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DYSREGULATION OF PERIPHERAL ENDOCANNABINOID LEVELS IN HYPERGLYCEMIA AND OBESITY: EFFECT OF HIGH FAT DIETS

Isabel Matias^{1,4}, Stefania Petrosino¹, Alessandro Racioppi¹, Raffaele Capasso², Angelo A. Izzo² and Vincenzo Di Marzo^{1#}

¹⁻³*Endocannabinoid Research Group*, ¹Institute of Biomolecular Chemistry and ²Institute of Cybernetics, Consiglio Nazionale delle Ricerche, Pozzuoli (NA), Italy; ³Department of Experimental Pharmacology, University of Naples “Federico II”, Naples, Italy. ⁴U862 Centre de Recherche INSERM, Institut François Magendie, Bordeaux, France.

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#Corresponding author. *Endocannabinoid Research Group*, Institute of Biomolecular Chemistry, Consiglio Nazionale delle Ricerche, Pozzuoli (NA), Italy. Tel.: +39-081-8675093; Fax.: +39-081-8041770; E-mail: vdimarzo@icmib.na.cnr.it

List of abbreviations

AEA, arachidonylethanolamide, anandamide; 2-AG, 2-arachidonoylglycerol; CB₁, cannabinoid receptor type 1; CB₂, cannabinoid receptor type 2; DAGL, diacylglycerol lipase; EC, endocannabinoid; FAAH, fatty acid amide hydrolase; HFD, high fat diet; STD, standard diet; TRPV1, transient receptor potential vanilloid type 1 channel.

Abstract

Increasing evidence indicates that endocannabinoid (EC) signalling is dysregulated during hyperglycemia and obesity, particularly at the level of anandamide (AEA) and/or 2-arachidonoylglycerol (2-AG) concentrations in tissues involved in the control of energy intake and processing, such as the liver, white adipose tissue and pancreas. Here we review this previous evidence and provide new data on the possible dysregulation of EC levels in organs with endocrine function (adrenal glands and thyroid), involved in energy expenditure (brown adipose tissue and skeletal muscle), or affected by the consequences of metabolic disorders (heart and kidney), obtained from mice fed for 3, 8 and 14 weeks with two different high fat diets (HFDs), with different fatty acid compositions and impact on fasting glucose levels. Statistically significant elevations (in the skeletal muscle, heart and kidney) or reductions (in the thyroid) of the levels of either AEA or 2-AG, or both, were found. Depending on the diet, these changes preceded or accompanied the development of overt obesity and/or hyperglycemia. In the adrenal gland, first a reduction and then an elevation of EC levels were observed. In the brown fat, a very early elevation of both AEA and 2-AG normalized levels was observed with one of the diets, whereas delayed decreases appeared to be caused by an increase of the amount of fat tissue weight induced by the HFDs. The potential implications of these and previous findings in the general framework of the proposed roles of the EC system in the control of metabolic, endocrine and cardiovascular and renal functions are discussed.

Introduction

Among the signalling systems suggested to play a role in the control of energy homeostasis and endocrine function, the endocannabinoid (EC) system is one of the most recently discovered (Pagotto et al., 2006; Wang et al., 2006). This system comprises: 1) the two cannabinoid receptors, CB₁ and CB₂ (Devane et al., 1988; Munro et al., 1993); 2) their endogenous ligands, named endocannabinoids (ECs), the best studied of which are anandamide (*N*-arachidonoyl-ethanolamine, AEA) and 2-arachidonoyl-glycerol (2-AG) (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995); and 3) the enzymes responsible for the formation of AEA and 2-AG and for their degradation, which have been identified not only in neurons, but also in peripheral cells including white adipocytes, rat insulinoma cells and mouse endocrine pancreas (Okamoto et al., 2004; Bisogno et al., 2003; Cravatt et al., 1996; Dinh et al., 2002; Engeli et al., 2005; Spoto et al., 2006; Matias et al., 2006; Starowicz et al., 2008). One of the best established functions of the EC system is the regulation of energy balance, at the level of both food-intake and peripheral control of metabolism. This was suggested by the observation that genetic and/or pharmacological impairment of cannabinoid CB₁ receptors causes inhibition of food-intake and reduction of body weight to a higher extent than that expected from the inhibitory effect on energy intake (Ravinet Trillou et al., 2003; Cota et al., 2003). Accordingly, CB₁ blockade normalizes several dysregulated metabolic parameters (i.e. low high-density-lipoprotein cholesterol, high triglycerides, low adiponectin, high glucose and insulin, etc.) in obese animals and patients in a way partly independent from weight loss (Cota et al., 2003; Després et al., 2005; Scheen et al., 2006), thus suggesting the existence of direct effects of ECs in tissues and cells controlling energy homeostasis such as adipocytes, pancreatic islets, hepatocytes and skeletal muscle, which all express CB₁ receptors (Cota et al., 2003; Bensaid et al., 2003; Osei-Hyiaman et al., 2005; Matias et al., 2006; Spoto et al., 2006; Roche et al., 2006; Pagotto et al., 2006; Juan-Pico et al., 2006; Starowicz et al., 2008). In particular, in adipocytes, ECs and CB₁ receptor levels increase during differentiation (Bensaid et al., 2003; Matias et al., 2006; Gasperi et al., 2007), and CB₁ stimulation leads to more rapid differentiation of

pre-adipocytes (Matias et al., 2006), stimulation of lipoprotein lipase activity (Cota et al., 2003), up-regulation of glucose uptake (Gasperi et al., 2007; Pagano et al., 2007), inhibition of AMP-activated protein kinase (AMPK) (Kola et al., 2005) and stimulation of fatty acid synthase (Osei-Hyiaman et al., 2005). These lipogenic actions of CB₁ in adipocytes explain in part why CB₁ knock-out mice fed with the same amount of food as wild-type mice still exhibit less fat mass (Cota et al., 2003). On the other hand, the role of the EC system in the endocrine pancreas is less well understood. In β -cells from isolated mouse pancreatic islets, both CB₁ (Nakata et al., 2007) and CB₂ (Juan-Pico et al., 2006) stimulation have been reported to inhibit insulin release, whereas in insulinoma cells grown in a high glucose concentration, CB₁, but not CB₂, stimulation causes enhancement of glucose-induced insulin release (Matias et al., 2006). In human β -cells, despite the relatively low abundance of CB₁ receptors, their stimulation also enhances insulin release (Bermudez-Silva et al., 2008).

The EC system also controls hormonal function, and is in turn regulated by several hormones, at the level of the hypothalamic-pituitary-adrenal (HPA) axis, the thyroid and in both female and male reproductive organs (Pagotto et al., 2006; Wang, et al. 2006). Worthwhile remembering are: 1) the mostly inhibitory effects of CB₁ receptor stimulation on corticotropin releasing hormone (CRH) in the hypothalamus, and subsequently on adrenocorticotrophic hormone (ACTH) and glucocorticoid plasma levels (Barna et al., 2004; Cota et al., 2007), and the direct suppression of adrenalin release from adrenal medullary cells (Niederhoffer et al., 2001); 2) the inhibitory actions on the release of both gonadotropin-releasing hormone (GnRH) from the hypothalamus (Gammon et al., 2005), and of prolactin and luteinizing hormone (LH), but not follicle stimulating hormone (FSH), from the pituitary (de Miguel et al., 1998; Wenger et al., 2001; Scorticati et al., 2004); 3) the effects of FSH on ECs levels in Sertoli cells (Rossi et al., 2007); 4) the inhibition of thyroid hormone release from the thyroid (Porcella et al., 2002); and 5) the effects of oestrus, ovulation and pregnancy on hypothalamic, pituitary, uterine and blood EC levels (Gonzalez et al., 2000; Lazzarin et al., 2004; Habayeb et al., 2004; Bradshaw et al., 2006). Given the several effects on metabolism exerted even by hormones not directly involved in the control of energy balance (such as for example the

estrogens), it is possible that some of these cross-talks between ECs and endocrine function also intervene in metabolic dysfunctions, particularly if the EC system becomes dysregulated.

Metabolic dysfunctions such as dyslipidemia and dyslipoproteinemia as well as insulin resistance and visceral adiposity and the associated inflammatory states, while being correlated with each other, clearly play a role in atherogenesis and the subsequent increased cardiovascular risk. Therefore, the EC system is likely to play a potentially important role in the cardiovascular consequences of these metabolic disorders, especially if these are caused in part by its dysfunction. However, the EC system also plays a direct role in the cardiovascular system by directly lowering blood pressure via both CB₁ and non CB₁-receptors in both endothelial and smooth muscle cells of the vasculature (Pacher et al., 2005), by reducing the accumulation of atherosclerotic plaques via CB₂ receptors (Steffens and Mach, 2006; Rajesh et al., 2007), and by exerting direct cardioprotective effects, again mostly via CB₂ receptors. In the heart, overactivation of CB₁ receptors seems instead to contribute to some pathological conditions such as cirrhosis-induced cardiac contractile dysfunctions (Batkai et al., 2007) and doxorubicin-induced damage (Mukhopadhyay et al., 2007). Local CB₁-mediated vascular effects of ECs also occur in the kidney, where these compounds also influence urine excretion although in a non-CB₁-mediated manner (Li et al., 2006). Importantly, chronic blockade of CB₁ receptors significantly reduces kidney hypertrophy, glomerulopathy and kidney failure in obese Zucker rats (Janiak et al., 2007).

