Abstract
Up-regulation of the endocannabinoid system in the uterus of leptin knockout (ob/ob) mice and implications for fertility

M. Maccarrone1,6, E. Fride2, T. Bisogno3, M. Bari1, M. G. Cascio3, N. Battista1, A. Finazzi Agro4, R. Suris2, R. Mechoulam5 and V. Di Marzo3,7

1 Department of Biomedical Sciences, University of Teramo, Piazza A. Moro 45, 64100 Teramo, Italy, 2 Departments of Behavioral Sciences and of Molecular Biology, College of Judea and Samaria, 44837 Ariel, Israel, 3 Endocannabinoid Research Group, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, Comprensorio Olivetti, Fabbricato 70, 80078 Pozzuoli (Napoli), 4 Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata", Via Montpellier 1, 00133 Rome, Italy and 5 Department of Natural Products, Hebrew University, 91120 Jerusalem, Israel

The levels of the endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) are under the negative control of leptin in the rodent hypothalamus. As leptin and endocannabinoids play opposite roles in the control of reproduction, we have investigated whether the impaired fertility typical of leptin-defective ob/ob mice is due, in part, to enhanced uterine endocannabinoid levels. We found that levels of both anandamide and 2-AG in the uterus of ob/ob mice are significantly elevated with respect to wild-type littersmates, due to reduced hydrolase activity in the case of anandamide, and to reduced monoaaclyglycerol lipase and enhanced diacylglycerol lipase activity in the case of 2-AG. Furthermore, the process mediating endocannabinoid cellular uptake was also impaired in ob/ob mice, whereas the levels of cannabinoid and anandamide receptors were not modified. Although ineffective in wild-type mice, treatment of ob/ob mice with lepton re-established endocannabinoid levels and enzyme activities back to the values observed in wild-type littersmates. Finally, treatment of ob/ob females with the CB1 receptor antagonist SR141716A did not improve their fertility, and inhibition of endocannabinoid inactivation with the endocannabinoid uptake inhibitor OMDM-1 in wild-type females did not result in impaired fertility.

Key words: anandamide/2-arachidonoylglycerol/fertility/leptin/ob/ob mice

Introduction
Leptin is the 16kDa non-glycosylated product of the obese (ob) gene, which is secreted by adipose cells, is released into the circulation and transported across the blood–brain barrier into the central nervous system, where it regulates energy homeostasis (Ahima and Flier, 2000). Leptin also serves systemic functions apart from those related to food intake and energy expenditure in mammals, including regulation of fertility (Cunningham et al., 1999; Ahima and Flier, 2000). Indeed the leptin receptor, which belongs to the class I cytokine superfamily, is present at all points along the hypothalamic–pituitary–gonadal axis (Cunningham et al., 1999). Furthermore, leptin has been found in preimplantation embryos (Antczak and Van Blerkom, 1997), but leptin mRNA was detected starting from the blastocyst stage (Kawamura et al., 2002). Leptin synthesis has been demonstrated in the placenta (Masuzaki et al., 1997) and uterine leptin levels are elevated in the pregnant mouse (Kawamura et al., 2002). Further, addition of leptin to embryo cultures promoted the development of preimplantation embryos (Kawamura et al., 2003). On the basis of these findings, it has been proposed that leptin secreted from the reproductive tract stimulates embryonal development at the preimplantation stage, until it is produced in sufficient amounts independently (Kawamura et al., 2003).

In fact, mice genetically defective in leptin (ob/ob knockout) are obese and infertile, and administration of exogenous leptin can reverse both defects (Cunningham et al., 1999; Ahima and Flier, 2000). It appears, however, that leptin regulates fertility in these animals at the premating stage, but is not required for the normal course of pregnancy and birth (Chehab, 2000). Recently, leptin has been shown to reduce the levels of anandamide [arachidonoylthelidonamide (AEA)] and the other endocannabinoid, 2-arachidonoylglycerol (2-AG), in the hypothalamus of ob/ob mice, suggesting that these compounds partake of the neural orexigenic circuitries down-regulated by leptin (Di Marzo et al., 2001; Kirkham et al., 2002).

