-AG, 2-arachidonoyl-glycerol; ACOX, acetyl-CoA oxidase; AdipoR1, adiponectin receptor 1; AEA, anandamide; CB receptor, cannabinoid receptor; DAGL, diacylglycerol lipase; ECS, endocannabinoid system; GH, growth hormone; GHR, GH receptor; HFD, high-fat diet; i.p., intraperitoneal(lly); MAGL, monoacylglycerol lipase; OEA, oleylethanolamide; PPAR, peroxisome-proliferator-activated receptor; RT-PCR, real-time PCR; qRT-PCR, quantitative RT-PCR; STD, standard/low-fat diet.

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However, the central action of CB₁ receptor antagonism has been associated with a higher risk of anxiety and depression [25]. This fact has led to the suspension of the marketing authorization for rimonabant, the first commercialized selective CB₁ receptor antagonist, by the EMEA (European Medicine Agency), highlighting the importance of limiting CB₁ receptor antagonism to peripheral organs in order to reduce the potential central risks. Thus further work is necessary to better understand the peripheral mechanisms occurring in adipose tissue, liver and muscle, as well as the possible interactions between these organs, in order to establish whether peripheral CB receptors are solely responsible for the metabolic changes observed in obese subjects treated with CB₁ receptor inverse agonists, leaving apart the hypothalamic control of the motivation to eat [26].

In the present study, we focused on skeletal muscles, which are an important tissue for glucose and fat oxidation, being an important site for insulin action [27]. However, despite the fact that AEA can modify the pathways regulating fatty acid oxidation in the skeletal muscle, probably via CB₁ receptors, suggesting that CB₁ receptor antagonism would have an important role in oxidative metabolism and energy regulation [28,29], there is still a general lack of clarity regarding the physiological functions and molecular mechanism implicated. In fact, there are almost no studies demonstrating the presence of endocannabinoid signalling proteins and their sensitivity to HFDs (high-fat diets). Therefore, in the present study, we have (i) investigated the presence of the endocannabinoid signalling machinery in skeletal muscle, (ii) analysed the impact of an HFD on lipid and glucose metabolism in skeletal muscle, and (iii) monitored the effects of the CB₁ receptor inverse agonist AM251 during an STD (standard/low-fat diet) and HFD on the endocannabinoid machinery and the genes related to lipid oxidative metabolism in skeletal muscle of rats. Among the many molecules involved in lipid metabolism of skeletal muscle, we evaluated changes in the gene and protein expression of relevant components of the ECS, such as the CB₁ and CB₂ receptors and some of the enzymes responsible for their synthesis [DAGL (diacylglycerol lipase) α and β] and degradation [MAGL (monoacylglycerol lipase)], as well as for PPARα, PPARγ, AdipoR1 (adiponectin receptor 1), ACOX (acetyl-CoA oxidase) and GHR [GH (growth hormone) receptor].

**EXPERIMENTAL**

**Animals**

All experimental procedures with animals were performed in compliance with the European Communities directive 86/609/ECC and Spanish legislation (BOE 252/34367-91, 2005) regulating animal research. Male Wistar rats (≈250 g; 10–12 weeks old) (Charles River) were housed in groups of two in cages maintained under standardized conditions in an animal facility (Servicio de Estabulario, Facultad de Medicina, Universidad de Málaga, Málaga, Spain) with a room temperature of 20 ± 2°C, 40 ± 5% relative humidity and a 12 h light/dark cycle with a dawn/dusk effect.

**Diet-induced obesity: measurement of food/caloric intake and body weight gain**

Rats (n = 16/group) were fed with two different types of diets for 12 weeks: an STD (10% fat; D1245B; Brogaarden) and an HFD (60% fat; D12492; Brogaarden) in order to induce obesity. The STD consisted of 3.85 kcal/g (with 20% protein, 70% carbohydrates and 10% fat; where 1 kcal ≈ 4.184 kJ) and the HFD had 5.24 kcal/g (of which 20% of the metabolizable energy content was protein, 20% carbohydrates and 60% fat).

The accumulated food/caloric intake by each rat and their body weight gain were measured every day for 12 weeks. Treatment was then administered when the weight curves showed a clear divergence and stabilization between both diets was achieved. This was accomplished after 10 weeks of the diet (Supplementary Figure S1 at http://www.BiochemJ.org/bj/433/bj4330175add.htm).

**Treatment**

From week 10 onwards (stabilization of weight curves), each group of diet-fed rats (n = 8/group) received a daily i.p. (intraperitoneal) injection of either vehicle (1 ml of 10% Tween in saline/kg of body weight) or the CB₁ receptor inverse agonist AM251 [N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] (Tocris Bioscience) at 3 mg/kg of body weight in 10% Tocrisolve over 14 days, while the diets remained unchanged. The accumulated food/caloric intake and the body weight gain were measured every day during the 14 days of treatment (Supplementary Figure S1).