Despite the well established metabolic function of ECs, our knowledge of their regulation under dietary conditions leading to hyperglycemia, obesity and increased cardiovascular risk is still limited. There is, however, evidence for the overactivity of the EC system (in terms of upregulation of either CB₁ receptor or EC levels, or both) during conditions of unbalanced energy homeostasis (e.g. obesity and hyperglycemia) (Di Marzo et al., 2005; Matias et al., 2007). This overactivity occurs at the level of both the hypothalamus (Di Marzo et al., 2001) and peripheral tissues, including the liver, pancreas, and epididymal adipose tissue in animals fed with a high fat diet (HFD), and in the visceral fat and blood of obese patients (Monteleone et al., 2005; Engeli et al.,

2005; Osei-Hyiaman et al., 2005; Matias et al., 2006; Bluher et al., 2006; Cote et al., 2007; Yan et al., 2007). We know that it is associated with, and it underlies in part, some of the metabolic dysfunctions that accompany obesity and hyperglycemia (Matias et al., 2006; Osei-Hyiaman et al., 2005; Cote et al., 2007), thus explaining why CB₁ receptor blockade is effective at reducing body weight in obese individuals (Cota et al., 2003). On the other hand, not in all tissues of hyperglycaemic and/or obese mice the levels of ECs increase following a high fat diet. In fact, a decrease has been observed in the subcutaneous fat and in the stomach of these mice (Starowicz et al., 2008; Di Marzo et al., 2008). The former effect, if present also in humans and in view of the pro-lipogenic effects of CB₁ receptors, might concur at reducing subcutaneous fat and enhancing abdominal fat, thus participating in the increased cardiometabolic risk accompanying abdominal obesity (Starowicz et al., 2008). On the other hand the reduction of EC signalling in the stomach might cause a reduction of ghrelin release (Cani et al., 2004), thus representing an adaptive response aimed at reducing food intake (Di Marzo et al., 2008). No studies have been carried out so far to investigate whether hyperglycemia and obesity cause by a HFD is accompanied by aberrant EC levels also: 1) in tissues involved in energy expenditure, i.e. the brown adipose tissue and the skeletal muscle; 2) in endocrine organs indirectly involved in the control of metabolism, i.e. the adrenal gland and thyroid; and 3) in tissues affected in the long term by the metabolic consequences of EC dysregulation, i.e. the heart and kidneys.

The possible biochemical mechanisms underlying aberrant EC levels in obesity are not completely understood. Impairment of FAAH expression or activity seems to correlate with obesity and overweight in humans (Sipe et al., 2005; but see also Jensen et al., 2007 for discrepant results) and with the elevated EC levels found in the liver of mice fed a HFD (Osei-Hyiaman et al., 2005) or in the blood of obese women (Engeli et al., 2005; Bluher et al., 2006). In HFD mice, dysregulation of the expression of both biosynthetic and degrading enzymes seems to account for at least part of the changes in EC levels observed in the pancreas and subcutaneous fat (Starowicz et al., 2008). Changes in the levels and functional activity of metabolic hormones that seemingly control EC

levels, such as leptin (Di Marzo et al., 2001; Maccarrone et al., 2005), insulin (Matias et al., 2006), glucocorticoids (Malcher-Lopes et al., 2006) and possibly ghrelin (Tucci et al., 2004), might underlie some of the effects of obesity and hyperglycemia on EC metabolic enzymes. It has also been found that changes in small intestine levels of AEA following food consumption or deprivation are due to biosynthetic precursor availability rather than to changes in the activity of biosynthesising and degrading enzymes (Petersen et al., 2006), thus raising the possibility that the fatty acid composition of the diet might influence peripheral EC levels, as it has been shown already for the brain (Berger et al., 2001; Watanabe et al., 2003), and for isolated adipocytes (Matias et al., 2007). Based on this background, and in order to provide some answers to the question of whether or not the dysregulation of the EC system occurs in all peripheral organs, and with what function, we have measured here, in mice fed for different periods of time either a standard diet (STD) or two different types of HFDs, the amounts of ECs, measured by using liquid chromatography-mass spectrometry, in the adrenal glands, thyroid, brown adipose tissue, skeletal muscle, heart and kidney.

Animals, materials and methods

Animals and diets

Male, seven-week old C57Bl/6J mice were purchased from Harlan Italy (Corezzana, MI). After one week acclimatization, animals were fed for 14 weeks different high fat diets (HFDs). These are: HFD1 (TD97366, Harlan Italy), containing 25.5% fat (49% of calories), 22% protein and 38.4% carbohydrate HFD2 (TD97070, Harlan Italy), containing 33.8% fat (59.9% of calories), 23.9% protein and 27.1% carbohydrate. In one set of experiments (i.e. brown fat experiments) mice were fed another HFD, i.e. HFD3 (D03082706, provided by Drs Piccioni Laboratories, Gessate, MI, Italy), containing 20% fat (40% of calories), 16.6% protein and 58.8% carbohydrate. Control mice received standard diet containing 5.7% fat (10.9% of calories), 18.9% protein and 57.3% carbohydrate (2018, Harlan Italy) (see Table 1 for the fatty acid compositions of the three diets).

Body weight was measured weekly. Mice were fed *ad libitum*, except for the 12-hr period immediately preceding the sacrifice, which occurred after 3, 8, and 14 weeks. An overnight, 12 hr fasting period was chosen because it is known that blood EC levels decrease post-prandially in human volunteers (Matias et al., 2006). Fasting plasma glucose levels were determined in 12-hours fasted animals using the glucose test kit with an automatic analyzer (Accu-Chek[®] Active, Roche) in blood samples obtained from tail vein (Darmani et al., 2005). Measurements were performed at time 0 and after 3, 8, and 14 weeks of dietary treatment. Experiments were also performed in Wistar rats (Harlan, Corezzana, MI, Italy) to compare basal expression of receptors and enzymes in α - and β -cells to those in the mouse. Rats received standard diet and were fed *ad libitum*, except for the 12-hr period immediately preceding the sacrifice and perfusion. After sacrifice, the tissues were removed and immediately immersed into liquid nitrogen, to be stored at -70° until extraction and purification of ECs. Visceral fat was taken from the mesenteric area.

EC measurement

Tissues from mice fed a standard diet (STD) and any of the three HFDs were removed and immediately frozen in liquid nitrogen until quantitative determination of ECs. The extraction, purification and quantification of AEA and 2-AG from tissues require several biochemical steps as described previously (Bisogno et al., 1997). First, tissues were dounce-homogenized and extracted with chloroform/methanol/Tris-HCl 50 mM pH 7.5 (2:1:1, v/v) containing internal standards ($[^2\text{H}]_8$ AEA and of $[^2\text{H}]_5$ 2-AG, 100 pmol each). The lipid-containing organic phase was dried down, weighed and pre-purified by open bed chromatography on silica gel. Fractions were obtained by eluting the column with 9:1 and 1:1 (by vol.) chloroform/methanol. After lipid extraction and separation, the pre-purified lipids were then analyzed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) by using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2010) quadrupole MS via a Shimadzu APCI interface as previously described (Di Marzo et al., 2001). The amounts of ECs in the tissues, quantified by isotope dilution with the above-mentioned deuterated standards, are expressed as

pmol or nmol per gram of wet tissue. In the case of the brown fat, where strong effects of the diet on the total amount of fat were observed, data are also expressed as total EC levels in nmol. Experiments with each HFD were carried out in different periods and have their separate control STDs. This explains why, although the STD was the same for each HFD, slightly different EC tissue concentrations between the two controls were found. In fact, several environmental (period of the year) and experimental (different internal standard stocks) factors might affect the evaluation of EC tissue concentrations.

Statistical Analysis

All quantitative results were expressed as mean $\pm$ SEM or $\pm$ SD. The statistical significance of differences in mean values was assessed by one-way ANOVA followed by the Bonferroni's post-hoc analysis.

Results and discussion

In this paper we have reviewed the previous evidence pointing to obesity and hyperglycemia-associated dysregulation of the EC system in key organs and tissues involved in energy homeostasis and have provided new data in tissues involved in energy expenditure, in endocrine organs involved in the control of metabolism and in peripheral organs that suffer the major consequences of prolonged metabolic disorders such as obesity and type 2 diabetes. We have also addressed the issue of whether a HFD and its fatty acid composition can cause such dysregulation, by comparing the effects of two very different HFDs on EC levels in the skeletal muscle, brown adipose tissue, adrenal gland, thyroid, heart and kidneys. We report data suggesting that a general peripheral dysregulation of EC levels is a hallmark of both high fat diet-induced hyperglycemia and obesity in a way that depends on the organ analysed, type of the diet and its duration.