AEA belongs to a group of endogenous lipids, which include amides, esters and ethers of long-chain polyunsaturated fatty acids, collectively termed ‘endocannabinoids’ (Mechoulam, 2002; De Petrocellis et al., 2004). It binds to type-1 and type-2 cannabinoid receptors (CB1R and CB2R), thus having many actions in the central nervous system (Fride, 2002a) and in the periphery (Parolaro et al., 2002). These activities of AEA are terminated by cellular uptake through an AEA membrane transporter (AMT) (Hillard and Jarrahian, 2003), followed by degradation to ethanolamine and arachidonic acid (AA) by the enzyme AEA hydrolase (fatty acid amide hydrolase,
FAAH) (Bisogno et al., 2002). Moreover, the checkpoint in AEA synthesis is thought to be the N-acyl-phosphatidylethanolamines hydrolysing phospholipase D (NAPE-PLD) (Moesgaard et al., 2000; Okamoto et al., 2004). 2-AG is synthesized by diacylglycerol (DAG) lipase (Bisogno et al., 2003) and its degradation occurs primarily through the activity of monoacylglycerol (MAG) lipase (Dinh et al., 2002), and possibly through FAAH (Bisogno et al., 2002). Together with AEA, 2-AG, N-arachidonoyldopamine, noladin and virodhamine, the SE proteins form the ‘endocannabinoid system’ (De Petrocellis et al., 2004).

Among the peripheral activities of AEA, the regulation of fertility has attracted growing interest (Maccarrone and Finazzi Agrò, 2004). In fact, low FAAH in circulating maternal lymphocytes has been shown to be an early (<8 weeks of gestation) predictor of spontaneous abortion in humans (Maccarrone et al., 2002a,b). Consistently, FAAH expression has been demonstrated to be under the control of fertility signals. Both progesterone and leptin, alone or synergistically, upregulate the FAAH gene by activating the promoter region through an Ikaros transcription factor or a STAT3 (signal transduction and activator of transcription 3) element, respectively (Maccarrone et al., 2003). As a consequence, blood levels of AEA are reduced (Maccarrone et al., 2002a) and hence the CB1R-dependent block of the release of leukaemia inhibitory factor (LIF) is removed (Maccarrone et al., 2002b). It should be recalled that in mammals LIF is necessary for embryo implantation and survival (Piccinni et al., 1998; Taupin et al., 1999). On the other hand, mouse uterus contains the highest amount of AEA as yet measured in any tissue (Paria and Dey, 2000), and uterine AEA can activate CB1 receptors in this organ, thus allowing epithelial changes needed for reproduction (Maccarrone et al., 2002a,b). Mouse uterus has also FAAH activity, which changes during the peri-implantation period (Paria and Dey, 2000), and possesses a functional AMT and a PLD similar to the NAPE-hydrolysing enzyme (Maccarrone et al., 2004). It is generally accepted that uterine metabolism of AEA can be critical for its activity on fertility. In fact, in vitro AEA leads mouse blastocysts to apoptosis (Maccarrone et al., 2002b; Paria and Dey, 2000). Conversely, within a very narrow concentration range AEA regulates blastocyst function and implantation by differentially modulating mitogen-activated protein kinase signalling and calcium channel activity via CB1 receptors (Wang et al., 2003). Therefore, uterine levels of AEA must be under tight control. To date, the mechanisms that allow the fine-tuning of the endogenous content of AEA in the uterus are neither understood nor is it known whether pro-fertility signals like leptin might partake in this stringent control. On this basis, we sought to investigate whether leptin mutant mice might have a dysregulated uterine endocannabinoid system. The biochemical analysis of the endocannabinoid system in ob/ob mice was further extended by functional experiments, aimed at clarifying the role of CB1 and endocannabinoid degradation in leptin-regulated pathways, allowing fertility in mice. In previous studies, fertility had been restored in sterile ob/ob (male and female) mice after chronic prematting treatment with leptin (Chehab et al., 1996). Moreover, in a separate series of experiments, suppression of uterine AEA, at the time and place of implantation, had been shown to be critical for implantation and survival of the blastocyst (Schmid et al., 1997; Paria and Dey, 2000). Therefore, in the present study, we also assessed the effect on mouse fertility of the selective pharmacological manipulation of either endocannabinoid action or levels. We administered SR141716A to ob/ob mice (experiment 1), or OMDM-1 to wild-type C57BL/6 mice (experiment 2), both before mating and until after implantation (at least 4 days after copulation).