**Sample collection**

AM251- and vehicle-treated (control) animals were anaeathetized (sodium pentobarbital; 50 mg/kg of body weight, i.p.) 2 h after the last treatment in a room separate from the other experimental animals. Blood samples were collected from the orbital cavity and centrifuged (1368 × g for 8 min at 4°C), and all plasma samples were frozen at −80°C for hormone analysis. Skeletal muscle from the abdominal wall was dissected. Part of each sample was fixed in 4% paraformaldehyde in PBS by immersion until immunohistochemical analysis. The remaining sample was briefly frozen at −80°C until RT-PCR (real-time PCR) analysis.

**RNA isolation and RT-PCR analysis**

RNA, reverse transcription and RT-PCR were performed as described in the Supplementary Materials and methods (http://www.BiochemJ.org/bj/433/bj4330175add.htm). Primers for the PCR (Table 1) were designed based on the GenBank® database sequences of rat reference mRNA and checked for specificity with BLAST software from the NCBI (National Center for Biotechnology Information) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Quantification was carried out with a standard curve run at the same time as the samples with each reaction run in duplicate. Absolute values from each sample were normalized with regard to the housekeeping gene *Actb* (β-actin).

**Western blot analysis**

In order to evaluate the presence of CB₁ and CB₂ receptors, DAGLα, DAGLβ, MAGL and PPARα, a Western blot analysis was performed. For protein detection, each blotted membrane lane was incubated separately with a specific primary rabbit polyclonal antibody, all of them diluted in the blocking buffer (PBS containing 0.1% Tween 20 and 2% (w/v) BSA) (Table 2). Western blot procedures using these antibodies were performed as described previously ([30], and see the Supplementary Materials and methods).
Immunohistochemistry
Paraffined tissue microarray blocks (Manual Tissue Arrayer MTA-1; Beecher Instruments) of rat skeletal muscle (abdominal) were analysed for the presence and quantification of CB₁, CB₂ receptors, DAGLα, DAGLβ, MAGL, and PPARα by immunohistochemistry and densitometry. The primary antibodies were rabbit polyclonal antibodies against CB₁ receptor, CB₂ receptor, DAGLα, MAGL, DAGLβ, and PPARα (Table 2). Immunohistochemical procedures using these antibodies were performed as described previously ([30], and see the Supplementary Material and methods section).

Statistical analysis
All data are means ± S.E.M. Differences between the different diets and treatments were analysed using a Student’s t test (two tailed, paired groups). The level of statistical significance was set at P values of less than 0.05.

RESULTS
In the present study, we investigated the effect of an HFD (60% fat) and the CB₁ receptor inverse agonist AM251 on glucose and lipid metabolism in skeletal muscle in a rat model of diet-induced obesity. We evaluated changes in the gene and protein expression of relevant molecules involved in skeletal muscle oxidative metabolism. The selected enzymes and receptors represent an assortment of molecules implicated in lipid and glucose metabolism within the skeletal muscle with a putative role in energy metabolism, including AdipoR1, ACOX, GHR, PPARα, and PPARγ, but also the gene and protein expression of relevant components of the ECS, such as the CB₁ and CB₂ receptors and the CB₁ receptor inverse agonist AM251 (in 10% Tocrisolve/kg of body weight) and degradation (MAGL). The endocannabinoid proteins were selected on the basis of abundance of mRNA using PCR studies. In order to control the effect of diet and treatment, we monitored the accumulated food/caloric intake and body weight gain.

Effect of diet on accumulated food/caloric intake and body weight gain
Figure 1 shows the accumulated food or caloric intake and body weight gain in the rats fed on either an STD (10% fat) or an HFD (60% fat) over the first 10 weeks of the feeding period. We observed that rats fed on the STD accumulated more food intake than rats fed on the HFD (Figure 1A), in contrast with the accumulated caloric intake (Figure 1B), and this difference was statistically significant from day 1 onwards. The lower food intake in the HFD-fed rats was due to the higher caloric/energy content in the HFD than the STD, as described in the Materials and methods section. Accumulated caloric intake correlated with the body weight increase (Figure 1C), but interestingly body weight gain also had a significant divergence in HFD-fed rats in comparison with STD-fed rats from week 4 onwards (30 days). When body weight gain began to be stabilized over time (after 10 weeks of either diet), AM251 treatment was scheduled for 14 additional days, while the diets remained unchanged.