Composition of the HFDs

The two HFDs (HFD1 and HFD2) were selected mostly based on their very different content of monounsaturated and, particularly, polyunsaturated fats (Table 1). In fact, the higher amount of total fat of HFD2 was mostly accounted for by higher amounts of esterified oleic acid (18:1), and, particularly, of linoleic acid (18:2) and α -linolenic acid (18:3). The ratio between ω 6 and ω 3 polyunsaturated fatty acids was, however, almost identical in both cases ($\sim$ 14) and higher than in the STD ($\sim$ 11). This allows to see effects that would depend not so much on the ratio between ω 6 and ω 3 polyunsaturated fatty acids, but rather on their absolute amounts, which have been suggested to indirectly affect EC levels in the brain and adipocytes via formation of arachidonic acid and docosahexaenoic acid, respectively (Berger et al., 2001; Watanabe et al., 2003; Matias et al., 2007). Also the total amounts of trans fatty acids were, however, higher in HFD2.

Effect of the two high fat diets on body weight, fasting glycemia levels and organ tissue weight

As shown in Fig. 1a, the two diets produced similar time-dependent effects on body weight, HFD2 being slightly more efficacious, possibly due to the presence of a higher amount of total fat in this diet. However, HFD2 was less effective at inducing hyperglycemia (Fig. 1b), which developed only after 14 weeks of this diet, when mice had become obese, whereas in the case of HFD1 the effect on fasting glucose was already visible after 3 weeks of diet. This difference between the two diets might have been due to the higher presence in HFD2 of precursors for ω 3 polyunsaturated fatty acid (i.e. α -linolenic acid), which are known to protect from insulin resistance (Nobukata et al., 2000; Carpentier et al., 2006). At any rate, the different time-course of hyperglycemia with HFD1 and HFD2 allowed us to correlate some of the effects observed with the two diets on EC levels to the development of hyperglycemia or obesity. No significant effect of the diets was found on the amounts of the tissues analysed except for the brown fat (Table 2, see below) and kidneys, where both HFD1 and HFD2 increased the weight at all time points (by 16.5, 26.0 and 10% and 24.0, 28.0 and 35.7%, respectively, at 3, 8 and 14 weeks, data not shown).

Tissues directly involved in energy expenditure: Skeletal muscle

The skeletal muscle is the tissue mostly responsible for glucose utilization and oxidation, ATP production and energy expenditure, especially during exercise, which, among other things, stimulates AMPK and the subsequent oxidation of both glucose and fatty acids. This tissue will use glucose coming from the liver or the diet and taken up via the action of glucose transporters, whose translocation to the plasma membrane of myotubes is stimulated by insulin. Despite evidence suggesting that an overactive EC system participates in decreased energy expenditure, no data on the regulation of EC levels in the skeletal muscle during hyperglycemia and obesity have ever been published.

With both diets employed here, we observed an early elevation of 2-AG levels in the mouse soleus muscle after 3 weeks of the regimen (Fig. 2), when mice are neither obese nor necessarily hyperglycemic (Fig. 1). After 8 weeks, this effect was not present any longer. However, after 14 weeks of HFD2, when the mice fed with this diet become not only overtly obese but also hyperglycemic, the stimulatory effect on 2-AG levels was observed again. The early elevation of the levels of 2-AG, which was the most abundant EC in all tissues analysed, might cause a stronger inhibition of AMPK1 α and, subsequently, of glucose uptake and oxidation from the skeletal muscle, where CB₁ receptors are present and functionally active (Cavuto et al., 2007), with subsequent overall reduced energy expenditure. This might contribute, in turn, to the high fasting glycemia observed already after 3 weeks of one of the two diets, and to the later development of obesity. On the other hand, the late elevation of 2-AG levels observed with HFD2 might explain the stronger stimulatory action observed with CB₁ receptor antagonists on AMPK1 α , glucose uptake and oxidation in the skeletal muscle in obese vs. lean individuals (Liu et al., 2005; Cavuto et al., 2007). Indeed, it was shown that also the expression of CB₁ receptors in the mouse soleus muscle increases when the animals are kept on a high-fat diet (Pagotto et al., 2006). The late effect observed on 2-AG levels with HFD2 might be due to the presence of significantly higher levels in this diet of linoleic acid with respect to STD and HFD1. In fact, linoleic acid is a precursor for

arachidonic acid, and the higher availability of the latter fatty acid might cause elevation of 2-AG levels (Matias et al., 2007). Furthermore, this second peak of 2-AG up-regulation coincides with the development of strong hyperglycemia with HFD2, which was stronger than the hyperglycemia observed with HFD1 at this time point.

Tissues directly involved in energy expenditure: brown fat

The brown adipose tissue in rodents is the organ mostly involved in maintaining the physiological temperature by consuming ATP during the course of futile cycles aiming at producing heat. Ion, substrate and protein degradation cycling contribute to these futile cycles. Importantly, several of the genes encoding for enzymes involved in these futile cycles, such as calcium binding proteins (sarcoplasmic reticulum Ca^{2+} -ATPase I, ryanodine receptor 1, parvalbumin and calsequestrin 1), enzymes regulating cellular glycogen concentrations (glycogen phosphorylase and glycogen synthase) and enzymes involved in amino acid degradation (cysteine dioxygenase, methylcrotonoyl-CoA carboxylase and methylmalonyl CoA mutase), are down-regulated in mice rendered obese following a high fat diet, and this up-regulation is reversed by the CB_1 receptor antagonist rimonabant, thus suggesting that an overactive EC system reduces futile cycles, thereby reducing energy expenditure, in the brown fat of obese mice (Jbilo et al., 2005). However, no data on the regulation of EC levels in the brown adipose tissue during hyperglycemia and obesity are available to date. Here we found that with one of the diets used, the HFD1, a strong up-regulation of both AEA and 2-AG levels is found in the mouse brown adipose tissue only after 3 weeks of the regimen (Fig. 3), when mice are hyperglycaemic but not yet obese (Fig. 1). This effect is not present anymore after 8 or 14 weeks of HFD1, when an apparent decrease of EC levels starts instead. In fact, also with HFD2 an apparent decrease of AEA levels was observed already with 3 weeks of the diet and throughout the duration of the experiment. However, with both HFD1, starting after 8 weeks, and HFD2, starting already at 3 weeks, the amounts of total brown fat in HFD mice was higher than in STD mice, as it might have been expected from the fact that chronic

HFDs increase the amount of all fat depots. This might have contributed to the apparent decrease of the levels of ECs, which were normalized in this study to the amount of wet tissue weight. If one considers, instead, the total amounts of ECs, these were not usually significantly decreased by the diets, and in fact were sometimes even increased, except for HFD2 at 14 weeks (Table 2).

In view of the pro-lipogenic action of the EC system in white adipocytes, the early increase of normalized EC levels found with HFD1 might represent an early response aimed at reducing dissipation of energy in futile cycles and at storing it in the white adipose tissue instead. The increase of the total amounts of ECs in this depot, found with both diets at 8 weeks, might thus contribute to the generally decreased energy expenditure through futile cycles observed in HFD vs. STD mice and thus explain the increase of futile cycle protein expression observed with rimobanant (Jbilo et al., 2005). On the other hand, we have no functional explanation for the decrease of ECs levels in brown fat observed with HFD2 after 14 weeks. For this reason we carried out another experiment with a third type of HFD (HFD3, Table 1), and found an increase of total 2-AG levels after 8, but not 14, weeks of the regimen (Table 2), in agreement with the data shown above for HFD1. We conclude that the effect observed at 14 week with HFD2 is strictly related to this type of diet, particularly in view of the inhibitory effect of ω 3-polyunsaturated fatty acids on 2-AG levels in adipocytes (Matias et al., 2007), and of the fact that HFD2 contains higher amounts of these fatty acids than STD, HFD1 and HFD3 (Table 1). On the other hand, this effect might also represent an adaptive response (aiming at burning more glucose with futile cycles) to the sudden development of hyperglycemia observed with HFD2 at 14 weeks, since with both HFD3 and HFD1 this phenomenon was not observed (Fig. 1 and data not shown).

Endocrine organs involved in metabolic control: thyroid

The thyroid hormones T3 and T4 are well known to control metabolism and defective or excessive production of these molecules is usually associated with increased or decreased body weight, respectively. Nevertheless, despite the ever increasing evidence that the EC system controls

metabolism, no data exist in the literature on the regulation of EC levels in the thyroid under conditions of unbalanced energy homeostasis. We found here that, whereas HFD2 induced an decrease of AEA levels in this organ after 3 and 8 weeks of the diet, with no significant changes after 14 weeks, a similar effect with HFD1 was observed only after 14 weeks (Fig. 4). Since ECs have been shown to decrease T3 and T4 release from the thyroid in lean rats by acting at CB₁ receptors (Porcella et al., 2002), it is possible that their decrease following HFDs represents an adaptive response aiming at increasing metabolic rate by disinhibiting thyroid hormone release. The onset and duration of this putative response, however, seem to be both dependent on the type of HFD administered. Interestingly, the loss of this putative compensatory action, observed after 14 weeks with HFD2, coincides with the late development of strong hyperglycemia following this diet.

