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Research Paper

Involvement of adipokines in rimonabant-mediated insulin sensitivity in *ob/ob* mice

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Abstract

Objectives It has been recently reported that blockade of type 1 cannabinoid (CB1) receptors by specific antagonists or genetic manipulation alleviates dyslipidaemia, hyperglycaemia and insulin resistance in animal models of obesity and type 2 diabetes. However, the precise role of adipokines in the insulin-sensitising effects of the CB1 antagonist rimonabant is not clear.

Methods *ob/ob* mice were treated with different doses of rimonabant and then subjected to an oral glucose tolerance test. The expression of different adipokines in white adipose tissue was analysed by quantitative real-time PCR.

Key findings Rimonabant (30 mg/kg) significantly inhibited body weight and fat pad weight gain ($P < 0.05$) and improved glucose tolerance. Gene expression analysis indicated that tumour necrosis factor-α, visfatin and retinol binding protein-4 were downregulated in the adipose tissue of *ob/ob* mice treated with rimonabant compared with controls, whereas adiponectin was significantly upregulated.

Conclusions Rimonabant-mediated alteration of adipokines in white adipose tissues may play a role in improving insulin sensitivity in obese animals.

Keywords adipokine; adiponectin; cannabinoid receptor 1; retinol binding protein-4; tumor necrosis factor-α; visfatin

Introduction

The endocannabinoids have been implicated in the regulation of food intake and peripheral energy metabolism.[1,2] Higher endocannabinoid tone has been observed in adipose tissues of obese patients.[3] Cannabinoid type 1 (CB1) receptors are expressed in adipocytes[1,4–8] and appear to be upregulated in the adipose tissue of animals with genetically modified or diet-induced obesity.[8–10] CB1 receptor activation induces adipocyte differentiation, increases the activity of lipoprotein lipase and stimulates lipogenesis *in vitro*, while blockade of CB1 receptors by rimonabant prevents these effects,[9,10] indicating the role of CB1 receptors in adipocytes. Deletion of CB1 receptors leads to leanness and resistance to diet-induced obesity.[1] These experimental results suggest that the endocannabinoid system is crucial for understanding of obesity and associated metabolic syndrome. One striking feature of the clinical trial with a CB1 receptor antagonist was the improvement in insulin resistance found after 1 year of treatment, indicating a role of the endocannabinoid system in glucose homeostasis.[11] Activation of CB1 receptors induces glucose intolerance in rats and this is reversed by the selective CB1 receptor antagonist AM251.[12] Thus, CB1 antagonists may be useful in diabetes-associated obesity.

Although the pathophysiological mechanisms that underlie the metabolic syndrome are incompletely understood, insulin resistance appears to be an important component.[13] Adipokines such as adiponectin, leptin, tumour necrosis factor (TNF)-α and visfatin are thought to provide important links between obesity, insulin resistance and inflammatory disorders, including cardiovascular diseases.[14] Adiponectin reverses insulin resistance in a mouse model of lipodystrophy and obesity,[15] and CB1 antagonists have been reported to normalise or increase the expression of adiponectin mRNA and secretion of adiponectin from adipose tissue of obese mice as well as cultured 3T3-L1 adipocytes.[10] Visfatin,

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produced preferentially in visceral adipose tissue of mice and humans, binds to and activates the insulin receptor, exerting insulin-mimetic effects both *in vitro* and *in vivo*.[16] Retinol binding protein-4 (RBP-4) is a recently identified novel adipokine secreted from adipocytes. Several studies have found a correlation between serum RBP-4 levels and the magnitude of insulin resistance in human subjects with obesity, impaired glucose tolerance or type 2 diabetes.[17,18] Hotamisligil *et al.* reported a strong positive correlation between the expression of TNF-α mRNA in white adipose tissue (WAT) and the extent of hyperinsulinaemia in obese patients.[19] Both CB1 receptors and adipokines have been suggested to play important roles in insulin resistance and metabolic syndrome. However, the precise role of adipokines in the insulin-sensitising effects of CB1 receptor antagonists is still elusive. The purpose of the present study was to understand the involvement of adipokines in insulin sensitivity, mediated through CB1 receptor blockade. To examine this, the oral glucose tolerance test was performed in *ob/ob* mice treated with rimonabant and expression of RBP-4, adiponectin, TNF-α and visfatin in WAT was determined.