Materials and methods

**Materials**

Chemicals were of the purest analytical grade. Leptin (mouse recombinant) and anandamide (AEA) were purchased from Sigma Chemical Co. (St Louis, MO). N-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR141716A) was a kind gift from Sanofi Recherche (Montpellier, France). The OMDM-1 was synthesized as described previously (Ortar et al., 2003). [3H]AEA (223 Ci/mmol) and [3H]CP55,940 (5-1,1'-dimethylheptyl)-2-[1R,5R-hydroxy-2R-(3-hydroxypropyl) cyclohexyl]-phenol, 126 Ci/mmol) were from NEN Life Science Products, Inc. (Boston, MA). [3H]AA (200 Ci/mmol) and N-[3H]arachidonoyl-phosphatidylethanolamine ([3H]NarPE, 200 Ci/mmol) were from ARC (St Louis, MO). [3H]2-AG was prepared from [3H]AA, as described recently (Cartoni et al., 2004). The labelled substrates for DAG and MAG lipases, i.e. sn-1-stearoyl-2[3H]arachidonoyl-glycerol (56 mCi/mmol) and 2[3H]arachidonoyl-glycerol (1 mCi/mmol) were purchased from Amersham Biosciences and synthesized in-house as previously described (Bisogno et al., 2003), respectively.

**Animals and treatments**

Leptin-deficient (B6.V-Lep<sup>ob</sup>,- ‘ob/ob’) and wild-type C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Wild-type and ob/ob mice (6 per group) received a single intravenous injection of 250 µg of mouse recombinant leptin, or vehicle in the controls, and were sacrificed 24 h later by decapitation (Di Marzo et al., 2001). Uteri were immediately collected as described (Maccarrone et al., 2004) and processed for biochemical analyses. All animal experimental protocols were approved by the local Committee on Animal Care and Use. Protocols met the guidelines of the US National Institutes of Health, detailed in the Guide for the Care and Use of Laboratory Animals, and were applied in conformity with the European Communities Council Directive of November 1986 (86/609/EEC) and the Institutional Ethics Committee. Two functional in vivo experiments were performed. In the first experiment, ob/ob female mice and C57BL6 controls were injected chronically (i.p.) with vehicle (EtOH:Cremophor: saline = 1:1:18) or with SR141716 (5 mg/kg, s.c.) for a 24-day period. In the second experiment, C57BL6 female mice were injected with the AEA uptake inhibitor OMDM-1 or vehicle for the same duration. Vehicle and SR141716-treated groups were matched for body weight before the first treatment.

The treatment period was divided into a premating (12 days), mating (8 days, 2 estrus cycles) and post-mating interval (4 days), in order to ensure treatment persisted into the implantation stage (when high levels of endocannabinoids interfere with implantation; see Schmid et al., 1997; Paria and Dey, 2000). The dose used for SR141716 (5 mg/kg, s.c.) for a 24-day period. In the second experiment, C57BL6 female mice were injected with the AEA uptake inhibitor OMDM-1 or vehicle for the same duration. Vehicle and SR141716-treated groups were matched for body weight before the first treatment.

Food intake was recorded in the ob/ob mice, by placing a pre-weighed amount of food in the cage, emptied of wood chips. The effects of chronic SR141716A and OMDM-1 on fertility were determined by their effects on the number of dams giving birth, litter size and pup birthweights. Effects of chronic treatment with SR141716 on ob/ob mice and of OMDM-1 on C57BL6 mice were assessed in the ‘tetrad’ [a series of four assays designed to reflect cannabinimetic effects, including horizontal and vertical movements in an open field, catalepsy on an elevated ring, hypothermia and analgesia on a hot plate (Martin et al., 1991; Fride and Mechoulam, 1993)], 15 days after the last injection. Ten ob/ob females and 12 C57BL6 females were used in experiment 1, whereas 16 C57BL6 females were used in experiment 2.

**Enzyme assays**

Fatty acid amide hydrolase (AEA amidohydrolase; E.C. 3.5.1.4; FAAH) activity was assayed at pH 9 with 10 µM [3H]AEA or 10 µM [3H]2-AG as substrate, by the reversed phase high-performance liquid chromatography method already described (Maccarrone et al., 2004). The FAAH activity was expressed as pmol [3H]AA released per min per mg protein.
The activity of NAPE-PLD (phosphatidylcholine phosphatidohydrolase, E.C. 3.1.4.4; NAPE-PLD) was assayed in homogenates of uteri (50 mg/test), using 100 μM [3H]NArPE as reported (Okamoto et al., 2004). The NAPE-PLD activity was expressed as pmol [3H]AEA released per min per mg protein. The activity of DAG lipase and MAG lipase was assayed as previously described (Bisogno et al., 2003). In brief, uterine tissue was homogenized in Tris–HCl buffer, pH 7, in a Dounce homogenizer. The homogenates were centrifuged at 4°C sequentially at 800 (5 min), 10 000 (25 min) and 100 000 g (70 min). The 10 000 g (for DAG lipase) and the 100 000 g (70 min, for MAG lipase) fractions were then incubated at pH 7 and 37°C for 15 min, with different radiolabelled substrates (i.e. for DAG lipase activity, with sn-1-stearoyl-2-[14C]arachidonoyl-glycerol; for MAG lipase activity, with synthetic 2-[3H]arachidonoyl-glycerol). After the incubation, lipids were extracted three times with two volumes of chloroform/methanol 2:1 (by vol), and the extracts lyophilized under vacuum. Extracts were then fractionated by thin layer chromatography on silica on polypropylene plates using chloroform/methanol/NH₄OH (85:15:0.1, by vol) as the eluting system. Under these conditions the Rf values of free fatty acids, MAG and DAGs were 0.25, 0.65 and 0.90, respectively. Bands corresponding to each class of lipids were cut and their radioactivity counted with a β-counter.