Endocrine organs involved in metabolic control: adrenal glands

Adrenalin, secreted by the adrenal glands, plays a key role in controlling energy homeostasis, particularly under conditions of stress, via multiple stimulatory effects on energy expenditure. Also in adrenal glands the two HFDs studied here seemed to affect only AEA levels. Interestingly, we observed, with both HFD1 and HFD2, an early decrease of the amounts of this EC, corresponding with the onset of hyperglycemia (Fig. 5). This decrease was maintained after 8 weeks with HFD1 but not with HFD2, with which we observed a reversal of the effect already at this time point, with a significant increase of AEA levels. This reversal was also observed with HFD1 after 14 weeks, when the effect of HFD2 was no longer statistically significant. In view of the suppression exerted by ECs and CB₁ receptors on adrenaline release from the adrenal glands (Niederhoffer et al., 2001), the early decrease of AEA levels might represent an adaptive response to high energy intake, aimed at enhancing adrenaline release and, therefore, at increasing lipolysis and energy expenditure and reducing lipogenesis. However, with longer exposures to HFDs, an overactivity of the EC system develops in this gland that might instead counteract adrenalin release, and thus contribute at reducing energy expenditure and at storing fat into the white adipose tissue.

Organs affected by the metabolic syndrome: heart

As mentioned in the Introduction, a dysregulated EC system, by enhancing the accumulation of fat in the visceral adipose organ and by reducing energy expenditure, contributes to the development of cardiometabolic risk factors such as dyslipidemia, dyslipoproteinemia, hyperglycemia and insulin sensitivity. Furthermore, ECs can directly affect cardiac function, and intervene in cardiac dysfunction, by acting at CB₁ and CB₂ receptors and, in the case of AEA, also at transient receptor potential vanilloid type-1 (TRPV1) channels (Zygmunt et al., 1999). However, little is known on the effects on the levels of ECs in the heart of HFDs causing enhanced cardiometabolic risk. Here we found that both HFD1, starting already 3 weeks, and HFD2, starting only 14 weeks, after the beginning of the dietary regimen, significantly increased cardiac EC levels (Fig. 6). By inhibiting cardiac AMPK (Kola et al., 2005), this elevation of CB₁ receptors might enhance lipogenesis in the heart, thus contributing to ectopic fat formation in this organ, which is *per se* an independent risk factor for cardiovascular events (Montani et al., 2004; Kankaanpää et al., 2006). On the other hand, enhanced AEA levels might act at TRPV1 receptors at the level of sensory neurons innervating the heart, and exert cardioprotective effects under ischemic conditions or during reperfusion (Zhong and Wang, 2007; Wang et al., 2005), whereas enhanced 2-AG levels might act at CB₂ receptors to reduce atherosclerosis in coronary arteries (Steffens et al., 2006), or to exert protective action against myocardial ischemia and hypoxia (Joyeux et al., 2002; Shmest et al., 2006). ECs, including AEA, may also elicit coronary vasodilation and a secondary increase in contractility via CB₁ receptors and potassium channels, or mediate delayed cardioprotection induced by nitric oxide, in the isolated rat heart (Wagner et al., 2005; 2006). Therefore, enhancement of EC levels, via several mechanisms, can both contribute at enhancing cardiovascular risk and reduce the consequences of cardiac ischemia. Interestingly, with both diets, the appearance of HFD-induced elevation of EC levels coincided with the appearance of hyperglycemia rather than obesity, thus possibly indicating a link with elevated fasting glucose levels and insulin resistance rather than body weight.

Organs affected by the metabolic syndrome: kidneys

A recent study showed that chronic blockade of CB₁ receptors could significantly reduce renal failure caused by obesity and type 2 diabetes in Zucker rats in a way partly independent from inhibitory effects on food intake (Janiak et al., 2007). However, in this in vivo study it was not possible to assess to what extent chronic CB₁ blockade exerts these protective effects also by directly acting at the level of the kidneys, or if these effects were simply the consequence of the general amelioration of the metabolic profile caused by this treatment. For this reason we analysed here EC levels in the kidneys of HFD mice and found, with both diets used, a strong elevation of AEA levels starting from 8 weeks of diet (Fig. 7). Among the possible implications of renal EC overactivity, a decreased glomerular filtration rate and increased renal blood flow, with subsequent glomerular hypertrophy, would be the most likely ones in view of the CB₁ receptor-mediated stimulatory effect by AEA on both afferent and, particularly, efferent arteriolae dilation (Koura et al., 2004). However, as in the case of the liver (Osei-Hyiaman et al., 2005) and the heart (see above), renal ectopic fat formation could also be a consequence of enhanced EC levels and contribute to kidney malfunctioning and cardiovascular disorders (Montani et al., 2004). Finally, elevated AEA levels might also affect diuresis and natriuresis via TRPV1 receptors (Zhu et al., 2007). Interestingly, with both diets, the appearance of HFD-induced elevation of EC levels coincided with the appearance of obesity rather than hyperglycemia, thus possibly indicating a link with elevated body weight rather than fasting glucose levels and insulin resistance. Finally, as both diets increased the weight of the kidneys, changes in total EC levels (not shown) were even more pronounced than changes in the levels normalized by tissue weight. We did not investigate whether this HFD-induced increase of kidney weight was due to perirenal fat accumulation or kidney hypertrophy.

Conclusions

We have reviewed here the evidence published so far suggesting the existence of a dysregulated peripheral EC system in mice made hyperglycaemic or obese as a consequence of prolonged HFDs. We have also described this phenomenon in six previously non-investigated organs, involved in different ways in metabolic dysfunctions and their consequences. We have shown that a HFD can not only be accompanied by generally aberrant EC levels in all of the organs analysed here and previously, but it does so with onset, duration and extent that depends on the type of diet, and, in particular, its fatty acid composition. Also the type of EC affected might depend on the HFD used. Although data are available in the literature suggesting that dietary fatty acids can influence EC levels in the brain (Berger et al., 2001; Watanabe et al., 2003) and adipocytes (Matias et al., 2007), we could not observe a strong correlation between the abundance of a certain fatty acid in the HFD and its effects on EC levels. However, in those organs where EC levels were first decreased and then increased by the HFD (i.e. the thyroid and adrenal gland), HFD2 produced this effect earlier. On the other hand, in some of the organs where an increase of EC levels was observed with HFD (i.e. the kidney and the heart), this effect was often extended also to 2-AG only when using HFD1. These tendencies might be due to the higher amounts, in HFD2 as compared to HFD1 and STD, of ω 3-polyunsaturated fatty acid precursors, whose potential inhibitory effects on both AEA and 2-AG levels might predominate over the stimulatory ones, on 2-AG levels only, of ω 6-polyunsaturated fatty acids (Matias et al., 2007), which are also more abundant in HFD2. In the brown fat (where interpretation of results is more problematic due to the effect of the diet on the amounts of this tissue), this hypothesis might also explain the late decrease of total EC levels observed with this diet. The only present findings that contradict this hypothesis relate to the skeletal muscle, where a second phase of 2-AG elevation was observed only with HFD2. However, this latter finding, like possibly others in the present study, might also be due to the fact that with HFD2, but not HFD1, hyperglycemia only develops after 14 weeks of the diet.

We have also discussed the potential sequelae of EC level dysregulation in hyperglycemia and obesity, at the level of both its contribution to the development of these metabolic disorders and of their consequences on cardiac and renal function (Fig. 8). Clearly, further and more detailed studies are necessary to fully appreciate the molecular causes and impact of HFD-induced EC level dysregulation. However, the present article strengthens the concept that aberrant EC levels in endocrine and non-endocrine organs involved in energy homeostasis, and in organs that are the ultimate targets of metabolism-derived pathological conditions, are involved in these conditions via potential alterations in CB₁, CB₂ and TRPV1 activity. This concept opens the way to new therapeutic strategies for the treatment of cardiometabolic risk based on the EC system, including, but not limited to, the use of the CB₁ receptor antagonists currently being developed by several pharmaceutical companies.

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Legends to Tables and Figures

Table 1. Fatty acid composition of the standard diet (STD) and the three high fat diets (HFD1, HFD2 and HFD3) used in this study. Only brown fat tissues were analysed from mice fed with HFD3. **Legend:** c, *cis*; t, *trans*.

Table 2. Weights (g) of brown fat and total (nmol) anandamide (AEA) and 2-arachidonoylglycerol (2-AG) levels in brown fat in mice fed with standard (control) and high fat diets HFD1, HFD2 and HFD3 for 3, 8 and 14 weeks. Data are means±SEM of N=4-5 separate determinations. *, P<0.05 as assessed by ANOVA followed by Bonferroni's post-hoc analysis. Experiments with HFD1 were carried out separately from experiments with HFD2 and HFD3 and this explains the different controls. The same STD was used for the two controls (see Table 1).

