The endocannabinoid anandamide during lactation increases body fat content and CB₁ receptor levels in mice adipose tissue

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INTRODUCTION

Present evidence from epidemiological and animal studies indicates that events occurring during fetal and/or neonatal life could have long-term health consequences.¹ Stress constitutes an early stimulus leading to overweight and metabolic alterations in adulthood associated with a perturbed endocannabinoid system (ECS), as previously reported.²

The ECS consists of Type 1 and 2 cannabinoid (CB₁, CB₂) receptors, which are present in several tissues including the central nervous system, adipose tissue, liver and pancreas.³⁵ Their endogenous ligands are known as endocannabinoids, with arachidonylethanolamide or anandamide (AEA) and 2-arachidonoylglycerol (2-AG) being the most studied agonists. These endocannabinoids have endocrine, autocrine and paracrine actions.⁶⁷ Stress elevates endocannabinoid levels in some areas of the central nervous system and activates CB₁R involved in the negative feedback mechanism to repress the activity of the hypothalamus–pituitary–adrenal axis.⁸ In addition, activation of CB₁R in some peripheral tissues has been related to overweight/obesity, insulin and leptin resistance and dyslipidemia.⁹¹⁰ We have previously demonstrated that oral administration of the AEA during lactation results in overweight, epididymal fat accumulation, hormonal disruption and a marked state of insulin resistance in adult mice. Moreover, a higher expression of CB₁R in epididymal fat of adult mice was also observed.¹¹

Adipocytes not only express CB₁R (a target for AEA), its activation is involved in adipocyte growth and differentiation, modulation of adipokine secretion and stimulation of lipogenesis.⁹¹²¹³ Nevertheless, these studies have been performed in cultured adipocytes, and there is no information whether some of these effects may be observed in vivo experiments.

With all these antecedents in mind, the aim of this study was to evaluate the in vivo effects of oral administration of anandamide during lactation on epididymal adipose tissue development and total body fat content, and whether these factors were associated with increased amounts of CB₁R immediately after weaning.

MATERIALS AND METHODS

Bioethics Committee for Animal Experimentation of the Institute of Nutrition and Food Technology, University of Chile, Santiago approved this study.

Animals

Synchronously primiparous pregnant Swiss CD-1 mice were housed under standardized conditions of humidity, temperature (22–24 °C) and a 12:12-h light–dark cycle. Animals had free access to purified deionized tap water and a standard diet.¹¹ From day 15, pregnant female mice were examined twice a day for the presence of pups. Newborn pups, 6–8 litters of homogeneous size (12–14 pups), were put together and male pups were separated from female pups. Subsequently, six male pups having homogeneous weights were randomly selected and assigned to a substitute mother for random cross-lactation. This procedure is used to homogenize genetic and behavioral factors affecting metabolic characteristics. Next, animals were distributed to constitute the following groups.

Control mice: from days 1 to 21 of lactation, pups were daily weighed and treated with an oral dose of soy oil (1 μl g⁻¹ of body weight) by means of an automatic pipette with an appropriate tip.

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AEA-treated mice: from days 1 to 21 of lactation, pups were daily weighed and treated with an oral dose of AEA 20 mg ml⁻¹ in soy oil (20 μg g⁻¹ body weight; Sigma Chem Co, St Louis, MO, USA).

After weaning, mice were killed and epididymal fat pads were extracted, weighed and quickly frozen until analysis.

**Body fat content.** Body fat content was determined by acid hydrolysis. After death, animals were frozen, pulverized and a 5 g aliquot of each mouse was homogenized with 20 ml of 6 N hydrochloric acid. The sample was subsequently heated and shaken during 45 min at 80 °C. Next, 1 g of celite was added and finally filtered under vacuum. The filter was dried at 103 ± 2 °C for 1 h, and fat previously absorbed in celite was extracted and quantified. This method is similar to that used to validate magnetic resonance spectroscopy for evaluating body composition.

**Western blot analysis of CB₁R.** Epididymal fat (100 mg) was homogenized in 500 μl of RIPA buffer (25 mM Tris-HCl, pH7.6, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS) in the presence of a protease inhibitor cocktail (Sigma Chem Co, P2714). Proteins were separated in a 10% SDS-polyacrylamide gel and subsequently transferred to a polyvinylidene fluoride membrane at 4 °C. Rabbit polyclonal antibody for CB₁R was used as the primary antibody (Cayman Chemical, Irvine, CA, USA), and an enzyme-conjugated anti-rabbit antibody was the secondary antibody (Bio-Rad, Ann Arbor, CA, USA). Appropriate positive and negative controls with brain tissue and the corresponding CB₁R antibody blocking peptide, respectively, were also performed (Cayman Chemical).