Materials and Methods

Animals

All animal experiments were carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines, using protocols approved by the Institutional Animal Ethics Committee.

This study was performed in 8–10-week-old female *ob/ob* mice procured from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed individually in ventilated cages and given pelleted food (Standard Rodent diet, NIN, Hyderabad, India) and water *ad libitum*, and were maintained at 25 ± 3°C and 50–70% humidity with a 12 h light–dark cycle.

Methodology

The animals were weighed and randomised into three groups of 12 each with similar mean body weight. The animals were given rimonabant, 3 or 30 mg/kg, or vehicle (0.5% v/v Tween 80) orally once daily for 14 days. On day 15, serum was collected. Half of the animals of each group were then anaesthetised, the abdomen opened and the epididymal, retroperitoneal, mesenteric and subcutaneous fat pads removed and weighed.[20] Samples of retroperitoneal WAT were flash frozen in liquid nitrogen for quantitative real-time PCR (qRT-PCR) analysis. Serum samples were stored at −70°C for later measurement of insulin and adiponectin. The remaining six animals in each group were subjected to an oral glucose tolerance test (OGTT), wherein an aqueous solution of glucose (3 g/kg in 10 ml) was administered orally after animals had been fasted for 18 h. Blood samples were taken via the retro-orbital sinus before (0 min) and 30, 60 and 120 min after glucose administration and glucose levels measured as described below.

RNA analysis and quantitative real-time PCR

Samples of WAT were homogenised in TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) using a Mixer 301 (Retsch, Haan, Germany) and total RNA was extracted following the manufacturer's protocol. Then, 1 μg total RNA from each sample was taken for first-strand cDNA synthesis using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). An equal amount of cDNA from each sample was taken for qRT-PCR using ABI-prism-7300 FAM-labelled Taqman probes viz. adiponectin, TNF-α and RBP-4 (all from Applied Biosystems). Taqman Universal Mastermix (Applied Biosystems) was used for expression profiling of the aforementioned target genes. The optimal primer concentration of visfatin for qRT-PCR was determined using the following combinations of forward and reverse primers: 50/50, 50/300, 50/900, 300/50, 300/300, 300/900, 900/50, 900/300 and 900/900 nmol/l. The concentration resulting in the lowest cycle threshold and best amplification efficiency was selected and used for qRT-PCR experiments (forward and reverse primers both 900 nmol/l). Amplification efficiency was determined by amplifying the different cDNA concentrations (10–100 ng) with the selected combination of forward and reverse primer. VIC-labelled mouse beta actin probe was co-amplified in each sample with every target gene(s) to normalise the results.

Serum measurements

Serum glucose levels were determined by the glucose oxidase/peroxidase (GOD/POD) method using a commercially available kit (Ranbaxy Laboratories, Gurgaon, India). Insulin (Linco Research Inc., St Charles, MO, USA) and adiponectin (B Bridge, Mountain View, CA, USA) levels in the serum were determined by ELISA according to the manufacturers' protocols.

Measurement of TNF-α in white adipose tissue

Samples of WAT were homogenised in ice-cold Tris buffer containing 1 mmol/l phenyl methyl sulfonyl fluoride for TNF-α estimation. TNF-α levels were measured by ELISA (BD Biosciences, San Jose, CA, USA) and were expressed per mg of tissue protein, measured using the biuret method (Pointe Scientific, Canton, MI, USA).

