# Chapter 4

## Effect of rimonabant, a selective cannabinoid CB1 receptor antagonist, on markers of inflammation and insulin resistance in *ob/ob* mice

## 4.1 Introduction

It is well established that the endocannabinoid (EC) system is involved in physiological regulation of many functions, including energy homeostasis, through activities in the central and peripheral nervous systems. Central cannabinoid-1 (CB1) receptors are distributed in the hypothalamus where they directly regulate orexigenic or anorexigenic signals [Cota et al., 2003]. Central activation of CB1 receptors modulates the energy balance and feeding behavior, resulting in stimulation of food intake [Kirkham et al., 2002]. However, they are also present in peripheral tissues including gut, [Coutts and Izzo, 2004] liver [Osei-Hyiaman et al., 2005] and white adipose tissue [Bluher et al., 2006].

There is mounting evidence for immunomodulatory effects of endocannabinoids, suggesting their crucial role in atherosclerotic inflammatory processes. Pacher and Ungvári (2008) shown that the activation of CB2 receptors inhibits human monocyte migration in response to classical chemoattractants, which are expressed in atherosclerotic plaques [Pacher and Ungvári, 2008] Furthermore, endothelial cells, macrophages, or platelets themselves increase their endocannabinoid synthesis during atherosclerosis, thus triggering platelet activation. These cells are also able to metabolize anandamide. Although some studies have also shown a possible pro-thrombotic effect of endocannabinoids, the majority of in vitro experimental evidence supports their possible anti-inflammatory role in atherosclerosis.

CB1 antagonist as an anti-obesity drug was reported to have potent anti-atherogenic effect. Recent experimental data suggests that a CB1 antagonist, might–in addition to their metabolic effects–exhibit anti-inflammatory properties in the vessel wall.

Furthermore, Dol-Gleizes et al showed that rimonabant has anti-atherosclerotic effects could primarily be related to its anti-inflammatory effect in LDLR–/– mice [Dol-Gleizes et al., 2009]. Higher endocannabinoid tone has been

observed in adipose tissues of obese patients [Di Marzo et al., 2005]. There are reports CB1 receptors are expressed in the white adipose tissue [Cota et al., 2003; Steinberg et al., 2007; Gary-Boboo et al., 2006; Matias et al., 2006], predominantly in mature adipocytes and appears to be upregulated in adipocytes of obese animals [Engell et al., 2005; Bensaid et al., 2003]. CB1 activation induces adipocyte differentiation, increases the activity of lipoprotein lipase and stimulates lipogenesis in vitro, while CB1 antagonism by rimonabant respectively blocks these effects [Engell et al., 2005; Bensaid et al., 2003]. Deletion of CB1 receptors lead to leanness and resistance to diet-induce obesity [Cota et al., 2003]. One striking feature of the clinical trial with a CB1 receptor antagonist was the improvement in insulin resistance found after 1 year of treatment, suggesting a role of the endocannabinoid system on glucose homeostasis [Van Gall et al., 2008]. Activation of CB1 receptors induces glucose intolerance in rats and is reversed by the selective CB1 receptor antagonist AM251 [Bermúdez-Siva et al., 2006]. Therefore, CB1 antagonists may be used in diabetes associated obesity.

Therefore, in the present study, we studied the effect of rimonabant a CB1 receptor antagonist to understand the adipokine regulation and their possible involvement in insulin resistance which is risk factor of cardiovascular risk.

## 4.2 Materials and Methods

### *4.2.1 Animals*

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

This study was performed in 8 to 10 weeks old female ob/ob mice. The animals were originally procured from the Jackson Laboratory Maine, USA. Mice were housed in individually ventilated cages and given pelleted food (Standard Rodent

diet, NIN, Hyderabad, India) and water ad libitum in a temperature (25±3°C) and humidity (50–70%)-controlled environment with a 12-h/12-h dark-light cycle.

### *4.2.2 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 periovarian,
retroperitoneal, mesenteric and subcutaneous fat pads removed and weighed.[Remessar et al., 2002] 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.