Analysis of anandamide uptake and binding to cannabinoid receptors

The uptake of 200 nM [3H]AEA by uterus through the AMT was studied as described (Maccarrone et al., 2004). The AMT activity was expressed as pmol [3H]AEA taken up per min per mg protein. For cannabinoid receptor (CBR) studies, membrane fractions were prepared from uterine extracts as reported (Maccarrone et al., 2004), quickly frozen in liquid nitrogen and stored at −80°C for no longer than 1 week. These membrane fractions were used in rapid filtration assays with the synthetic cannabinoid [3H]CP55,940 (400 pM), as described previously (Maccarrone et al., 2004). The same filtration assays were used to analyse the binding of [3H]AEA to uterine membranes. In this case, ligand was used at 400 nM (Maccarrone, 2004). Unspecific binding was assessed in the presence of 10 μM ‘cold’ agonist (Maccarrone et al., 2004). Receptor binding was expressed as fmol [3H]agonist bound per mg protein.

Analysis of the uterine levels of endocannabinoids

Tissues (30–45 mg wet weight/data point) were dounce-homogenized with chloroform/methanol/Tris–HCl 50 mM, pH 7.4 (1:1:1, by vol) containing 50 pmol of d₈-AEA, 50 pmol of d₄-palmitoylethanolamide and 100 pmol of d₆-arachidonylethanolamide as internal standards. The homogenates were centrifuged at 100 000 g for 70 min. The 100 000 g fractions were extracted with chloroform/methanol/Tris–HCl (1:4:1, by vol) to remove the hydrophilic fraction. The extracts were subsequently fractionated by thin layer chromatography on silica on polypropylene plates using chloroform/methanol/NH₄OH (85:15:0.1, by vol) as the eluting system. Under these conditions the Rf values of free fatty acids, MAG and DAGs were 0.25, 0.65 and 0.90, respectively. Bands corresponding to each class of lipids were cut and their radioactivity counted with a β-counter.

Figure 1. The endocannabinoid system in the uterus of leptin-deficient mice. The binding of 400 pM [3H]CP55,940 and of 400 nM [3H]AEA (A), the activity of AMT (B), the activity of FAAH (C) and the activity of NAPE-PLD (D) were assayed in mouse uterus of wild-type (WT) or leptin knockout (KO) animals, as such or injected with 250 mg leptin (+ leptin). The 200 nM [3H]AEA, 10 μM [3H]AEA or 100 μM [3H]NArPE were used as AMT, FAAH or NAPE-PLD substrates, respectively. In all panels, *denotes P < 0.01 versus WT, **denotes P < 0.01 versus KO (P > 0.05 in all other cases). Results are means and SD values.
d5-2-AG (Cayman Chemicals) as internal standards. Lipid-containing organic phase was dried down, weighed and pre-purified by open-bed chromatography on silica gel, and analysed by liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry using a Shimadzu high-performance liquid chromatography apparatus (LC-10ADVP) coupled to a Shimadzu quadrupole MS via a Shimadzu APCI interface (Maccarrone et al., 2002a). Positive ion MS analyses were carried out in the selected ion monitoring (SIM) mode as described previously (Di Marzo et al., 2001). The temperature of the APCI source was 400°C; high-performance liquid chromatography column was a Phenomenex (5µm, 150 × 4.5 mm) reverse phase column, eluted as described (Di Marzo et al., 2001). AEA (retention time, 14.5 min), PEA (retention time, 18.5 min), and 2-AG (retention time, 15.5 min) quasi-molecular ions were quantified by isotope dilution with the above-mentioned deuterated standards and their amounts in pmols normalized per mg of protein extract.