Statistical analysis

All values are given as means ± SEM. Statistical analysis of the data was done by one-way analysis of variance followed by Dunnett's multiple comparison test to identify differences between the groups. Difference was considered significant at $P < 0.05$. All analyses were performed using GraphPad software (version 4.0).

Results

Effect of rimonabant on body weight

Two weeks' treatment with 30 mg/kg rimonabant significantly decreased body weight gain in *ob/ob* mice compared with vehicle-treated animals (Figure 1). In parallel with reduction in body weight, subcutaneous and retroperitoneal fat pads were significantly reduced in weight in mice treated

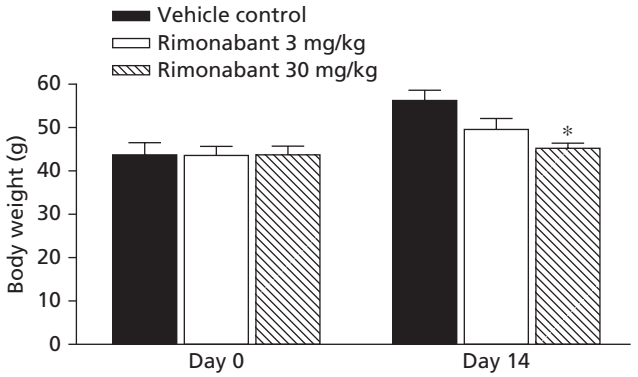

Figure 1 Effect of rimonabant on body weight in *ob/ob* mice. Values are means ± SEM (n = 12). $^{*}P < 0.05$ vs vehicle control on day 14.

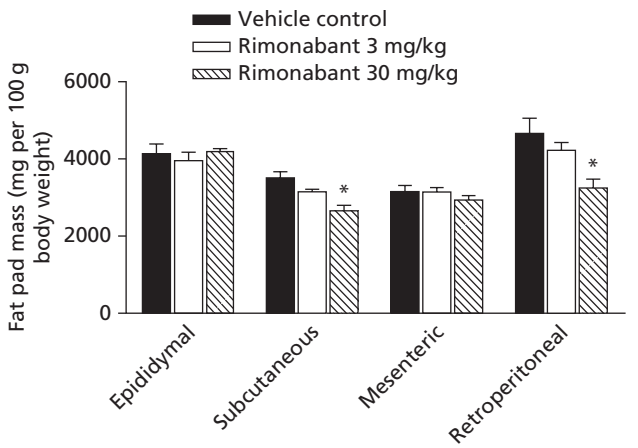

Figure 2 Fat pad weights in *ob/ob* mice treated with rimonabant for 14 days. Values are means ± SEM (n = 6). $^{*}P < 0.05$ vs vehicle control.

with 30 mg/kg rimonabant compared with control animals (Figure 2). Treatment with the 3 mg/kg dose slightly decreased body weight and fat pad weight but this was not significant when compared with control animals.

Effects of rimonabant on serum glucose, insulin and glucose tolerance

Rimonabant had a dose-dependent effect on the OGTT (data not shown) from which 30 mg/kg was identified as the optimal dose; 3 mg/kg showed no significant effect. As shown in Table 1, fasted serum glucose and insulin concentrations were significantly decreased in mice treated with rimonabant 30 mg/kg compared with control animals. However, no change in serum glucose or insulin was observed in the 3 mg/kg dose group. Serum glucose concentrations were significantly lower in the 30 mg/kg dose group than vehicle-treated animals at all time points after the oral glucose load (Figure 3). Based on the OGTT data, it was worth investigating how adipokines are modulated at these two dose levels of rimonabant.