### *4.2.3 RNA analysis and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)*

White adipose tissue samples were homogenized in TRIzol reagent (Invitrogen, Life Technologies, Carsbad, CA, USA) using Mixer 301 (Retsch, Germany) and total RNA was extracted following the manufacturer's protocol. 1 µg total RNA from each sample was taken for first strand cDNA synthesis using High Capacity cDNA archive kit (Applied Biosystems, USA Part No 4322171). cDNA from each

sample was taken for Quantitative Real Time PCR using ABI-prism-7300. FAM labeled Taqman probes viz Adiponectin [Mm00456425_m1], TNF-alpha (Mm00443258_m1) and RBP-4 [Mm00803265_m1]. Taqman Universal Mastermix (Cat. No: 430437) were procured from Applied Biosystems (Foster City, CA, USA) for expression profiling of above mentioned target genes.

Optimal Primer Concentration of Visfatin for qRT-PCR was determined using the following combination of forward and reverse primers: 50/50, 50/300, 50/900, 300/50, 300/300, 300/900, 900/50, 900/300 and 900/900 nM. The concentration resulting in the lowest cycle threshold and best amplification efficiency was selected and used for qRT-PCR experiments (Forward primer 900 nM, Reverse primer 900 nM). Amplification efficiency was determined by amplifying the different cDNA concentrations (10-100 ng) with the selected combination of forward and reverse primer. VIC labeled, housekeeping gene, Mouse beta actin (Part No: 4352341E) probe was coamplified, in each sample with every target gene[s], for normalizing the results.

### *4.2.4 Serum measurements*

Serum glucose levels were determined by the GOD/POD method using biochemical kit (Ranbaxy laboratories Ltd, India). Insulin (Linco Research, Inc. USA) and adiponectin (B Bridge, CA, USA) levels in the serum were determined by ELISA methods specified by the manufacturer.

### *4.2.5 Measurement of TNF alpha in WAT*

Visceral white adipose tissues were homogenized using ice-cold TRIS buffer containing 1mM phenyl methyl sulphonyl fluoride for TNF alpha estimation. TNF alpha levels were measured by ELISA method using kit from BD Biosciences, USA and were expressed per mg of tissue protein, measured using Biuret method (Pointe Scientific, USA).

### *4.2.6 Statistical Analysis*

All data were expressed as means ± SEM. Statistical analysis of the data was done by one-way analysis of variance (ANOVA) and followed by Dunnett's multiple comparison tests to identify the differences between the groups. Difference was considered significant when $P < 0.05$. All analyses were performed using graph pad soft ware (Version 4.0).

## 4.3 Results

### *4.3.1 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 4.1A). In parallel with reduction in body weight, subcutaneous and retroperitoneal fat pads were significantly reduced in weight in mice treated with 30 mg/kg rimonabant compared with control animals (Figure 4.1B). 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.