Statistical analysis
Data reported from in vitro data are the mean (± SD) of at least three independent determinations, each in duplicate. Statistical analysis was performed by the non-parametric Mann–Whitney U-test, elaborating experimental data by means of the InStat 3 program (GraphPAD Software for Science, San Diego, CA). In vivo data were analysed using 2-way analysis of variance (for feeding and body weight), t-test for cannabimimetic assays and birthweight, and χ²-tests for fertility measures.

Results
The endocannabinoid system in the uterus of ob/ob mice
Leptin-deficient mice showed the same ability as wild-type littersmates to bind the synthetic cannabinoid [3H]CP55,940, which has high affinity to both CB1 and CB2 receptors (Pertwee, 1999). Treatment of both groups with exogenous leptin, under the same conditions shown to restore fertility in ob/ob mice (Cunningham et al., 1999; Ahima and Flier, 2000), was ineffective (Figure 1A). Also the binding of [3H]AEA, which can activate cannabinoid and non-CBRs (Di Marzo et al., 2002), was the same in uterine membranes from wild-type and knockout animals and was not affected by intravenous injection of leptin (Figure 1A). Unlike AEA-binding receptors, the uptake of AEA through a putative AMT was significantly reduced (down to ~35% of the controls) in ob/ob mice compared to wild-type littersmates, and injection of exogenous leptin fully reversed this effect (Figure 1B). However, treatment of wild-type animals with leptin under the same experimental conditions was ineffective on AMT (Figure 1B). Interestingly, FAAH activity mirrored the activity of AMT, showing a marked decrease (down to ~20% of the controls) in ob/ob mice and a full recovery in leptin-treated knockout animals (Figure 1C). Again, the FAAH activities in wild-type mice treated or not with exogenous leptin were superimposable on each other (Figure 1C). Finally, the activity of NAPE-PLD was almost identical in all groups of mice, suggesting that leptin had no effect on AEA synthesis (Figure 1D).

Although the activity of NAPE-PLD was assayed under conditions found to be optimal and specific for the NAPE-hydrolysing enzyme (Moesgaard et al., 2000; Okamoto et al., 2004), to date there are no specific inhibitors of this enzyme that might allow us to extend further its analysis and to conclusively assess its contribution to AEA metabolism. At any rate, the presence of NAPE-PLD, shown in mouse uterus for the first time, seems to indicate that this organ is able to synthesize AEA, extending a recent report on mouse brain (Okamoto et al., 2004).

In keeping with the biochemical data, uterus of leptin-deficient mice had endogenous levels of AEA ~4-fold higher than wild-type littersmates, and injection of exogenous leptin fully prevented this rise in AEA (Figure 2A). On the other hand, the amount of PEA, a poor substrate for FAAH (Maccarrone et al., 2002b) not recognized by the AEA transporter, was not significantly different in ob/ob mice compared to wild-type littersmates (Figure 2A). Additionally, neither AEA nor PEA were affected by injection of recombinant leptin in wild-type animals (Figure 2A); instead, the endogenous level of 2-AG was approximately doubled in the uterus of knockout mice, an effect fully reversed by exogenous leptin in ob/ob mice but not in wild-type animals (Figure 2B). In line with this observation, hydrolysis of [3H]2-AG by either MAG lipase or FAAH was markedly reduced in leptin-deficient mice compared to wild-type littersmates (Table I). Again, leptin injection fully reversed or, in the case of the MAG lipase, significantly attenuated these changes in ob/ob mice, without affecting the MAG lipase and the FAAH activity in wild-type animals (Table I). In addition, the activity of DAG lipase was almost doubled in leptin-deficient mice and was significantly reduced by exogenous leptin, which instead was ineffective in wild-type mice (Table I).

Figure 2. Uterine levels of endogenous cannabinoids in leptin-deficient mice. The levels of AEA and of PEA (A), and those of 2-AG (B) were measured in the same samples as in Figure 1. In both panels, *denotes P < 0.01 versus WT, **denotes P < 0.01 versus KO (P > 0.05 in all other cases). Results are means and SD values.
Table I. Activity of DAG lipase and hydrolysis of [3H]2-AG by monoacylglycerol (MAG) lipases and fatty acid amidase hydrolase (FAAH) in the uterus of wild-type and leptin knockout mice