Figure 1. Body weight (measured every week) and fasting glucose levels (measured before sacrifice) of mice fed with standard (STD) and high fat diets HFD1 and HFD2 for 3, 8 and 14 weeks. Data are means±SEM of N=8 separate determinations. *, P<0.05 as assessed by ANOVA followed by Bonferroni's post-hoc analysis. Starting from the 1st week the body weights of the HFD-fed animals were always significantly different from those fed with a STD.

Figure 2. Tissue concentrations of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in the soleus muscle of mice fed with standard control diet (STD) or high fat diets HFD1 (A) and HFD2 (B). Experiments with each diet were carried out in different periods and have their separate STD controls. This explains slightly different values between the two controls were observed despite the fact that the same STD was used for the two sets of experiments. Data are means±SEM of N=4-5 separate determinations. *, P<0.05; ***, P<0.005 as assessed by ANOVA followed by Bonferroni's post-hoc analysis.

Figure 3. Tissue concentrations of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in the brown adipose tissue of mice fed with standard control diet (STD) or high fat diets HFD1 (A) and HFD2 (B). Experiments with each diet were carried out in different periods and have their separate STD controls. This explains slightly different values between the two controls were observed

despite the fact that the same STD was used for the two sets of experiments. Data are means $\pm$ SEM of N=4-5 separate determinations. *, P<0.05; ****, P<0.001 as assessed by ANOVA followed by Bonferroni's post-hoc analysis.

Figure 4. Tissue concentrations of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in the thyroid of mice fed with standard control diet (STD) or high fat diets HFD1 (A) and HFD2 (B). Experiments with each diet were carried out in different periods and have their separate STD controls. This explains slightly different values between the two controls were observed despite the fact that the same STD was used for the two sets of experiments. Data are means $\pm$ SEM of N=4-5 separate determinations. *, P<0.05; **, P<0.01 as assessed by ANOVA followed by Bonferroni's post-hoc analysis.

Figure 5. Tissue concentrations of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in the adrenal glands of mice fed with standard control diet (STD) or high fat diets HFD1 (A) and HFD2 (B). Experiments with each diet were carried out in different periods and have their separate STD controls. This explains slightly different values between the two controls were observed despite the fact that the same STD was used for the two sets of experiments. Data are means $\pm$ SEM of N=4-5 separate determinations. *, P<0.05; ***, P<0.005 as assessed by ANOVA followed by Bonferroni's post-hoc analysis.

Figure 6. Tissue concentrations of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in the heart of mice fed with standard control diet (STD) or high fat diets HFD1 (A) and HFD2 (B). Experiments with each diet were carried out in different periods and have their separate STD controls. This explains slightly different values between the two controls were observed despite the fact that the same STD was used for the two sets of experiments. Data are means $\pm$ SEM of N=4-5 separate determinations. **, P<0.01; ***, P<0.005; ****, P<0.001 as assessed by ANOVA followed by Bonferroni's post-hoc analysis.

Figure 7. Tissue concentrations of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in the kidneys of mice fed with standard control diet (STD) or high fat diets HFD1 (A) and HFD2 (B).

Experiments with each diet were carried out in different periods and have their separate STD controls. This explains slightly different values between the two controls were observed despite the fact that the same STD was used for the two sets of experiments. Data are means $\pm$ SEM of N=4-5 separate determinations. *, P<0.05; **, P<0.01; ***, P<0.005; ****, P<0.001 as assessed by ANOVA followed by Bonferroni's post-hoc analysis.

Figure 8. Summary of the effects of high fat diet on endocannabinoid (EC) levels in various tissues involved in metabolic control and in the consequences of metabolic disorders. Abbreviations: AMPK, AMP kinase ; FAS, fatty acid synthase; UCP-1, uncoupling protein-1.

Table 1

	HFD1	HFD2	HFD3	STD
Total Fat g/kg	250	335,1	200	57
Saturated Fat g/kg	142.81	139.08	119.05	1.95
C4:0 Butyric	-	-	6.13	-
C6:0 Caproic	0.42	0.5	3.63	-
C8:0 Caprylic	5.25	6.75	2.11	-
C10:0 Capric	4.2	5.4	4.74	-
C12:0 Lauric	32.66	40.14	5.31	0.26
C14:0 Myristic	17.69	15.53	19.01	0.06
C16:0 Palmitic	47.7	37.92	50.75	-
C17:0 Margaric	0.01	0.23	-	-
C18:0 Stearic	32.57	30.5	23.29	1.5
C20:0 Arachidic	0.09	1.05	-	0.1
C22:0 Behenic	-	0.9	-	0.03
C24:0 Lignoceric	-	0.13	-	-
Monounsaturated Fat g/kg	76.46	106.7	56.86	12.84
16:1 undifferentiated	6.74	0,05	4.25	0.07
16:1 c	0.02	-	-	-
16:1 t	-	-	-	-
17:01	-	-	-	-
18:1 undifferentiated	67.13	105.9	49.82	12.59
18:1 c	5.47	74.3	-	-
18:1 t	-	26.39	-	-
20:01	0.5	0.22	0.02	0.17
22:1 undifferentiated	-	-	-	0.01
22:1 c	-	-	-	-
22:1 t	-	-	-	-
24:1 c	-	-	-	-
Polyunsaturated Fat g/kg	18.6	70.49	12.82	34.11
16:2 undifferentiated	-	-	-	-
18:2 undifferentiated	16.92	65.87	9.37	-
18:2 n-6 c,c	10.6	58.24	-	31.35
18:2 c,t	-	-	-	-
18:2 t,c	-	-	-	-
18:2 t,t	-	-	-	-
18:2 i	0.1	5.88	-	-
18:2 t	-	-	-	-
18:03	1.19	4.62	3.43	-
18:3 n-3, c,c,c	0.23	4.62	-	2.76
18:3 n-6, c,c,c	-	-	-	-
Total t- fatty acids g/kg	0.1	32.28	-	-
Total t-monoenoic FA g/kg	-	26.42	-	-
Total trans-polyenoic FA g/kg	0.1	5.86	-	-
Total ω-3 fatty acids g/kg	1.2	4.62	3.43	2.76
Total ω-6 fatty acids g/kg	16.9	65.87	9.37	31.35

Table 2

	3 weeks			8 weeks			14 weeks		
	Fat amount (g)	Total AEA (nmol)	Total 2-AG (nmol)	Fat amount (g)	Total AEA (nmol)	Total 2-AG (nmol)	Fat amount (g)	Total AEA (nmol)	Total 2-AG (nmol)
Control 1	54.7±7.1	1.3±0.2	169.6±5.5	50.7±8.3	1.4±0.1	162.2±25.3	56.3±20.7	1.0±0.2	202.7±45.0
HFD1	68.2±6.0	2.8±0.3*	381.9±54.6*	99.0±4.9*	2.5±0.3*	346.5±29.7*	128.0±7.1*	1.3±0.1	217.6±38.4
Control 2	35.0±2.7	0.7±0.01	84.0±21.0	28.4±2.0	0.9±0.06	56.8±17.0	50.1±5.8	0.8±0.1	145.3±10.0
HFD2	57.5±17.6	0.4±0.1	161.0±34.5	84.5±6.9*	0.7±0.08	143.7±33.8*	88.8±7.1*	0.8±0.06	79.9±8.9*
HFD3	42.1±7.6	0.7±0.1	134.7±12.6	43.5±9.4	1.0±0.02	134.9±47.9*	65.8±4.6	0.8±0.1	134.9±9.9

