CB1 antagonism exerts specific molecular effects on visceral and subcutaneous fat and reverses liver steatosis in diet-induced obese mice.

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OBESTITY results from an imbalance between energy intake and expenditure and is characterized by increased body weight and abnormal development of adipose tissue with excessive fat storage (1). Recently, evidence has accumulated for the overactivity of the endocannabinoid system (ECS) during conditions of unbalanced energy homeostasis (2). The ECS consists of the cannabinoid receptors (CBs), their endogenous ligands (the endocannabinoids), and the enzyme proteins catalyzing the endocannabinoid formation and degradation (3). Activation of central CB1 receptors clearly promotes food intake and weight gain (4–6). Accordingly, pharmacological antagonism of CB1 has been shown to improve several pathological features associated with obesity, including overweight, hyperinsulinemia, insulin resistance, hyperglycemia, and dyslipidemia in obese rodents (7–9) and humans (10,11).

Even if the reduction in food intake induced by central CB1 blockade may be the main initial cause of body weight loss and associated beneficial effects, several data collected from animal and human studies indicate that peripheral CB1 may also directly control lipid metabolism (12–14). Thus, an activation of ECS has been recently reported in peripheral tissues of animal models of obesity (15,16) and associated with visceral fat obesity in humans (17,18). Consequently, it has been proposed that the long-term effects of CB1 antagonism are resolved by stimulation of energy expenditure and by peripheral effects related to adipose tissue, liver, skeletal muscle, and pancreas physiology (19–21).

In the present work, we tested the effects of CB1 antagonism on the regulation of the liver and adipose tissue lipid metabolism in a mouse model of diet-induced obesity. We first examined the global impact of CB1 antagonism on plasma parameters and liver steatosis, which were primarily altered by long-term feeding of a high-sucrose high-fat (HSHF) diet. Next, we examined whether CB1 inactivation was associated with biochemical and molecular alterations in the liver and adipose tissue (distinguishing visceral and subcutaneous fat depots) that could account for an improvement of liver lipid metabolism.

RESEARCH DESIGN AND METHODS
Official French regulations (no. 87848) for the use and care of laboratory animals were followed throughout the experimental period. The experimental protocol was approved by the local ethic committee for animal experimentation (no. BX0622). Four-week-old C57BL/6 male mice (Elevage Janvier, Le Genest Saint Isle, France) were housed in individual plastic cages and adapted to a standard diet (AO4; UAR, Epinay-sur-Orge, France) for 1 week. A series of mice was maintained on the standard diet (CON group; n = 5), while another series was subjected to an HSHF diet containing casein 20%, corn starch 13%, sucrose 29.3%, cellulose 5%, maltodextrin 2.2%, lard 20%, soya oil 2.5%, mineral 205B SAFE 7%, vitamin 200 SAFE 1% (ref. 235HF SAFE; Auguy, France). After 19 weeks, HSHF animals that were not both overweight and hyperinsulinemic were excluded from the study. Selected mice were maintained on an HSHF diet and received orally either 10 mg · kg⁻¹ · day⁻¹ of SR141716 (HSHF + SR series; n = 14) or vehicle (HSHF series; n = 10). The CB1-specific antagonist SR141716 (Rimonabant) was supplied by sanofi-aventis (Paris, France). Animals had free access to fresh food and water throughout the experimental period. Mice were fed deprived 4 h before anesthesia with ketamine/xylazine (7.5 mg · 1 kg⁻¹ · 100 g body wt⁻¹) and tissue handling. Epididymal and inguinal fat were surgically removed as representatives of visceral and subcutaneous fat, respectively (22). Tissue samples were frozen in liquid nitrogen pending further analyses.