Effect of AM251 treatment on accumulated food/caloric intake and body weight gain
After a feeding period of 10 weeks, rats received a daily dose of either vehicle (1 ml of 10% Tocrisolve/kg of body weight) or the CB₁ receptor inverse agonist AM251 (in 10% Tocrisolve) (3 mg/kg of body weight) for 14 days (Figure 2). We observed that treatment with AM251 reduced the accumulated food and

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**Table 1 Primer sequences used for RT-PCR**

<table>
<thead>
<tr>
<th>Gene symbol (name)</th>
<th>Oligosense (5′→3′)</th>
<th>Oligoantisense (5′→3′)</th>
<th>GenBank® accession no.</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
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<tr>
<td>Actb (β-actin)</td>
<td>CAGGCTGTGTTCTCCCTGTA</td>
<td>GCTGTGGTGGTAAGCCTGTA</td>
<td>NM_031144.2</td>
<td>203</td>
<td>51.4</td>
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<tr>
<td>Chn2 (CB₁ receptor)</td>
<td>AGACCTTCTCTCGTGGCTG</td>
<td>GTCAGGGTATGGCAAAGCTG</td>
<td>NM_012784.2</td>
<td>314</td>
<td>58.4</td>
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<tr>
<td>Cnr2 (CB₂ receptor)</td>
<td>GGAACCTGCTGATCTGGCTG</td>
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<td>NM_020543.3</td>
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<tr>
<td>Dagla (DAGLα)</td>
<td>GGATGCTGCTGCTTCCA</td>
<td>AGACTGACATCCAAACCTG</td>
<td>NM_001005886</td>
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<td>60.5</td>
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<tr>
<td>Dalgβ (DAGLβ)</td>
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<td>AGGTCATGTGCTGCTGAGA</td>
<td>NM_00107120.1</td>
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<td>59.8</td>
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<tr>
<td>Mgl (MAGL)</td>
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<td>58.1</td>
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<tr>
<td>Ppara (PPARα)</td>
<td>TGGTGCCCTTTCTGATGAC</td>
<td>GCTTGAGCACGTCGACAAATC</td>
<td>NM_013196.1</td>
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<td>58.9</td>
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<tr>
<td>Pparγ (PPARγ)</td>
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<td>Acox1 (ACOX)</td>
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<tr>
<td>Ghr (GHR)</td>
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<td>ACACACCATCAGGCAGAAG</td>
<td>NM_027587.1</td>
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<tr>
<td>Cnr1 (CB1 receptor)</td>
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<td>GTACAGCGATGGCAGCTGCTG</td>
<td>NM_013196.1</td>
<td>370</td>
<td>58.9</td>
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**Table 2 Antibodies used in the present study with the dilutions and molecular mass**

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<th>Protein</th>
<th>UniProt number</th>
<th>Source of antibody</th>
<th>Antibody dilution</th>
<th>Western blotting</th>
<th>Immunohistochemistry</th>
<th>Molecular mass (kDa)</th>
</tr>
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<td>CB₁ receptor</td>
<td>P20272</td>
<td>Abcam (ab23703)</td>
<td>1:100</td>
<td>1:100</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>CB₂ receptor</td>
<td>Q8Q9N9</td>
<td>ABR (PA1-746A)</td>
<td>1:200</td>
<td>1:100</td>
<td></td>
<td>55</td>
</tr>
<tr>
<td>DAGLα</td>
<td>Q0YLM1</td>
<td>Produced in our laboratory [30]</td>
<td>1:100</td>
<td>1:100</td>
<td></td>
<td>120</td>
</tr>
<tr>
<td>DAGLβ</td>
<td>P0C1S9</td>
<td>Produced in our laboratory [30]</td>
<td>1:100</td>
<td>1:100</td>
<td></td>
<td>73.6</td>
</tr>
<tr>
<td>MAGL</td>
<td>Q8RH41</td>
<td>Cayman (#100035)</td>
<td>1:200</td>
<td>1:100</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>PPARα</td>
<td>P37230</td>
<td>ABR (MA1-822)</td>
<td>1:200</td>
<td>1:100</td>
<td></td>
<td>52</td>
</tr>
</tbody>
</table>
We observed that rats fed with an STD accumulated more food intake than rats fed on an HFD (A), in contrast with the accumulated caloric intake (B), and this difference was statistically significant from day 1 onwards. Body weight gain showed a significant divergence in HFD-fed rats in comparison with STD-fed rats from week 4 onwards (C). When body weight gain had stabilized over time (for 10 weeks at least), AM251 treatment was administered, while the diets remained unchanged. Values are means ± S.E.M. (16 animals per diet group). *P < 0.05 and **P < 0.01 compared with STD-fed rats, as determined using a Student’s t test.