Table 1 Effects of rimonabant on fasted serum glucose, insulin, adiponectin and tissue TNF-α levels after 14 days' treatment

	Rimonabant		
	Vehicle control	3 mg/kg	30 mg/kg
Glucose (mg/dl)	108.4 ± 3.9	95.7 ± 4.0	83.7 ± 2.6*
Insulin (ng/ml)	1.66 ± 0.11	1.56 ± 0.08	1.36 ± 0.04*
Adiponectin (μg/ml)	22.36 ± 0.66	24.1 ± 0.92	28.11 ± 0.88*
TNF-α (pg/mg)	86.23 ± 6.98	56.82 ± 3.95*	49.25 ± 2.67*

TNF-α, tumour necrosis factor α. Values are means ± SEM (n = 6). $^{*}P < 0.05$ vs vehicle control group.

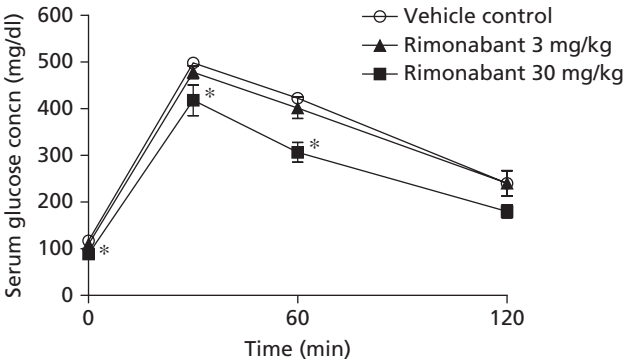

Figure 3 Effect of rimonabant treatment on the glucose tolerance test in *ob/ob* mice. Values are means ± SEM (n = 6). $^{*}P < 0.05$ vs vehicle-treated control *ob/ob* mice.

Effect of rimonabant on serum adiponectin and tissue TNF-α levels

Serum adiponectin level was significantly increased in mice treated with 30 mg/kg rimonabant. Serum TNF-α was found to be below the detectable limit so we measured TNF-α levels in adipose tissue. Rimonabant treatment caused a significant reduction in adipose TNF-α levels compared with the control group even at 3 mg/kg (Table 1).

Effect of rimonabant on the expression of adipokine genes in visceral adipose tissue

RBP-4 mRNA levels were significantly decreased by 30 mg/kg rimonabant compared with the vehicle control group (Figure 4a) whereas adiponectin mRNA levels were significantly increased at this dose (Figure 4b). The expression of adiponectin and RBP-4 mRNA in mice treated with 3 mg/kg rimonabant was not significantly different from that in control animals, although there was a tendency towards increasing mRNA levels of adiponectin and a decrease in RBP-4 in WAT. Expression of TNF-α and visfatin mRNA was significantly decreased after rimonabant treatment compared with the control group (Figure 4c and d).

Discussion

Blockade of CB1 receptors not only modulates feeding behaviour but also adipocyte biology and affects systemic glucose and lipid metabolism. Substantial data demonstrate