Serum and tissue parameters. Serum parameters were determined using commercial kits (glucose RTU, TG PAP150, and cholesterol RTU from
BioMérieux [Marcy l’Etoile, France] for glucose, triglycerides, and cholesterol assay, respectively; nonesterified fatty acid C from Wako Pure Chemical Industries [Richmond, VA] for free fatty acid [FFA] assay; and mouse insulin and adiponectin enzyme-linked immunosorbent assay kits from AbCys [Paris, France]). Liver malonyl-CoA concentration was determined by high-performance liquid chromatography as previously described (23). Liver total lipids were extracted according to the method of Folch et al. (24). After mixing thoroughly, 1.0 ml of organic phase was transferred to a clean tube containing 1 ml of 1% Triton X-100 in chloroform and dried using nitrogen. The residue was resolubilized in 0.25 ml distilled water and used for the determination of triglycerides and cholesterol as in serum. For determination of adiponectin content in adipose tissue, samples were homogenized in 10 volume of PBS. After centrifugation (10 min at 12,000g, 4°C), the supernatants were carefully collected through the fat cake, diluted to 1/40,000 in PBS, and used for adiponectine measurements as in serum.

Fatty acid oxidation and apolipoprotein A and B secretion. Freshly removed livers from five HSHF and five HSHF+SR mice were sliced using a Brendel/Vitron slicer (Tucson, AZ), and thin slices were used to measure 11C]palmitic acid oxidation and apolipoprotein (apo) A and B secretion as previously described (25).

\[^{3}H\]-cholesteryl ether-HDL uptake. Liver slices, prepared as described above, were also intended for HDL uptake. First, an HDL fraction was isolated from human plasma by sequential flotation ultracentrifugations (26). HDL was radiolabeled with 3H]-cholesteryl ether (CE) combining 3H]-cholesteryl he-dacyl ether with 1,2-phosphatidylcholine and butylhydroxytoluene in a 500:1:6 molar ratio and sonicating to form liposomes. HDL-[3H]CE was obtained by centrifugation (10 min at 12,000g, 4°C), the supernatants were carefully collected through the fat cake, diluted to 1/40,000 in PBS, and used for HDL uptake measurements as in serum.

RESULTS

Body and organ weights. Baseline body weights of the three groups of mice were comparable. After 19 weeks, body weights of HSHF animals were significantly higher than that of control mice (Fig. 1). From week 20 to 26, the body weight of HSHF+SR mice rapidly decreased to become similar to that of CON mice at week 26 (Fig. 1). Consistent with that, the masses of both epididymal and inguinal fat pads differed in the order HSHF > HSHF+SR > CON mice (Table 1). Concomitantly, the liver weight was greater in HSHF and less in HSHF+SR than in CON mice (Table 1).

Serum and liver parameters. At the end of the experiment, serum glucose concentration of HSHF mice was not different from CON mice despite an increase in insulin levels, indicating that the HSHF mice were in the early stage of developing insulin resistance (Table 1). Likewise, FFAs and total cholesterol levels were higher in HSHF than in CON mice. Surprisingly, HSHF mice had 50% lower plasma triglyceride levels than CON mice, suggesting an increase in triglyceride clearance by the liver and adipose tissue. In parallel with fat mass expansion, serum adiponectin levels were less in mice fed with the HSHF diet than in control mice. Interestingly, insulin and adiponectin levels in HSHF+SR mice were not different from the control group (Table 1). Serum FFA concentration was less in HSHF+SR than in HSHF mice, while glucose, triglycerides, and cholesterol levels did not differ between these groups. In the liver, administration of HSHF diet induced a steatosis with a fivefold increase in triglyceride.
Adiponectin content in visceral and subcutaneous adipose tissue homogenates prepared as described in research design and methods. Results are expressed as means ± SE. Values with different superscript letters (a, b, c) are statistically different at \( P < 0.05 \).