The presence of the ECS in skeletal muscle

Before evaluating any changes in the gene and protein expression of relevant molecules involved in skeletal muscle caloric intake in both diets (Figures 2A and 2B) from 4–5 days of treatment onwards, and this correlated with a significant body weight decrease (Figure 2C). Interestingly, the body weight decrease was more marked in HFD-fed rats than in STD-fed rats, i.e. we observed a significant decrease in HFD-fed rats from day 1 of treatment onwards, whereas we did not detect a significant body weight decrease in STD-fed rats until day 8 of treatment. This may be due to the fact that, despite AM251 treatment, the accumulated food consumed continued to be slightly higher in STD-fed rats than in HFD-fed rats.
oxidative metabolism by qRT-PCR (quantitative RT-PCR) and immunohistochemistry, we analysed the presence of CB₁ receptor, CB₂ receptor, DAGLα, DAGLβ, MAGL and PPARα protein and gene expression by Western blotting and RT-PCR. Figure 3(A) shows a comparative study of the gene expression distribution for both skeletal muscle and adipose tissue, represented as the number of copies of PCR product from STD-fed rats. We observed lower levels of gene expression for CB₁ receptor (15-fold; \( P < 0.01 \)), CB₂ receptor (6-fold; \( P < 0.05 \)), DAGLα (5-fold) and DAGLβ (3.5-fold) in the skeletal muscle when compared with the levels obtained in the adipose tissue. In contrast, MAGL and PPARα gene expression were higher in skeletal muscle than in adipose tissue (7.5-fold; \( P < 0.01 \); and 2-fold respectively). In order to demonstrate that the antibodies recognized the corresponding antigens in rat skeletal muscle, we performed a Western blot analysis. Membrane protein extracts of skeletal muscle from Wistar male rat revealed prominent immunoreactive bands of the expected molecular masses of 60 kDa for CB₁ receptor, 55 kDa for CB₂ receptor, 120 kDa for DAGLα, 74 kDa for DAGLβ, 37 kDa for MAGL and 52 kDa for PPARα (Figure 3B).

**Effect of diet and AM251 treatment on gene expression of the molecules implicated in skeletal muscle glucose and lipid metabolism**

Gene expression of receptors and enzymes involved in metabolism of skeletal muscles as well as the components of the ECS showed significant changes according to the diet used (STD compared with HFD) on the levels of CB₂ receptor, PPARα and MAGL (Figure 4). The expression of both CB₂ receptor and PPARα was reduced significantly when using an HFD as shown in Figures 4(B) and 4(E). Meanwhile, MAGL expression, the 2-AG-degradation enzyme, was increased significantly in HFD-fed rats (Figure 4F). Thus the tendency for the synthesis enzyme DAGLα and the receptors analysed was to decrease, which was the opposite of the gene expression of the degradation enzyme MAGL, suggesting the existence of a lower production of 2-AG in the skeletal muscle and desensitization of the receptors.

Regarding the use of the STD with AM251 treatment (Figure 5), most of the genes studied showed a decrease in their expression levels after treatment with the inverse agonist, being statistically significant only for CB₂ receptor, PPARγ and AdipoR1 (Figures 5B, 5G and 5I). However, as in the case of the HFD, MAGL gene expression tended to increase, which was the opposite of that found for the other genes analysed.

We observed a differential response in the gene expression of the molecules studied in rats fed on an HFD and treated with AM251 (Figure 6). Changes observed in the STD-fed rats were not observed, and CB₁ and CB₂ receptor gene expression tended to be normalized (up-regulated) to levels in the STD-fed rats, again in contrast with the tendency of MAGL gene expression. PPARγ, GHR, AdipoR1 and ACOX tended to be down-regulated.

**Effect of diet and AM251 treatment on protein expression of the molecules implicated in skeletal muscle glucose and lipid metabolism**

Figure 7 shows representative high-magnification images of skeletal muscle immunostained for CB₁ receptor, CB₂
The expression of both CB1 receptor (B) and PPARγ (G) was significantly reduced when using an HFD. MAGL expression was significantly increased in HFD-fed rats (F). Absolute values from each sample were normalized with regard to the housekeeping gene β-actin. Values are means ± S.E.M. (eight animals per diet group). *P < 0.05 compared with STD-fed rats, as determined using a Student’s t test.

Most of the genes studied had a decrease in their expression levels after treatment, being statistically significant for CB2 receptor (B), PPARγ (G) and AdipoR1 (I) gene expression only. MAGL gene expression had a tendency to increase. Absolute values from each sample were normalized with regard to the housekeeping gene β-actin. Values are means ± S.E.M. (eight animals per treated group). *P < 0.05 compared with vehicle (Veh), as determined using a Student’s t test.

had a significant decrease in their protein expression levels in HFD-fed rats (Figures 8B and 8F). On the other hand, after AM251 treatment, DAGLα and DAGLβ protein levels were significantly decreased in HFD- and STD-fed rats respectively (Figures 8C and 8D).
Figure 6  Effect of the CB1 receptor inverse agonist AM251 with the HFD on
the gene expression of the molecules implicated in skeletal muscle glucose
and lipid metabolism

We observed no significant changes in the gene expression, but CB1 (A) and CB2 (B) receptor
gene expression tended to be normalized (up-regulated) to that of levels in the STD-fed rats.
Absolute values from each sample were normalized with regard to the housekeeping gene
β-actin. Values are means ± S.E.M. (eight animals per treated group). Veh, vehicle.