Figure 1

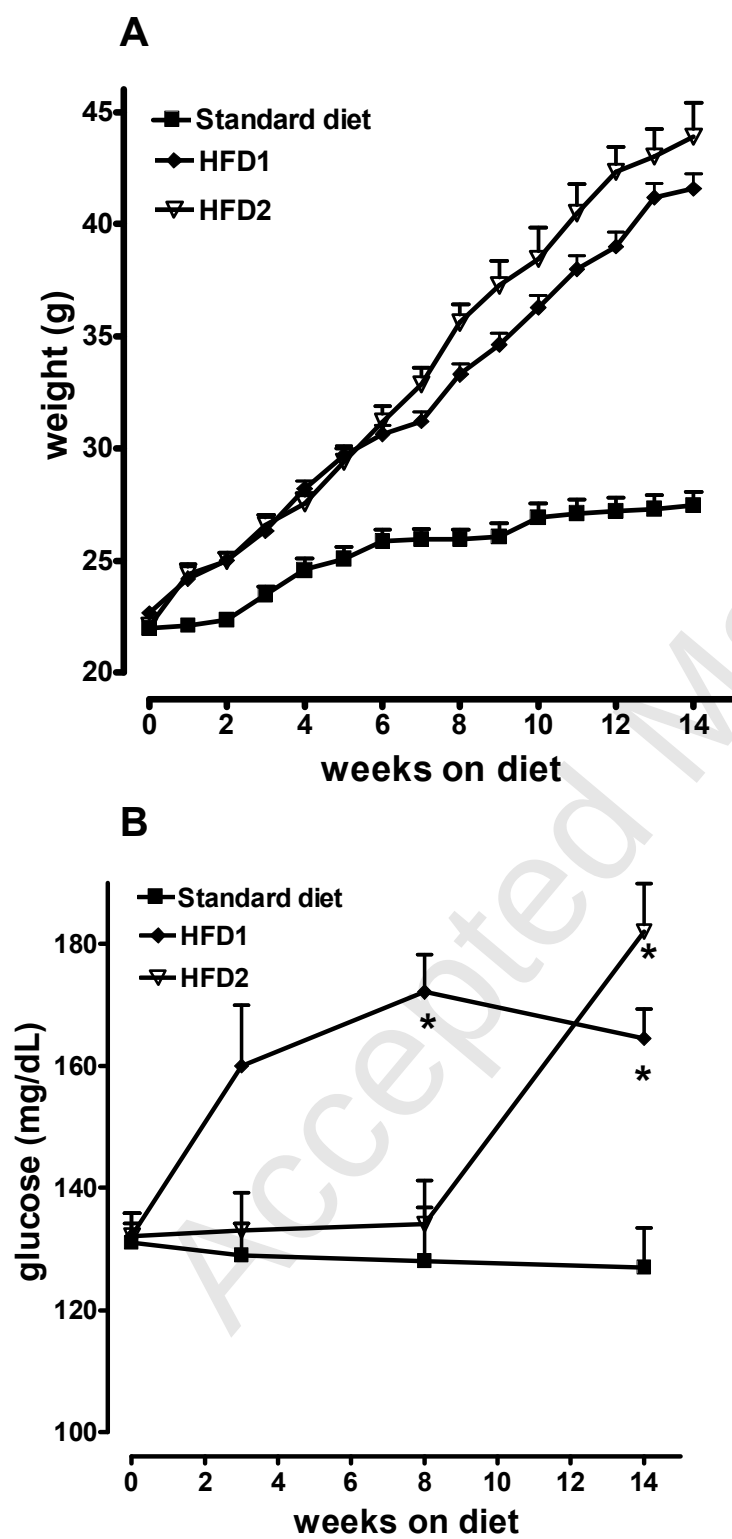


Figure 2

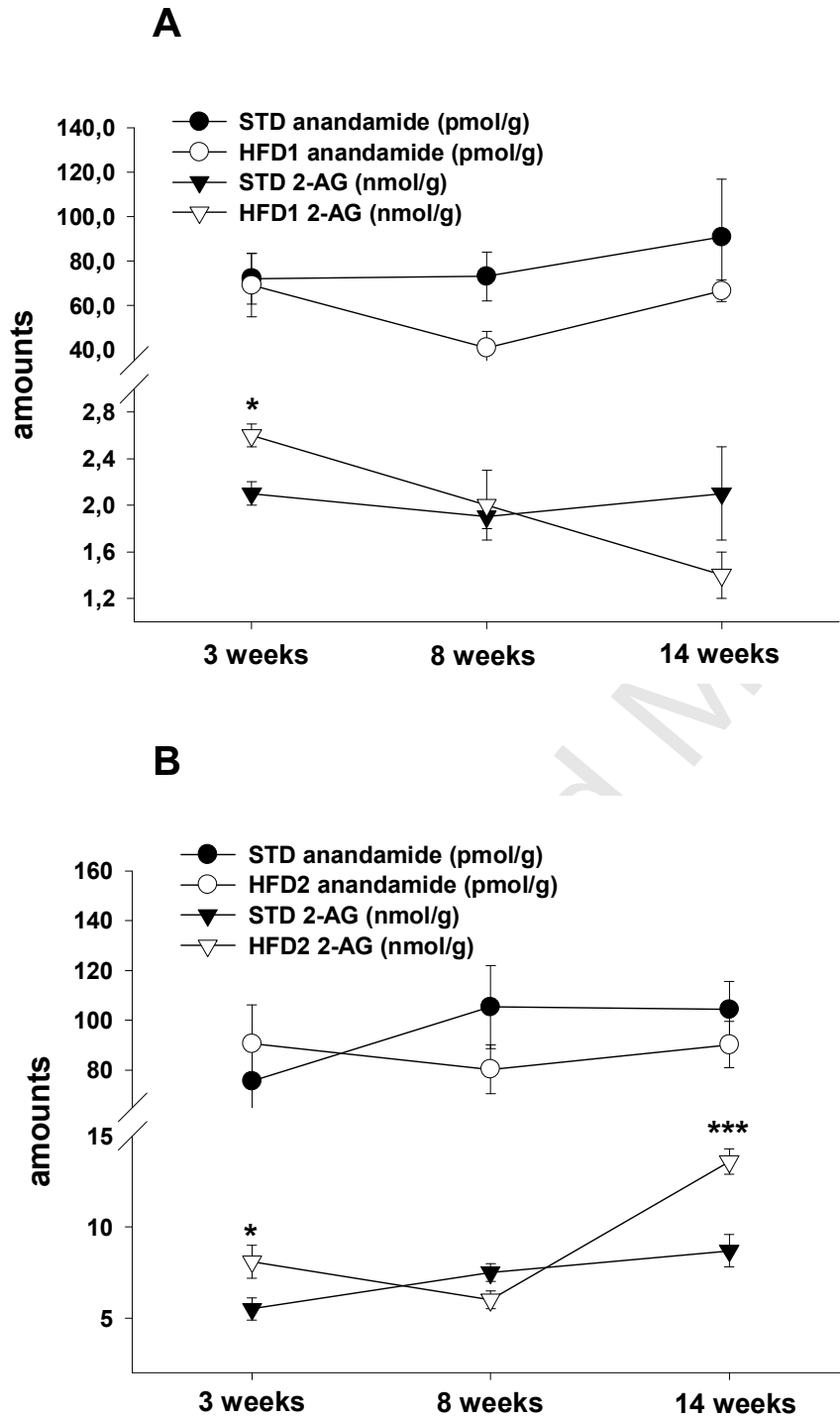


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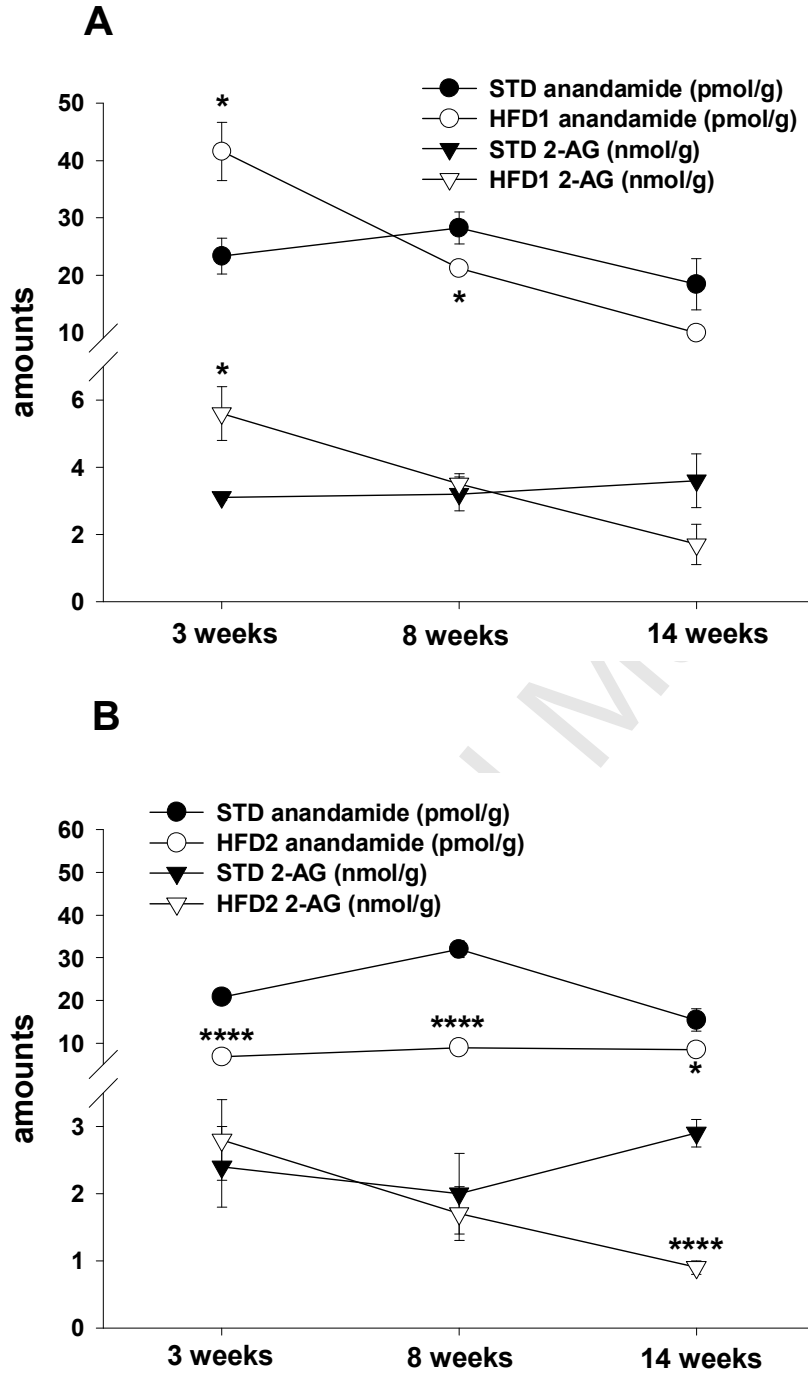