<table>
<thead>
<tr>
<th>Sample</th>
<th>DAG lipase activity (pmol/min per mg protein)</th>
<th>MAG lipase (pmol/min per mg protein)</th>
<th>FAAH activity (pmol/min per mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>4.4 ± 0.6</td>
<td>142.7 ± 30.8</td>
<td>330 ± 35</td>
</tr>
<tr>
<td>Knockout (ob/ob)</td>
<td>8.1 ± 1.1*</td>
<td>4.4 ± 3.1**</td>
<td>70 ± 10**</td>
</tr>
<tr>
<td>Wild-type + leptin</td>
<td>6.6 ± 1.0</td>
<td>166.0 ± 24.3</td>
<td>310 ± 30</td>
</tr>
<tr>
<td>Knockout + leptin</td>
<td>5.4 ± 1.0*</td>
<td>28.7 ± 6.5**</td>
<td>235 ± 25*</td>
</tr>
</tbody>
</table>

*Denotes P < 0.05 versus wild-type.
**Denotes P < 0.01 versus wild-type.
Denotes P < 0.05 versus knockout.
Denotes P < 0.01 versus knockout (P > 0.05 in all other cases, n = 4).
Values are means ± SEM.

Figure 3. Food intake (A) and body weights (B) of ob/ob female mice during daily treatment with SR141716A. SR141716A was injected daily s.c. at a dose of 5 mg/kg. Food intake was measured daily for the first 13 days of treatment (until the mating period). Mice were weighed every day throughout the experiment. On day 13 of treatment each ob/ob female was mated with a proven C57BL/6 male for eight nights. The last four injections were administered after cessation of mating. Results are means and SEM values (veh, vehicle; SR1, SR141716A).

Effect of a CB1 receptor antagonist and of an AMT inhibitor on fertility and body weight

SR141716 induced a significant decrease in food intake (Figure 3A, recorded for 13 days) and body weight loss (Figure 3B, recorded for 21 days) in ob/ob females. Three weeks after the last injection of SR141716, the weight difference had disappeared [mean ± SEM (g): vehicle, 79.10 ± 2.87 and SR141716, 80.50 ± 3.51]. The performance in the ‘tetrad’ was no different in SR141716-treated compared to control ob/ob mice, 15 days after the last injection (data not shown). Further, SR141716 had no effect on fertility (none of the females gave birth).

No consistent changes in body weights were recorded after treatment with 5 mg/kg OMDM-1 (data not shown). However, as presented in Table II, 15 days after the last treatment, OMDM-1-treated dams were hyperactive in the open field [mean ± SEM: vehicle, 76 ± 8.0 - (n = 9); OMDM-1, 144 ± 13.0 - (n = 8); P < 0.001] and exhibited reduced catalepsy with OMDM-1 (vehicle 37 ± 6.5, OMDM-1 18 ± 2.8, P < 0.05). The number of dams treated with OMDM-1 giving birth (8 of 16 dams), was lower than that of controls (11 of 16 dams), but this difference was not significant (χ² = 1.16, P = 0.14). Litter sizes tended to be higher in OMDM-1-treated dams, but the difference was not significant. In contrast, birthweights were significantly elevated in the offspring of OMDM-1-treated mice, and body weights of 3-week-old pups were still higher in pups whose mothers had been injected with OMDM-1 (Table II). Thus the chronic treatments with SR141716 and OMDM-1 exerted observable effects on the dams and/or on their offspring.

Discussion

In this study, we show that leptin-deficient (ob/ob) mice exhibit markedly enhanced uterine endocannabinoid levels due to reduced degradation by AMT and FAAH, in the case of AEA, and to both reduced degradation by MAG lipase and enhanced biosynthesis by DAG lipase, in the case of 2-AG. To date, AEA levels in the uterus of wild-type mice have been measured only during pregnancy and, based on the assumption that the same amount of tissue yields amounts of phospholipids and proteins in the same order of magnitude, the levels we found here were significantly lower than those reported previously on the first day of pregnancy (Schmid et al., 1997). It remains to be established whether this difference is due to the different analytical procedures used in the two studies or to an up-regulation of AEA levels immediately following fertilization. By contrast, 2-AG levels have never been measured in the mouse uterus. At any rate, the levels of endocannabinoids increase 2–5-fold in ob/ob mice, whereas cannabinoid CB1 receptors and other possible AEA-binding sites are not affected. Administration of exogenous leptin under conditions which restore fertility also restores normal endocannabinoid levels in ob/ob mice, while it is ineffective in wild-type littermates; overall, these data suggest that leptin tonically down-regulates endocannabinoid signalling in vivo.