DISCUSSION

Over recent years, animal models of obesity and clinical trials with the cannabinoid CB1 blocker rimonabant have
demonstrated that CB1 receptor antagonism helps to improve several cardiometabolic risk factors, insulin sensitivity and
glucose uptake, which are found to be deregulated in obesity states and related disorders [24]. Independently of the lowering
effects on food intake by central CB1 receptor antagonism of CB1 receptors, the long-term effects on body weight, visceral
fat reduction and metabolic regulation appeared to be mediated by stimulation of energy expenditure and by effects on glucose
and lipid metabolism in peripheral organs, such as the liver, adipose tissue and skeletal muscle [29,31]. The skeletal muscle
is known to be the primary site for nutrient oxidation, including fatty acid oxidation and glucose uptake, and it has been suggested
that insulin resistance and impaired glucose uptake seem to be associated with a loss of oxidative capacity in this tissue in
response to an increase in dietary fat intake [28]. Insulin resistance in the skeletal muscle might ultimately play an important role in
the pathogenesis of the metabolic syndrome and Type 2 diabetes [32,33]. Consequently, blocking of CB1 receptors in skeletal
muscle will have a direct effect on energy expenditure and oxidative metabolism, as it has been demonstrated in various
studies with rimonabant [16,34]. However, owing to a lack of clarity regarding the molecular mechanisms implicated, this area
would benefit from further investigation. This lack of knowledge starts from the absence of studies addressing the overall
expression in skeletal muscle and changes of endocannabinoid signalling machinery, which has been addressed in the present
experimental design.

In the present study, we aimed to identify molecular changes occurring within the metabolic pathways of skeletal muscle
by an HFD (60% fat) and the subsequent response to a subchronic treatment with the CB1 receptor inverse agonist AM251. This issue is important because several findings suggest that endocannabinoids might affect insulin resistance in skeletal muscle, as well as participating in the metabolic consequences (i.e. insulin resistance) of high-fat-induced obesity. In accordance with previous results [5,15,35], we have confirmed by immunohistochemistry, Western blotting and qRT-PCR that, in addition to the cannabinoid receptors CB1 and CB2, skeletal muscle cells express the enzymes to locally produce (DAGLα and DAGLβ) and degrade (MAGL) endogenous cannabinoids (Figures 3 and 7). These observations give biological support to an endocannabinoid regulation of genes controlling skeletal muscle metabolism. Indeed, in our present study, we observed that the most important changes induced by an HFD on several molecules implicated in lipid metabolism in the skeletal muscle of male Wistar rats were at the transcriptional level. As it would be expected, the HFD caused an important weight gain in these animals. At such a level, chronic treatment with an HFD significantly reduced the gene expression of CB2 receptors and the nuclear receptor PPARα. We detected the same effect at the protein level by immunohistochemistry of both CB2 receptors and PPARα. In contrast, the gene expression of MAGL, the 2-AG-degradation enzyme with lipolytic action, was significantly increased as a result of feeding the animals with this type of diet. Although we have focussed the present study on the ECS, we have also detected that the protein expression of NAPE-PLD (N-acylphosphatidylethanolamine-phospholipase D), the enzyme that synthesizes the non-cannabinoid PPARα agonist OEA, and FAAH (fatty acid amide hydrolase), the enzyme that degrades it, were significantly reduced in HFD-fed rats (results not shown).

This regulation of MAGL can be explained by an ECS overactivity caused with the use of fatty diets, as it has been observed in previous studies and/or in conditions of unbalanced energy homeostasis (obesity and Type 2 diabetes) [23,31,36,37]. A relevant previous study [38] found elevated levels of 2-AG in the soleus muscle of mice after an HFD, particularly when an HFD with a high amount of saturated fat is used. Such studies
Arose after results in the brain showed that DAGLα was found to be a major biosynthetic enzyme for 2-AG in the nervous system, revealing a regulatory role for this enzyme in retrograde synaptic plasticity and adult neurogenesis [39,40]. However, the dysregulated ECS levels found in obesity and/or hyperglycaemic states could be affected differently depending on the type of diet and tissue involved. Whatever the circumstances, protein expression followed the same pattern as the gene expression results, but differences in the regulatory pathways involved in mRNA and protein stability in the muscle cell could be responsible for the observed variations within the experiments.