Figure 4

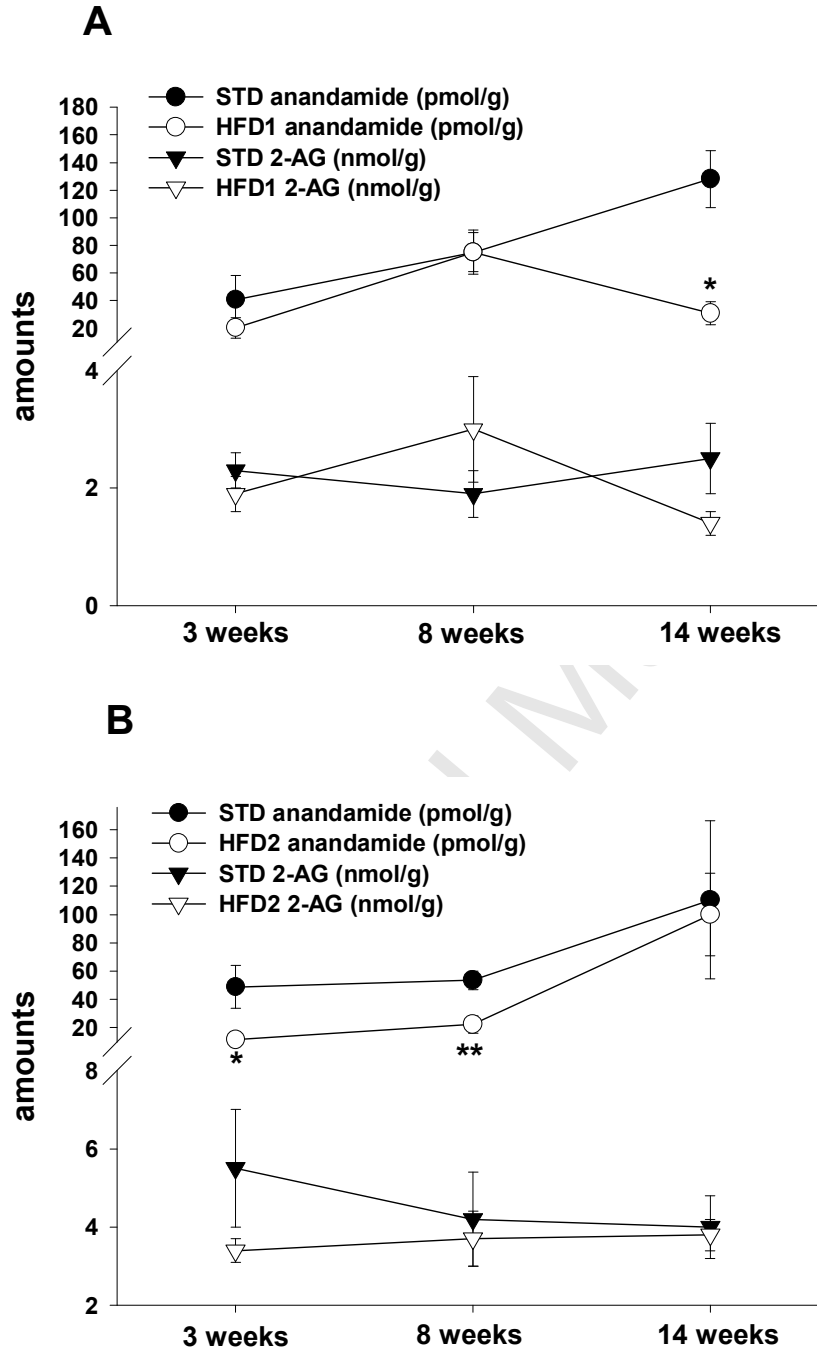


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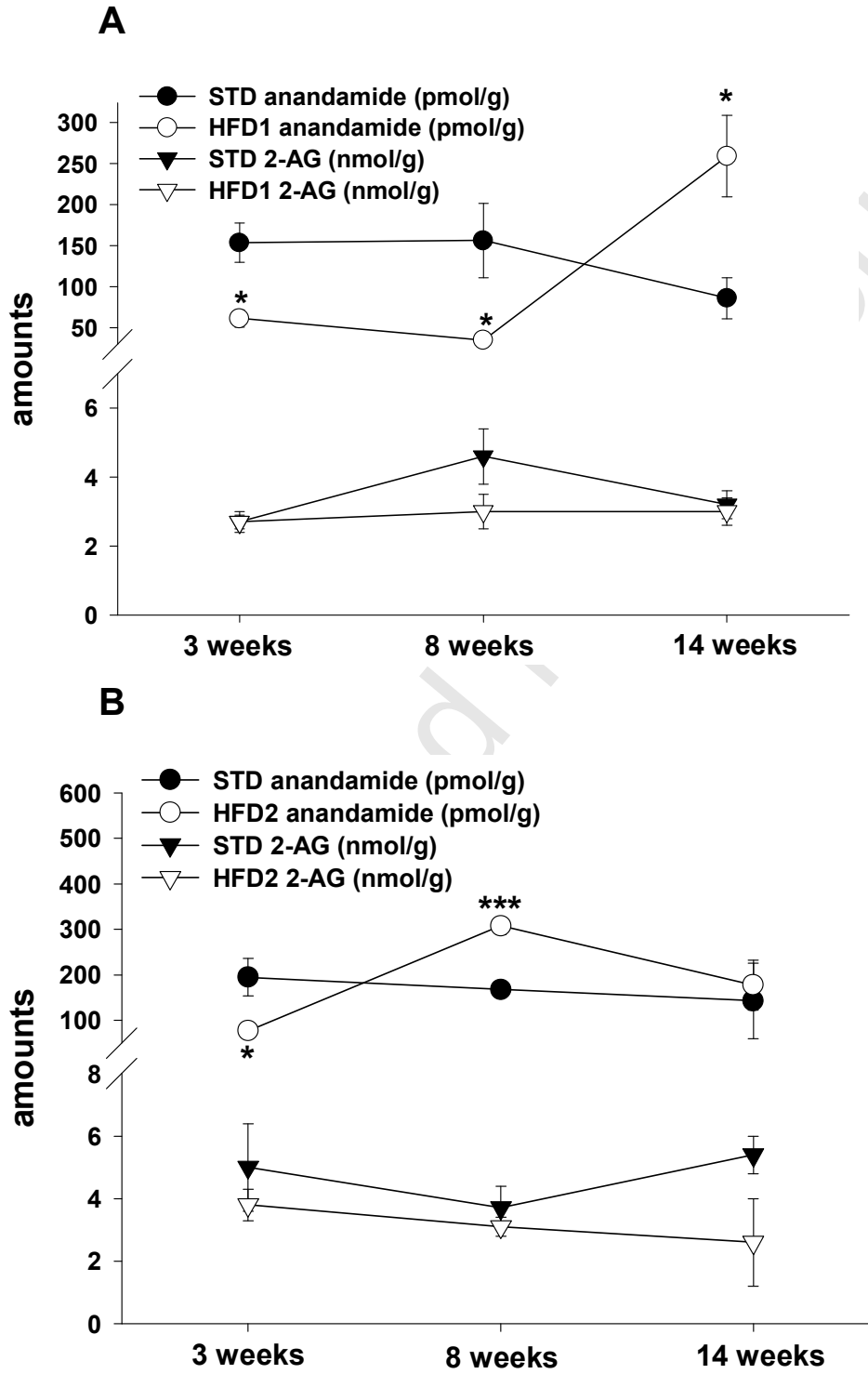