**FIG. 2.** Effect of CB1 antagonism on adiponectin concentration in visceral (A) and subcutaneous (B) fat. Mice were fed an HSHF diet for 25 weeks receiving during the last 6 weeks either 10 mg \( \cdot \) kg \(^{-1} \) \( \cdot \) day \(^{-1} \) of the CB1-specific antagonist SR141716 (HSF+SR; \( n = 14 \)) or the vehicle (HSF; \( n = 10 \)). In parallel, a series of mice was maintained on a control diet (CON; \( n = 5 \)). Adiponectin concentration was measured in adipose tissue homogenates prepared as described in research design and methods. Results are expressed as means ± SE. Values with different superscript letters (a, b, c) are statistically different at \( P < 0.05 \).
**DISCUSSION**

In this study, the effects of CB1 antagonism were tested on mice previously exposed to a long-term HSHF diet (19 weeks) with a lipid content and fatty acid composition nearly similar to the human Western diet. Administration of HSHF diet-induced obesity, liver fat accumulation and peripheral insulin resistance as indicated by the elevation of plasma insulin and FFA levels. A significant number of experimental reports describe beneficial effects of CB1 antagonism on insulin resistance and fatty liver in mice and humans, and these data strongly indicate that the ECS has a major role in the regulation of lipid metabolism not only at the central but also at the peripheral level (rev. in 28). From our mouse model of obesity, we provided further evidence that CB1 blockade causes peripheral metabolic and molecular changes in liver and adipose tissue associated with the reversion of fatty liver. We particularly showed that the lipid metabolism of visceral and subcutaneous adipocytes was differentially regulated in response to diet-induced obesity and to CB1 antagonism.

**Effects of CB1 antagonism on liver lipid metabolism.** Our findings clearly indicate that the strong upregulation of liver CB1 primarily induced by an HSHF diet is fully reversed by the treatment with SR141716, suggesting that the metabolic improvements observed could be mediated by the blockade of these receptors. This concept is supported by other studies using CB1−/− mice, demonstrating that ECS overactivity occurs in the liver of animals fed a high-fat diet and that hepatic CB1 are required for the development of diet induced steatosis (14,29). In line with this, the normalization of liver parameters related to carbohydrate and lipid metabolism such as PEPCK, G6P, ACC, and SCD-1 mRNA levels after treatment with CB1 antagonist strongly suggests that these adaptations correspond to a normalization of liver insulin responsiveness as evoked in muscles of rimonabant-treated ob/ob mice (21). The activation of hepatic CB1 receptors has been recently associated with an increase in de novo lipogenesis, suggesting that this metabolic pathway participates to steatosis development in conditions of ECS overactivity (30).

Unlike this finding, we observed no stimulation of the liver expression of ACC and FAS in HSHF animals, which were quite hyperinsulinemic. In the works of Osei-Hyiaman et al. (30), the stimulation of ECS consisted of an acute injection of CB1 agonist to control animals, while in our study, ECS activation was induced for a much longer period using an HSHF diet. Since the diet contained high proportions of saturated fatty acids, it can be hypothesized that the provision of a diet rich in preformed saturated fatty acids led to the reduced expression of mRNA for lipogenic genes. The inhibitory effect of palmitoyl-CoA on ACC demonstrated by Ogihara et al. (31) supports this concept. The induction of the SCD-1 gene and the increase in monounsaturated fatty acid content in the liver of HSHF mice (data not shown) indicate that saturated fatty acid delivery to the liver was increased. Indeed, in HSHF mice, the expression of these genes was either less than CON mice (FAS and ACC1) or unchanged (FAT/CD36, HSL, and CPT-I), suggesting different sensitivity and function of subcutaneous versus visceral adipocytes in conditions of insulin resistance (Fig. 4B). Interestingly, in visceral fat, all genes that were upregulated by HSHF feeding were significantly downregulated after treatment with CB1 antagonist except FAT/CD36 (Fig. 4A).
the liver steatosis appears to be mainly due to an enhanced delivery of FFAs to the liver rather to an increase in de novo lipogenesis. Aside from direct effects on the liver, steatosis might have also been reduced indirectly by the limitation of the influx of fatty acids originating from adipose tissue. The gene expression profile of visceral adipose tissue is consistent with an hyperactivation of lipid metabolism as suggested by the strong upregulation