Fatty acids and eicosanoids are natural ligands for PPARs and, considering that the chemical structure of endocannabinoids is derived from arachidonic acid and that the anorectic lipid OEA acts on PPARα, this can lead to the hypothesis that endocannabinoids might also act through binding to PPARs, as well as the endocannabinoid receptors. Previous evidence [41] suggests that endocannabinoids are natural activators of PPARα, which could mediate many of the biological effects of cannabinoids, including feeding and lipid metabolism. According to our present results, energy overload caused by an HFD diet could be the reason for PPARα to be negatively regulated in skeletal muscle. Conversely, the degrading enzyme MAGL would be up-regulated in order to compensate for the overactivation of endocannabinoids as a result of chronic feeding with an HFD.

In our present experiments, diet exerted more important effects on skeletal muscle metabolism than treatment with the inverse agonist AM251. However, AM251 caused an important weight loss in these animals, as would be expected from a CB1 receptor blocker given results from previous studies [21,24]. Such weight loss would be reflected on muscle cell dynamics, which will require further investigation. High endocannabinoid levels are associated with insulin resistance and with a decrease in glucose uptake by skeletal muscle cells. Rimonabant has been found to reverse this situation [16] and that may account for the better use of energy in these HFD-fed animals treated with AM251. The blockade of cannabinoid receptors and the reversion of DAGL/MAGL overexpression may account for this. It is important to mention two studies based on the possible role played by CB1 receptors in modulating insulin sensitivity within the skeletal muscle cells. In the first one [42], the authors found that treatment with the CB1 receptor inverse agonist SR141716 alone improved insulin sensitivity in skeletal muscle, as determined by its ability to enhance both insulin-stimulated PKB (protein
kinase B)- and ERK1/2 (extracellular-signal-regulated protein kinase 1/2)-directed signalling. In the other study [43], the authors found that treatment of skeletal muscle cells with AEA increased Akt (ser308) phosphorylation, thus disturbing insulin signalling at an early point in the cascade. Studying these signalling pathways would have added important information regarding insulin resistance and glucose uptake in rats fed on an HFD.

In relation to any effect of treatment with AM251 on gene expression, we observed changes in the levels of the CB1 receptor, the nuclear receptor PPARγ and the adiponectin receptor AdipoR1 in animals fed on an STD (10% fat) and treated with AM251 at a dose of 3 mg/kg of body weight. However, this dose of AM251 did not have any effect when animals were fed on an HFD. It is possible that, despite the blockade of ECS signalling in the muscle, it may not be sufficient to normalize the changes observed after a short AM251 treatment period and that extended periods of administration or additional diet modifications might be necessary to observe changes in the genes selected. The immunohistochemical results showed that there was a contrasting tendency for the synthesis enzymes DAGLα and DAGLβ to the decrease and increase respectively after treatment with AM251. This could suggest the existence of mechanisms implicated in maintaining the balance of the levels of 2-AG in skeletal muscle.

In addition to the ECS, we have studied the effects of diet on two hormonal modulators of muscle function, namely adiponectin and GH. Adiponectin, produced in adipose tissue, helps improve hyperglycaemia, hyperinsulinaemia and insulin resistance via activation of AdipoR1, which is abundantly expressed in skeletal muscle [44]. It is important to note that skeletal muscle is one of the most important sites for the actions of adiponectin and insulin, and it has also been observed that PPARγ agonists have a marked effect on plasma adiponectin [45]. However, although acute doses of AM251 resulted in an increase in insulin in plasma (results not shown), plasma adiponectin levels remain unchanged after subchronic treatment with AM251. Some reports have suggested that CB1 receptor antagonism with rimonabant might exert a direct effect on adiponectin by stimulating its mRNA expression [46]. In contrast, other studies have not found any difference in plasma adiponectin levels between animals treated with a CB1 receptor antagonist and control animals, or in animals fed with an HFD or a control diet [47,48]. Whether chronic treatment with AM251 is capable of inducing alterations in plasma adiponectin production in adipose tissue remains to be determined conclusively.

The observed effects of AM251 on the muscle might be indirect and a mere consequence of an AM251-induced reversal of an HFD-induced increase in body weight; however, the opposite, i.e. AM251 having an intrinsic effect on skeletal muscle, cannot be disregarded. It is important to note that we did not find any significant changes in CB1 receptor and DAGLα gene expression in either the liver or adipose tissue (Supplementary Table S1 at http://www.BiochemJ.org/bj/433/bj4330175add.htm). This could be the reason why no significant changes were seen in other genes included in our present study (i.e. ACOX, GHR, etc.). As an example, it has been proposed that increased lipid synthesis in the liver may produce insulin resistance in other tissues such as muscle. Overactive endocannabinoids produce liver lipogenesis and could account for this hypothesis. In addition, endocannabinoids may also influence insulin secretion directly in islet β-cells via CB1 [14] and CB2 [13] receptors.