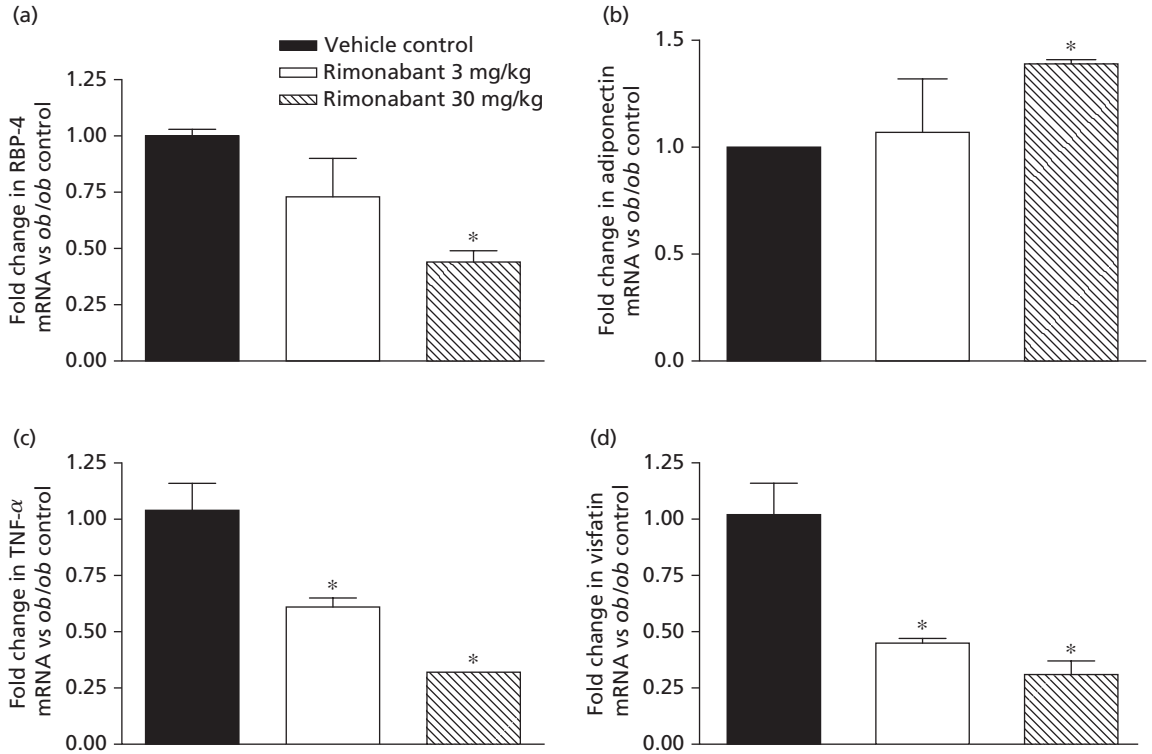

Figure 4 Expression of (a) RBP-4, (b) adiponectin, (c) tumour necrosis factor (TNF-)-α and (d) visfatin mRNA in white adipose tissue of *ob/ob* mice, determined by quantitative real-time PCR. The bars represent the fold change in the treatment groups compared with the vehicle control group, mean ± SEM (n = 6). *$P < 0.05$ vs untreated group.

the role of endocannabinoids on insulin resistance, and that CB1 blockade improves insulin sensitivity.[12] However, the precise role of adipose-derived cytokines in the insulin-sensitising effect of rimonabant is still not clear. In this study we have investigated the effect of rimonabant on insulin and WAT mRNA expression and serum levels of various adipokines in *ob/ob* mice, a model of obesity. Two weeks' treatment with rimonabant 30 mg/kg decreased body weight, fat pad weight, fasting plasma glucose and insulin and improved OGTT, which emphasises the insulin-sensitising effects of rimonabant. Trillou *et al.* reported similar findings in a mouse model of diet-induced obesity.[21]

To investigate the molecular mechanism of rimonabant-mediated insulin sensitivity, we examined RBP-4 expression in WAT. RBP-4 is overexpressed in WAT of adipose-specific GLUT4 knockout mice, which are insulin resistant, and underexpressed in WAT of transgenic mice over-expressing GLUT4 in adipose tissues, which have enhanced insulin sensitivity, suggesting that adipocyte-derived RBP-4 may act as an insulin resistance factor.[17] The expression and secretion of RBP-4 are positively regulated by insulin.[22] Higher RBP-4 levels found in diabetic patients were normalised by treatment with a thiazolidinedione.[23] In the present study, 2 weeks' treatment with rimonabant 30 mg/kg decreased WAT expression of RBP-4 mRNA in *ob/ob* mice. This is the first report of the effect of rimonabant on RBP-4 expression in rodents. It is possible that the decrease in RBP-4 expression by rimonabant may contribute to its insulin-sensitising effect.