FIG. 3. Effect of CB1 antagonism on the mRNA expression of CB1 and genes involved in carbohydrate and lipid metabolism in the liver. Mice were fed an HSHF diet for 25 weeks receiving during the last 6 weeks either 10 mg · kg⁻¹ · day⁻¹ of the CB1-specific antagonist SR141716 (HSHF + SR) or the vehicle (HSHF). In parallel, a series of mice was maintained on a control diet (CON). For each gene, a standard curve was established from four cDNA dilutions (1/10–1/10,000) and used to determine the relative gene expression after normalization with a geometric average of 18S and TATA box binding protein expression. Results are expressed as means ± SE (n = 5 per group). Values with different superscript letters (a, b, c) are statistically different at P < 0.05.
FIG. 4. Effect of CB1 antagonism treatment on the mRNA expression of CB1 and genes involved in adipocyte metabolism in epididymal (A) and inguinal (B) fat. Mice were fed an HSHF diet for 25 weeks receiving during the last 6 weeks either 10 mg · kg⁻¹ · day⁻¹ of the CB1-specific antagonist SR141716 (HSHF+SR) or the vehicle (HSHF). In parallel, a series of mice was maintained on a control diet (CON). For each gene, a standard curve was established from four cDNA dilutions (1/10–1/10,000) and used to determine the relative gene expression after normalization with a geometric average of 18S and TATA box binding protein expression. Results are expressed as means ± SE (n = 5 per group). Values with different superscript letters (a, b, c) are statistically different at P < 0.05.
of genes involved in transport, synthesis, oxidation, and release of fatty acids. Altogether, these data suggest that the reversion of liver steatosis induced by the treatment with CB1 antagonist was associated with an improvement of adipose tissue metabolism.

In line with an improvement of cardiovascular risk in type 2 diabetic patients treated with rimonabant (11,32), our findings support the possibility that CB1 antagonism is associated with an alteration of liver HDL catabolism. Previous studies (33) showed that overexpression of SR-BI in the liver, while reducing plasma HDL cholesterol levels, reduced atherosclerosis in mice, suggesting that hepatic SR-BI overexpression may promote reverse cholesterol transport. Accordingly, the increase in SR-BI and hepatic lipase expression induced by CB1 antagonism may be associated with a modification of HDL size and kinetics (34) and thereby explain the increase in HDL-CE uptake observed in our model of liver slices. Additional studies are currently under investigation to clearly identify the direct effects of CB1 antagonism on liver lipid metabolism.

**Effects of CB1 antagonism on visceral fat.** Recently, evidence has accumulated from animal and human studies (17,35–37) that obesity is also associated with overactivation of ECS in visceral fat. Concordant findings from this study and from literature support the view that CB1 blockade exerts specific effects on visceral fat metabolism that could be associated with the reduction of liver triglyceride content. Hence, the coordinated upregulation of genes acting at different levels of the lipogenic pathway and that of the nuclear activator PPARγ strongly suggested that an HSHF diet favored triglyceride synthesis and thereby formation of enlarged visceral fat deposits. Adipocyte hypertrophy in obesity is consecutive to a deficit in adipogenesis (38), and the limitation of fat stores would promote ectopic lipid deposition in liver and skeletal muscle, leading to decreased insulin action in these tissues (39). Remarkably, the fact that CB1 antagonism totally or partially normalized the expression levels of lipogenic genes in adipocytes may limit the accumulation of intracellular lipid droplets and give rise to smaller cells and reduction of visceral fat mass as also suggested in (40). The decrease in HSL expression consecutive to CB1 antagonism is of particular importance since excessive HSL-dependent fat lipolysis leads to an increased release of FFAs into the circulation, which in turn has deleterious effects on insulin sensitivity (41).