Leptin-deficient mice showed similar decreases in the activity of AMT compared with that of FAAH (Figure 1B,C). It seems
noteworthy that AEA uptake by AMT may be a direct consequence of the activity of FAAH, because intracellular degradation of AEA keeps the concentration gradient which drives the facilitated diffusion of this endocannabinoid through the plasma-membrane of the cell (Deutsch et al., 2001). Yet, the relationship between AMT and FAAH is still under debate (Hillard and Jarrahian, 2003), and only recently it has been confirmed that a mechanism different from FAAH is responsible for AEA uptake and release in many cells (Ligresti et al., 2004; Ronesi et al., 2004). At any rate, the observation that both AMT and FAAH were down-regulated in ob/ob mice supports their critical role in controlling the endogenous tone of AEA. In fact, AEA levels were higher in the uterus of ob/ob mice compared to their wild-type littermates, and injection of exogenous leptin reduced these levels to the wild-type background (Figure 2A). These data are in keeping with previous observations in transgenic mice with a disrupted FAAH-encoding gene (Cravatt et al., 2001). Along the same line, we have recently reported an inverse correlation between FAAH activity and AEA levels in the blood of pregnant women (Maccarrone et al., 2002a). In the present study, the endogenous levels of 2-AG were also higher in ob/ob than in wild-type animals, and again exogenous leptin reduced the concentration of 2-AG to the background values. This effect seems to be due to a reduced 2-AG degradation by FAAH and the MAG lipase and a higher 2-AG synthesis by the DAG lipase (Table I). Therefore, leptin control of 2-AG metabolism is at variance with that of AEA, whose endogenous levels appeared to be under the control of FAAH only (Figure 1A–D). In addition, unlike AEA and 2-AG, the levels of the anandamide congener PEA were not affected (Figure 2A), possibly reflecting its slow degradation by FAAH. (Maccarrone et al., 2002a). This observation is in keeping with the previous observation that PEA, unlike AEA, does not modulate the release of cytokines critical for fertility like LIF (Maccarrone et al., 2002b). Finally, the reduction of AEA degradation in leptin-deficient compared with wild-type mice and the ability of exogenous leptin to reverse this change in ob/ob mice, but not in wild-type littermates (Figure 1B,C), suggests the existence of a tonic control of FAAH by leptin in vivo. This hypothesis is strengthened by the lack of effect of exogenous leptin in wild-type mice (Figure 1B,C), which already have enough circulating leptin to saturate the binding sites on leptin receptors (Ahima and Flier, 2000), and to tonically reduce endocannabinoid levels.

Of interest is the observation that the binding of $[^{3}H]$CP55,940, a CB1 receptor agonist (Pertwee, 1999), and $[^{3}H]$AEA, which might bind also to non-CB1 receptors (Di Marzo et al., 2002), was almost superimposable in uterine membranes, remained the same in wild-type and ob/ob mice and was not affected by exogenous leptin (Figure 1A). Therefore, it can be concluded that in the uterus CB1 receptors and AEA binding to yet-to-be-identified receptors are not under the control of leptin. Given the widely reported inhibitory effects of endocannabinoids on embryo implantation in mice and the pro-apoptotic action of AEA on mouse blastocysts (Maccarrone et al., 2002b), it was tempting to speculate that the dysregulation of the endocannabinoid system shown here in the uterus of leptin-deficient mice is partly responsible for the impaired fertility typical of ob/ob mice. Indeed, the reduced uterine FAAH activity reported here in these mice mirrors changes previously observed in circulating lymphocytes of women experiencing spontaneous abortion (Maccarrone et al., 2002a). Along these lines, decreased FAAH activity and increased AEA levels in the uterus (Figures 1C and 2A) of ob/ob infertile mice reflect decreased FAAH and increased AEA content in the blood of miscarrying women (Maccarrone et al., 2002a). Therefore, it can be suggested that peripheral lymphocytes mirror reproductive organs and that the relationship between low FAAH activity—high AEA content and impairment of gestation is of a cause–effect type. Also, FAAH localization in the endometrial epithelium of mouse uterus (Maccarrone et al., 2002b), and in human amniotic epithelial cells, chorionic cytotrophoblast and placenta (Park et al., 2003), is suggestive of a role in controlling AEA tone. In keeping with this hypothesis, mouse blastocysts have been recently shown to activate uterine FAAH by releasing a lipid compound, which fastens AEA cleavage at the site of implantation, thus protecting the embryo against the noxious effects of this endocannabinoid (Maccarrone et al., 2004).