To further explore the effects of rimonabant, we measured adiponectin mRNA in adipose tissue and its circulating levels. Our results showed that both adiponectin mRNA and circulating levels were upregulated by rimonabant at 30 mg/kg. Lower expression of adiponectin has been reported in obese individuals[24] and patients with type 2 diabetes.[25] Physiological doses of adiponectin improve insulin resistance in mouse models of obesity and type 2 diabetes.[26] In the present study, the observed increase in adipose expression of adiponectin after rimonabant treatment is in accordance with a previous study in Zucker fa/fa rats.[10] Elevated adiponectin expression correlated well with the levels in serum at the 30 mg/kg dose of rimonabant, which were in parallel with a fall in plasma glucose, insulin and improved glucose tolerance. Therefore, upregulation of adiponectin along with lowering of RBP-4 by rimonabant may lead to an improvement in insulin sensitivity. In a recent study, Lim *et al*. reported that exercise caused an increase in adiponectin and a decrease in RBP-4 levels and may lead to insulin sensitisation in young and middle-aged women.[27]

Overexpression of TNF-α is associated with increased adiposity and has been implicated in causing insulin resistance through inhibition of insulin receptor tyrosine kinase activity in adipose tissue.[19,28] We have observed significantly higher expression of TNF-α mRNA in obese *ob/ob* mice compared with lean C57BL/6J mice (data not shown). In the present study, 2 weeks' treatment with rimonabant lowered TNF-α mRNA expression and protein levels in visceral adipose tissue, even at the lower 3 mg/kg

dose. Similar reduction in lipopolysaccharide-induced serum TNF-α by rimonabant has been reported.[29] TNF-α inhibits insulin action in multiple ways – impairing phosphorylation of serine residues on insulin receptor substrate-1 (IRS-1) and activity of insulin receptor, and decreasing expression of IRS and GLUT-4.[28,30] A two-fold increase in insulin-stimulated tyrosine phosphorylation of the insulin receptor in the adipose tissue of TNF-α knockout mice has been reported, suggesting that insulin receptor signalling is an important target for TNF-α.[31] Inhibition of TNF-α by rimonabant may therefore improve insulin signalling.

Visfatin, another adipocytokine known to alleviate insulin resistance, exhibited an insulin-mimetic effect,[16] although Pagano *et al*. could not find a positive correlation between visfatin and insulin sensitivity.[32] The current study demonstrates for the first time that visfatin mRNA expression was significantly reduced after rimonabant treatment. Several reports describe visfatin as a new marker of inflammation. Visfatin expression is increased in different inflammatory conditions like atherosclerosis and inflammatory bowel disease.[33,34] In addition, visfatin expression is detected in synovial fibroblasts of patients with rheumatoid arthritis, and visfatin itself activates nuclear factor κB and related cytokines in cultured synovial fibroblasts.[35] The parallel suppression of visfatin and TNF-α by rimonabant even at a low dose emphasises its anti-inflammatory properties. Our data support previous finding of reduction in serum levels of RANTES and MCP-1 after long-term treatment with rimonabant in Zucker fa/fa rats.[36]

Global CB1 blockade enhances insulin sensitivity or glucose utilisation; however, blockade of central nervous system CB1 receptors did not improve insulin sensitivity, indicating involvement of peripheral CB1 receptors.[37] This is further supported by the increased glucose uptake of adipocytes *in vitro* following CB1 receptor stimulation.[7,38] Here it may be speculated that the improvement in insulin resistance produced by rimonabant is due to its direct effect on adipocytes to cause modulation of adipokines. However, further in-vitro studies are required to explain this phenomenon. These findings give a new insight into the development of peripheral CB1 antagonists as a possible target in the treatment of type 2 diabetes.

Conclusions

The insulin-sensitising effects of rimonabant in *ob/ob* mice may involve an increase in adiponectin, with concomitant decreases in expression of RBP-4 and TNF-α. Furthermore, our results indicate that rimonabant suppresses pro-inflammatory cytokines in adipose tissue, which may attenuate the low-grade inflammation due to excess adiposity and thus the metabolic syndrome.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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