It has been suggested recently that obesity-induced inflammation of adipose tissue may directly activate ECS (42). This could result in a protective response against inflammation as described in colon (43). From our research, it appears that ECS activation induced by an HSHF diet is also associated with an increase in TNF-α in adipose tissue. This interaction between inflammation and ECS needs to be further explored to determine whether inflammation causes ECS activation or vice versa. However, the concentration-dependent stimulation of lipolysis by TNF-α demonstrated in rodent and human fat cells is considered to be an important pathogenetic factor in the development of insulin resistance and type 2 diabetes (44). Therefore, it is reasonable to suggest that the reduction of TNF-α expression in visceral adipose tissue of HSHF mice treated with CB1 antagonist is linked to the normalization of adipocyte metabolism and to underlying effects on lipid and carbohydrate metabolism.

**Effects of an HSHF diet and CB1 antagonism in subcutaneous versus visceral fat.** This study also provides new information regarding the impact of HSHF diet and subsequent CB1 antagonism on the regulation of lipid metabolism in subcutaneous compared with visceral adipose tissue. Taken together, our findings give molecular evidence that 1) an HSHF diet causes deleterious effects in visceral adipose tissue that were not observed in subcutaneous fat and 2) CB1 blockade is able to reverse the molecular changes primarily induced by an HSHF diet in visceral adipose tissue and to exert specific effects on subcutaneous adipocytes. These discrepancies in gene regulation between visceral and subcutaneous adipocytes in response to high-fat diet and CB1 antagonism are consistent with a different degree of ECS activation in these tissues. This consideration is supported by several recent findings (36,37,45,46) indicating differences in endocannabinoid levels between epididymal and subcutaneous fat. In addition, the overexpression of PPARγ2, FAS, and ACC gene in epididymal fat of obese mice is also in favor of the activation of ECS in this tissue since it has been reported that CB1 activation stimulates lipogenesis by increasing PPARγ and lipogenic enzyme expression in adipocytes and liver (30,36,47). Collectively, data suggest that ECS is more activated in epididymal than in subcutaneous fat in our mice model of obesity and it can be predicted that antagonism of CB1 was more effective in the tissue presenting elevated levels of endocannabinoids (36,45).

Interestingly, an HSHF diet or CB1 antagonist treatment induced nearly similar effects on the amounts of epididymal and subcutaneous fat, suggesting that molecular and metabolic differences observed are not solely related to the modification of the fat depot size. In contrast, the induction of TNF-α expression by an HSHF diet was far less important in subcutaneous than in visceral fat, and the treatment of obese mice with CB1 antagonist induced the complete normalization of TNF-α expression only in subcutaneous fat, whereas inflammation remains high in visceral adipocytes. Concomitantly, the normalization of TNF-α mRNA levels in subcutaneous fat is associated with an increase in adiponectin content in this tissue. An inverse relationship between circulating adiponectin and TNF-α has already been evoked (48), suggesting that adipose tissue inflammation could alter adiponectin production. The increased expression of PPARγ2 induced by CB1 antagonism in subcutaneous fat may also correspond to an activation of adipocyte differentiation (49) and thereby of adiponectin secretion (50). In addition, our findings regarding the adiponectin content in visceral and subcutaneous fat suggest that the normalization of adiponectin plasma levels induced by CB1 antagonism may be exclusively associated with an increased production of this adipokine by subcutaneous adipocytes.

In conclusion, this study indicates that treating obese mice with a CB1 antagonist exerts beneficial effects on liver steatosis and various lipid parameters, providing supportive evidence that the hyperactivity of ECS associated with obesity was adjusted by the antagonism of CB1. This notion is further supported by data from an ongoing study indicating that CB1 antagonism exerts no effects on body weight, fat mass, and liver triglyceride content in control mice (T.J., L.Dj., L.De., J.G., B.V., and P.D.; personal data). Our findings are also consistent with a contribution of peripheral CB1 and suggest different degrees of ECS activity in visceral and subcutaneous fat. In this way,
the improvement of visceral adipose tissue metabolism appears to be a determining factor for the normalization of plasma parameters and the regression of liver steatosis. Therefore, future studies should investigate the direct effects of CB1 antagonism on the liver to precise the respective implication of ECS and products secreted by adipose tissue in the regulation of lipid metabolism.

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