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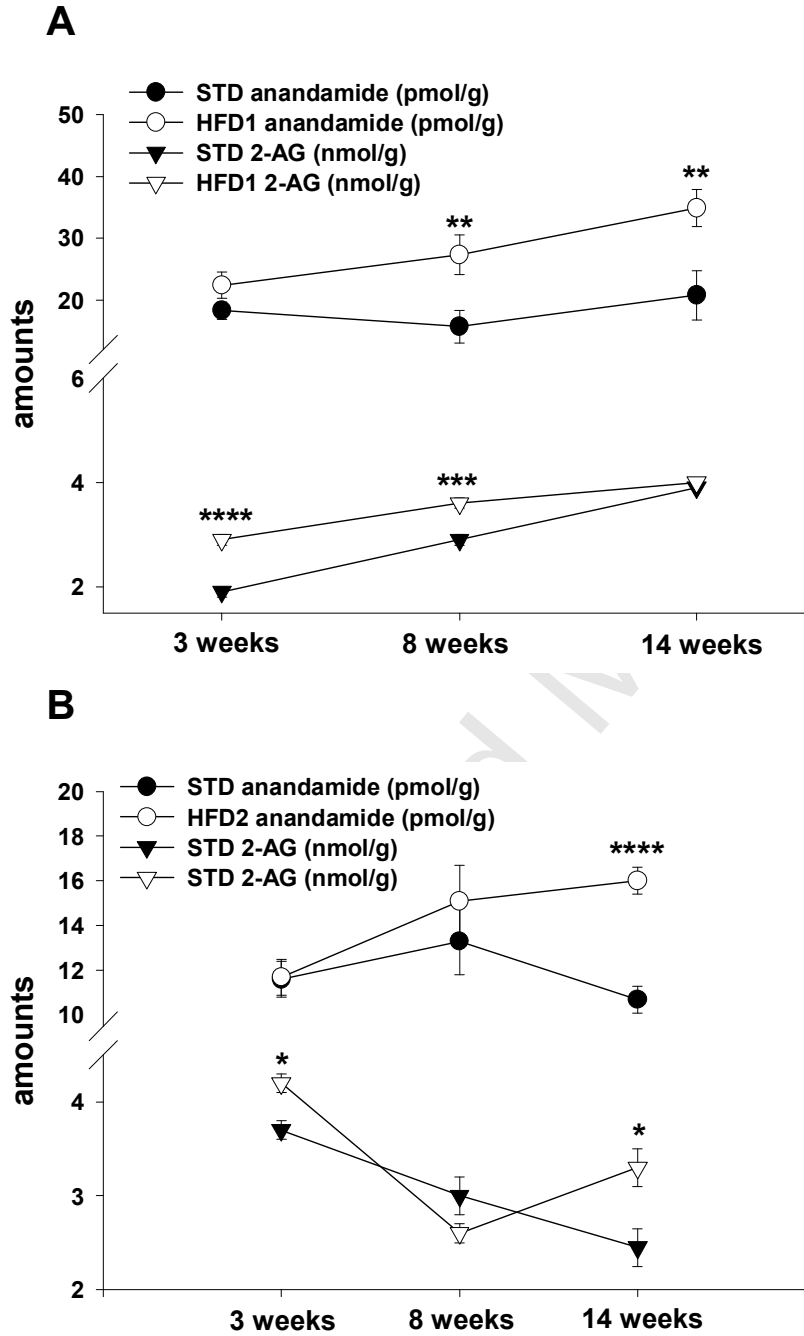


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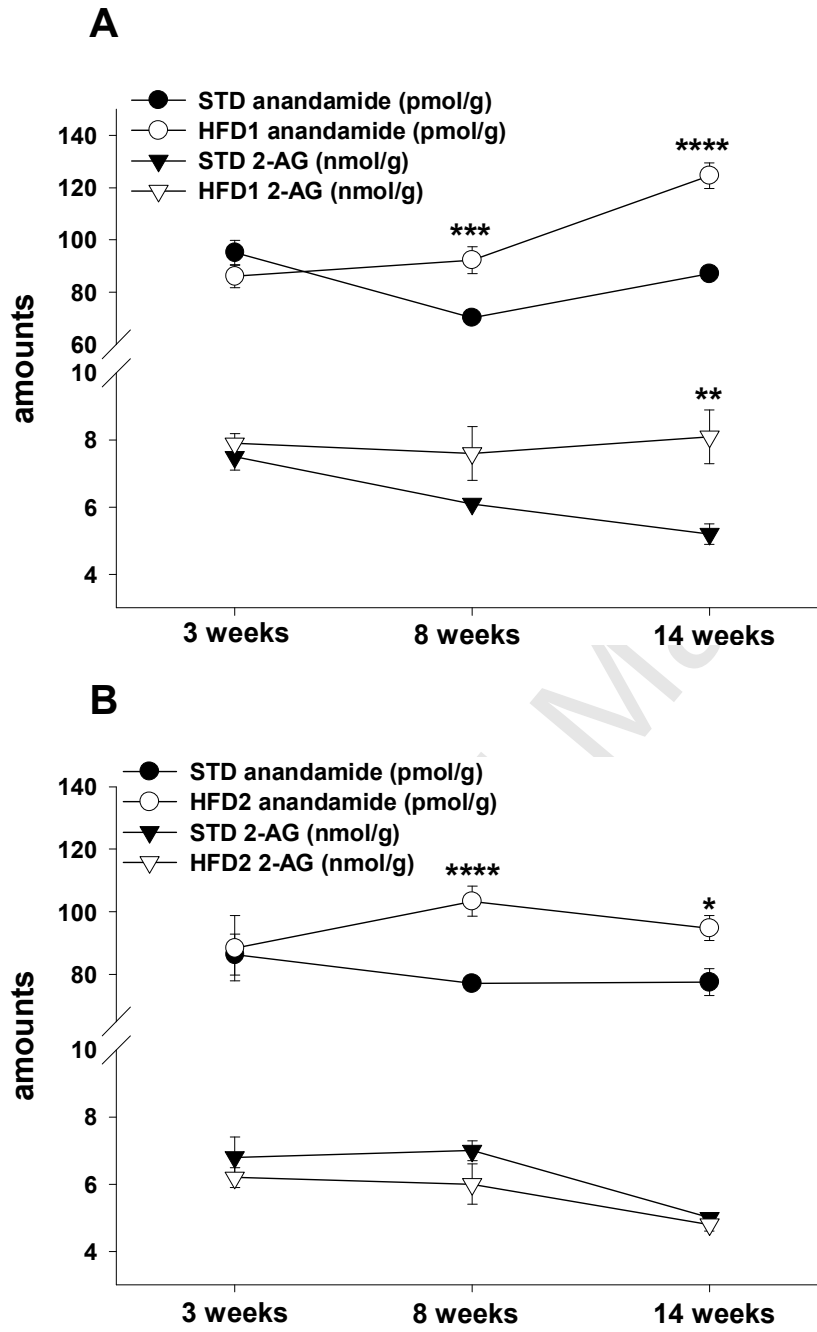
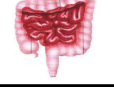
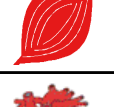
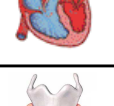
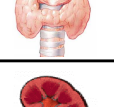
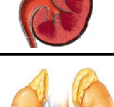


Figure 8

	Site of Action	Effect of CB ₁ activation	Effects of HFD	Potential Consequences
	<i>Hypothalamus</i>	↑ Food intake	↑ ECs	↑ Body weight / Waist circumference
	<i>Visceral Adipose Tissue</i>	↑ Lipogenesis (FAS) ↑ PPAR γ expression ↑ Glucose uptake ↓ AMPK activity ↓ Adiponectin	↑ ECs	↑ Energy storage ↑ Dyslipidemia ↑ Insulin resistance ↑ Waist circumference
	<i>Subcutaneous Adipose Tissue</i>	See above	↓ ECs	↓ Subcutaneous fat ↑ Visceral and ectopic fat
	<i>Brown Adipose Tissue</i>	↓ UCP1-1 ↓ Futile cycle	↑ ECs	↓ Thermogenesis ↓ Energy expenditure
	<i>Pancreas</i>	↓ ↑ Insulin release	↑ ECs	↑ Hyperinsulinemia
	<i>Liver</i>	↑ Lipogenesis	↑ ECs ↑ CB ₁	↑ Dyslipidemia ↑ Dyslipoproteinemia ↑ Steatosis
	<i>Gastrointestinal Tract</i>	↓ Satiety ↑ Food assimilation		↑ Body weight / Waist circumference
	<i>Muscle</i>	↓ Glucose uptake ↓ Glucose oxidation	↑ ECs ↑ CB ₁	↑ Insulin resistance ↓ Energy expenditure
	<i>Heart</i>	↓ AMPK activity ↑ Lipogenesis	↑ ECs	↑ Pericardial fat? ↑ Cardiovascular risk
	<i>Thyroid</i>	↓ Thyroid hormones (T3 and T4)	↓ ECs	↑ Metabolic rate
	<i>Kidneys</i>	↑ Renal failure ↑ Lipogenesis ↓ Glomerular filtration	↑ ECs	↑ Hypertrophy? ↑ Perirenal fat? ↑ Cardiovascular risk
	<i>Adrenal Glands</i>	↓ Adrenaline release	↓ ECs (early) ↑ ECs (late)	↑ Lipolysis (early) ↓ Lipolysis (late)