**Adipocyte size.** Epididymal fat pads were warmed in warm saline and fixed in 4% Böuin solution for 48 h, and then included in solid paraffin and sliced in 10-μm-thick sections, which were mounted onto gelatin-coated glass slides. The morphometric analysis (estimated mean volume) was carried out under light microscopy. Six slices for each animal and three randomly selected fields per slice were considered.

**Statistical analysis**

Data are expressed as mean ± s.e.m. Shapiro–Wilks and Levene tests were carried out to evaluate normal distribution and variance homogeneity. When appropriate, the Mann–Whitney U-Test statistical analysis was performed. Significance was set at P < 0.05. All analyses were conducted using Stata 10.1 statistical package (StataCorp LP, College Station, TX, USA).

**RESULTS**

**Body and epididymal fat contents**

Figure 1a shows that 21-day-old AEA-treated mice had significantly higher epididymal fat content compared with control animals together with higher adipocyte size (12.1 ± 0.9 × 10³ vs 6.8 ± 0.3 × 10³ μm³, respectively; P < 0.05). Body fat content was determined in 21- and 150-day-old AEA-treated and control animals. Our previous results have demonstrated that AEA-treated, 21-day-old animals had no significant differences on body weight in comparison with control mice. Notwithstanding, body fat content was 31% higher in the AEA-treated mice (11.2 ± 0.6% vs 14.7 ± 1.0%; mean ± s.e.m.; n = 6; P < 0.05; Figure 1b). The accumulation of body fat was a remarkable progressive process during life span of AEA-treated mice during lactation, being 322% higher in 150-day-old AEA-treated animals than in control animals (28.8 ± 0.5% vs 8.9 ± 1.3%, respectively). This fat accumulation was reflected in the body weight of AEA-treated animals, due only in part, to a higher accumulative food intake after weaning.

**DISCUSSION**

This in vivo study shows for the first time that mice treated with anandamide during lactation show increased epididymal and total body fat content, in addition to larger adipocytes in epididymal adipose tissue concomitant to higher levels of CB₁R at weaning. In a previous report, it has been shown that adult female mice treated with a low dose of AEA (0.001 mg kg⁻¹) increased food intake. In this way, it may be argued that our lactating animals may program long-term body weight and fat content owing to a higher consumption of maternal milk during lactation. However, as previously stated, the effect of AEA on appetite may be variable and even absent depending on the dose and experimental design. As our AEA-treated animals did not show any difference in body weight in comparison with control animals during lactation, our results indicate that effects of early AEA treatment on long-term adult body weight and fat content are likely owing to an increased amount and/or overactivity of CB₁R in visceral adipose tissue.
Several in vitro studies have previously shown that the endocannabinoid system is involved in adipogenesis and lipogenesis of the adipose tissue. It has been shown that AEA increased the expression and activation of PPAR-γ, leading to differentiation of fibroblasts to adipocytes. Moreover, CB1R activation with the synthetic agonist WIN-55,212-2 stimulated several enzymes associated with intracellular lipid accumulation, including augmented lipoprotein lipase activity in cultured adipocytes. If we consider that administration of AEA during lactation increased CB1R levels in adipose tissue in 21-day-old mice, which are maintained until adulthood, this condition could be involved in the progressive fat accumulation observed from 21- until 150-day-old AEA-treated animals, likely involved in insulin resistance and dyslipidemia, as we have previously shown.

Diet may be a factor involved in elevated levels of endocannabinoids in different tissues. In fact, it has been recently shown that increasing dietary linoleic acid (LNA) elevates arachidonic acid and therefore endocannabinoid levels and adiposity in mice. In humans, an epidemiological report has linked increased intake of LNA over time to increased prevalence of obesity and postulated that arachidonic acid-induced elevation of the endocannabinoid 2-AG may have altered the energy balance.

If we consider that human milk contains endocannabinoids, mothers with a higher intake of LNA could enrich their milk with endocannabinoids, in this way having a chance to induce an early programming effect on body fat accumulation during life span. Early availability of endocannabinoids and its suggested involvement in programming body fat development, leading to unhealthy conditions during adulthood, guarantee further research in this area.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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