As a final note, the regulatory mechanisms may be different at rest and during exercise, may change as the exercise intensity increases, and this could be influential in endocannabinoid production [31,49]. It would be interesting to repeat this type of experiment combining exercise and diet in its original design. Regulation of skeletal muscle fat and glucose metabolism is clearly multifactorial, and different mechanisms may dominate in different conditions; besides, potential variations may exist between individuals in response to stimulating or blocking CB1 receptors. This could cause differences in response to treatment with CB1 receptor antagonists between different obese states. In conclusion, we have provided findings identifying important relevant players involved in the signalling pathways of CB1 receptor antagonism in skeletal muscle and determined the extent of changes in this system associated with either an HFD or CB1 receptor blockade.

Figure 8 Quantification of CB1 receptor, CB2 receptor, DAGLα, DAGLβ, MAGL and PPARα immunoreactivity in skeletal muscle in the four rat groups

Protein expression levels of CB2 receptor (B) and PPARα (F) were significantly decreased in HFD-fed rats. On the other hand, after AM251 treatment, DAGLα (C) and DAGLβ (D) protein levels were significantly increased in HFD- and STD-fed rats respectively. Values are means ± S.E.M. (eight animals per group). *P < 0.05 compared with the STD; #P < 0.05 compared with vehicle; &P < 0.05 compared with vehicle, as determined using a Student’s t test.

AUTHOR CONTRIBUTION
Juan Suárez, Francisco Bermúdez-Silva, Pedro Fernández-Llèbrez and Fernando Rodríguez de Fonseca designed the experiments; Ana Crespillo, Juan Suárez, Patricia Rivera, Margarita Vida, Monica Alonso and Antonia Serrano performed the laboratory work; Ana Palomino, Miguel Lucena, Margarita Pérez-Martín and Manuel Macias provided technical support; and Ana Crespillo, Juan Suárez, Francisco Bermúdez-Silva and Fernando Rodríguez de Fonseca interpreted the results and wrote the manuscript.

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REFERENCES


SUPPLEMENTARY ONLINE DATA

Expression of the cannabinoid system in muscle: effects of a high-fat diet and CB₁ receptor blockade


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MATERIALS AND METHODS

RNA isolation and RT-PCR analysis

RNA from skeletal muscle and adipose tissue samples was extracted using TRIzol®; according to the manufacturer’s instruction (Gibco BRL Life Technologies). Tissue portions of skeletal muscle and adipose tissue (~100 mg) were homogenized with an IKA-Ultra-Turrax® T8 homogenizer (IKA-Werke). To ensure the purity of the mRNA sequences excluding molecules smaller than 200 nucleotides, RNA samples were isolated with RNAeasy MinElute cleanup-kit (Qiagen), which included digestion on a DNase I column (RNase-free DNase Set; Qiagen). Total mRNA concentration was quantified using a spectrophotometer (Eppendorf® BioPhotometer) to ensure A₂₆₀/A₂₈₀ ratios of 1.8–2.0.

Purified RNA and random hexamers were utilized to generate first-strand cDNA using transcriptor reverse transcriptase (Roche Applied Science). Negative controls included reverse transcription reactions omitting reverse transcriptase. qRT-PCR was performed using an iCycler System (Bio-Rad) and the SYBR Green detection format (FastStart Universal Master kit; Roche). Each reaction was run in duplicate and contained 5 μl of cDNA template and 0.4 μM of primers in a final reaction volume of 15 μl. Cycling parameters were 95°C for 3 min and 30 s to activate DNA polymerase, followed by 45 cycles at 95°C for 15 s, an annealing temperature for 30 s and a final extension step of 72°C for 15 s in which fluorescence was acquired. Melting curve analysis was performed to ensure only a single product was amplified.

Western blot analysis

Skeletal muscle samples (~100 mg) were homogenized in 50 mM Hepes (pH 8)/0.32 M sucrose buffer to obtain membrane and cytoplasmic protein extracts. The homogenate was centrifuged at 800 g for 10 min at 4°C and the supernatant centrifuged at 40000 g for 30 min. The pellets were resuspended in 50 mM Hepes (pH 8) buffer and pulverized using a homogenizer. Membrane protein concentration was measured using the Bradford protein assay.