Against this background, we decided to investigate the possibility that enhanced endocannabinoid signalling in ob/ob mice contributes to their lack of fertility. We did this by: (1) studying the effect of the CB1 receptor antagonist SR141716A (and hence of reverting the enhanced endocannabinoid signalling in these animals) on the fertility of female ob/ob mice; and (2) by mimicking the enhanced endocannabinoid signalling observed in ob/ob mice also in wild-type mice via the administration of a selective AMT inhibitor, OMDM-1 (Ortar et al., 2003; de Lago et al., 2004), and monitoring its effect on reproduction. The two substances proved to have biological activity: SR141716 caused significant effects on the body weight and food intake of ob/ob mice, which is in accordance with decreased food intake and body weight in rodents treated with 1–10 mg/kg SR141716 (Colombo et al., 1998; Round et al., 2001; Fride, 2002b), and OMDM-1 displayed significant effects on behaviour even 15 days after cessation of treatment.

Fertility had been restored in ob/ob (male and female) mice after chronic premating treatment with leptin (Chehab et al., 1996). Moreover, in a separate series of experiments, suppression of uterine AEA at the time and place of implantation had been shown to be critical for implantation and survival of the blastocyst (Schmid et al., 1997; Paria and Dey, 2000). Therefore, we administered SR141716 to ob/ob mice, or OMDM-1 to wild-type C57BL/6 mice, both before mating and until after implantation. In our first experiment, none of the ob/ob females gave birth. In the second experiment, fewer OMDM-1-treated C57BL/6 dams gave birth compared to vehicle controls, but the difference was not significant. Interestingly, the premating and early gestational treatment affected embryonal

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<th>Table II. Behaviour and fertility: effects of chronic treatment of C57BL/6 female mice with OMDM-1</th>
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<td>Motor activity (score)</td>
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<td>Vehicle</td>
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C57BL/6 female mice were injected with OMDM-1 (5 mg/kg) or vehicle for a period of 24 days. Every day, body weight and rectal temperature (YSI, Yellow Springs, OH) were assessed. Vehicle and OMDM-1-treated groups were matched for body weight before the first treatment. The treatment period was divided into a premating period (12 days), mating (10 days, 2–3 estrus cycles) and post-mating interval (4 days). Thus, on day 13 of treatment, two females (1 vehicle and 1 OMDM-1-treated female) were mated with 1 (C57BL/6) male. On day 12 after the onset of mating, females were removed but continued to receive four daily injections of OMDM-1 in order to ensure that every female received the antagonist during the implantation stage. Motor activity and catalepsy were assessed 15 days after the last treatment. *Denotes P < 0.001 versus vehicle. **Denotes P < 0.05 versus vehicle. Values are means ± SEM.
development, since the birthweights and the offspring’s body weights at 3 weeks of age were increased after OMDM-1, despite a tendency for increased litter size after OMDM-1 treatment. The implication of this finding is currently being investigated.

In conclusion, the results reported here represent the first evidence that in the mouse uterus leptin controls AE2 degradation by AMT and FAAH, suggesting that cleavage, but not synthesis or receptor binding of AE2, might be the target for new therapies of mammalian defects in reproduction. The cellular source of endocannabinoids in the uterus is not known at present, but, based on our current data, it will have to be looked for among those cells that express at the same time leptin receptors, FAAH and the MAG lipase. It is noteworthy, however, that data reported here extend to the mouse uterus previous findings on human peripheral lymphocytes, where the FAAH gene is up-regulated by leptin through a STAT3-dependent activation of the promoter region (Maccarrone et al., 2003). Although human and mouse FAAH genes (localized on chromosomes 1 and 4, respectively) share 84% sequence identity and have a conserved genomic structure (Wan et al., 1998), the mouse promoter does not contain the same STAT3-binding element present in the human gene (Maccarrone et al., 2003). Therefore, the molecular mechanisms leading to enhancement of FAAH activity by leptin in mouse uterus remain to be elucidated and might be based on the engagement of different transcription factors (Maccarrone and Finazzi Agrò, 2004). It is noteworthy that, independent of the regulatory mechanism, the final effect of leptin is always the enhancement of FAAH activity, and that this effect has been conserved across species in tissues critically involved in reproduction, such as the human immune system and the mouse reproductive organs. Finally, our present findings have provided the first example of the regulation by leptin of the activity of the enzymes involved in the regulation of 2-AG levels. Whether the effects of leptin on 2-AG levels are exerted by impacting on the regulatory regions of the MAGL and DAGL genes will have to be established in future studies awaiting the characterization of these genes.

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