For immunoblotting, equivalent amounts of membrane proteins (20 μg) were separated by SDS-PAGE on 10% (w/v) polyacrylamide gels, transferred on to nitrocellulose membranes (Bio-Rad), and controlled by Ponczeau Red staining. The membranes were pre-incubated for 1 h at room temperature with blocking buffer [PBS containing 0.1% Tween 20 and 2% (w/v) BSA]. For protein detection, each blotted membrane lane was incubated separately with the specific primary antibody (see Table 2 of the main paper). This incubation was done overnight at 4°C, followed by several washes in PBST [PBS and 1% (v/v) Tween 20]. Then, the membranes were incubated with a peroxidase-conjugated goat anti-(rabbit IgG H+L) antibody (Promega) diluted 1:2500, or a peroxidase-conjugated goat anti-(mouse IgG) antibody (Promega) diluted 1:2500, for 1 h at room temperature and protected from light. Coloured marker proteins with defined molecular masses were used for molecular mass determination (ECL Western Blotting Molecular Weight Markers; Amersham Biosciences). After extensive washing in PBST, the membranes were incubated for 1 min with the Western Blotting Luminol Reagent kit (Santa Cruz Biotechnology), and the specific protein bands were visualized and quantified by chemiluminescence using an imaging AutoChem™ II UVP BioImagin System (LTF Labortechnik). Western blots showed that each primary antibody detected a protein of the expected molecular mass.

Immunohistochemistry

The skeletal muscle (abdominal) samples were fixed in 4% paraformaldehyde in PBS by immersion and embedded in paraffin. In order to perform immunoreactivity one at a time in a parallel way, tissue blocks were organized in a paraffined tissue microarray block (Manual Tissue Arrayer MTA-1; Beecher Instruments) containing 64 cylinders of paraffined tissue samples with a diameter of 1 mm each (two replicates per sample, four groups, and eight animals each). Tissue microarray blocks were cut into 5 μm-thick sections using a Microm HM325 microtome. Sections were mounted on to glass slides with the positively charged surface (DAKO). Sections were dewaxed, washed several times with PBS and incubated in 3% (v/v) H₂O₂ in PBS for 20 min in dark at room temperature in order to inactivate endogenous peroxidase. After three washes in PBS for 5 min, antigen retrieval was achieved by incubating in sodium citrate (pH 6) for 20 min at 80°C. A background blocking solution containing 10% (v/v) donkey serum, 0.3% Triton X-100 and 0.1% sodium azide was used to incubate the sections for 1 h, which was then followed by an overnight incubation at room temperature with the primary antibodies (see Table 2 of the main paper). The following day sections were washed three times with PBS, incubated in a biotin-labelled donkey anti-(rabbit IgG) antibody (Amersham Biosciences) diluted 1:500 for 1 h, washed again in PBS, and incubated in ExtrAvidin peroxidase (Sigma) diluted 1:2000 in the dark at room temperature for 1 h. After three
Figure S1  Treatment schedule over the experimental period

Table S1  Effect of the CB₁ receptor inverse agonist AM251 on CB₁ receptor and DAGLα gene expression in liver and adipose tissue of diet-induced obese male Wistar rats

Rats were fed on an STD or HFD for 10 weeks and then treated with vehicle or AM251 (3 mg/kg of body weight per day) for 14 days. The gene expression levels were analysed in the four groups 2 h after the last dose by RT-PCR, as described in the Materials and methods section of the main paper. Expression was normalized with respect to endogenous SP1 and is expressed relative to the corresponding control levels. Values are means ± S.E.M. (n = 6–8 animals per group).

**P < 0.01 compared with the vehicle-treated STD group, as determined using a Student’s t test.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Group</th>
<th>STD-Vehicle</th>
<th>HFD-Vehicle</th>
<th>STD-AM251</th>
<th>HFD-AM251</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver CB₁ receptor</td>
<td>1.14 ± 0.27</td>
<td>0.98 ± 0.16</td>
<td>0.92 ± 0.23</td>
<td>1.14 ± 0.17</td>
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</tr>
<tr>
<td>DAGLα</td>
<td>0.82 ± 0.06</td>
<td>1.21 ± 0.18</td>
<td>0.65 ± 0.12</td>
<td>0.99 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>Adipose tissue CB₁ receptor</td>
<td>0.62 ± 0.12</td>
<td>1.64 ± 0.23**</td>
<td>0.92 ± 0.15</td>
<td>1.53 ± 0.34</td>
<td></td>
</tr>
<tr>
<td>DAGLα</td>
<td>1.06 ± 0.19</td>
<td>0.65 ± 0.18</td>
<td>0.53 ± 0.33</td>
<td>0.86 ± 0.14</td>
<td></td>
</tr>
</tbody>
</table>

Digital high-resolution microphotographs of the skeletal muscle were taken under the same conditions of light and brightness/contrast by an Olympus BX41 microscope equipped with an Olympus DP70 digital. Quantification of immunostaining was carried out by measuring densitometry of the selected areas using the analysis software ImageJ 1.38x (National Institutes of Health). Representative digital images were mounted and labelled using Adobe PageMaker.