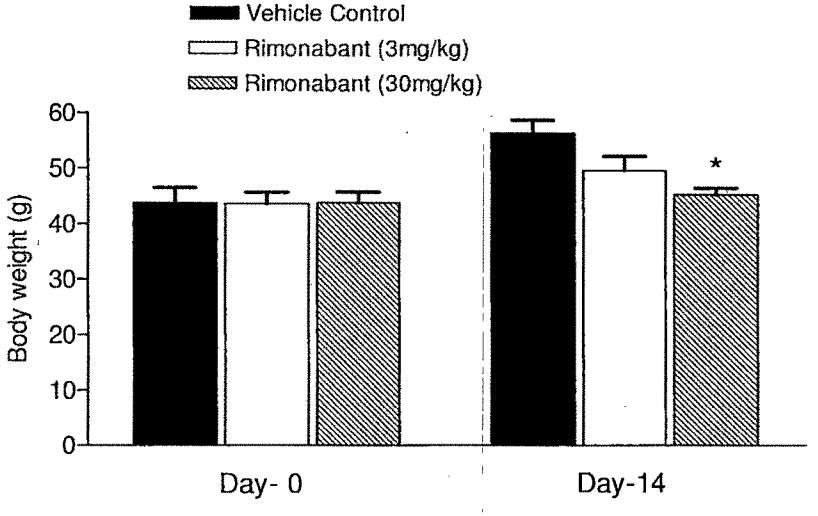


Fig. 4.1A

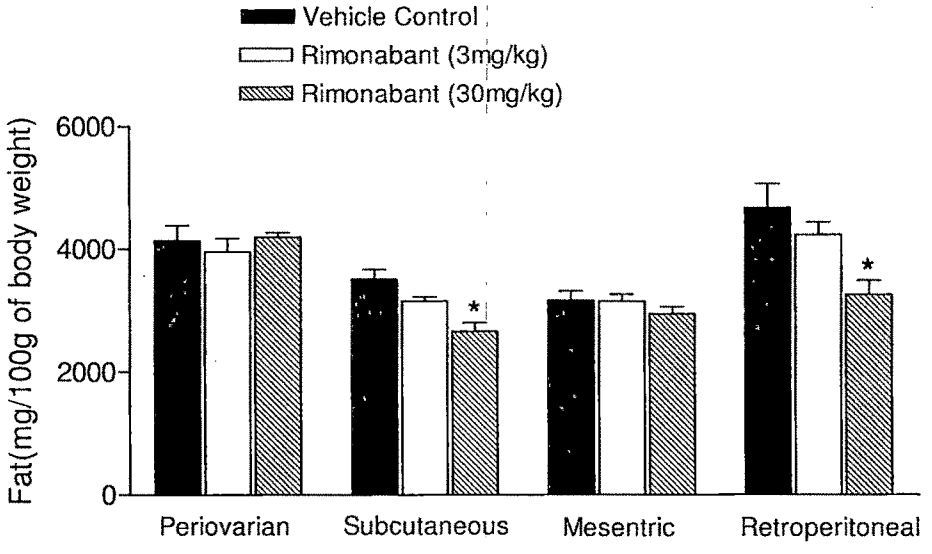


Fig. 4.1B

Fig. 4.1 Effects of rimonabant, on body weight (A) and fat pad (B) in ob/ob mice. Body weights were measured on day 0 (treatment initiation) and at the end of the treatment period (i.e day 14). Values are Mean ± SEM. ($n$ = 12 for body weight and 6 mice/group for fat pad measurement). * P < 0.05 when compared with vehicle control on day 14.

### *4.3.2 Effects of rimonabant on serum glucose, insulin and glucose tolerance*

As shown in Table 4.1, 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 4.2). Based on the OGTT data, it was worth investigating how adipokines are modulated at 3 and 30mg/kg dose levels of rimonabant.

Table 4.1: Effects of rimonabant, on serum glucose, insulin, adiponectin and tissue TNF alpha levels after 14 days treatment.

| | Vehicle control | 3mg/kg | 30mg/kg |
|---|---|---|---|
| Glucose (mg/dl) | 108.4 ± 3.9 | 95.7 ± 4.00 | 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 alpha (pg/ml) | 86.23 ± 6.98 | 56.8 ± 3.95* | 49.25 ± 2.67* |

The values are expressed as mean ± SEM. (n=6 per group). The drug and vehicle treatments were carried out once a day for 14 days. * $P < 0.05$ when compared with vehicle control group.

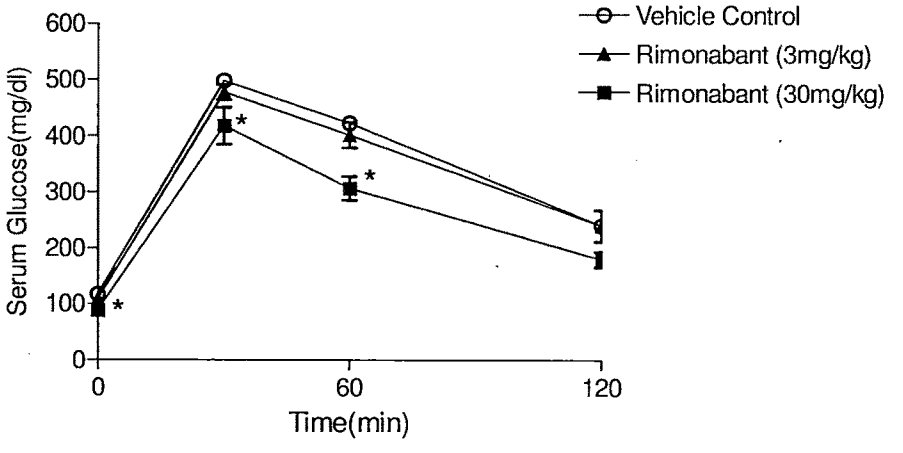


Fig. 4.2: Insulin sensitivity was measured using oral glucose tolerance test in ob/ob mice. Effect of rimonabant treatment on serial glucose increase after glucose load in ob/ob mice. Values are Mean ± SEM. ($n$ = 6 mice/group). * $P < 0.05$ as compared to vehicle-treated control ob/ob mice.

### *4.3.3 Effect of rimonabant on serum adiponectin and tissue TNF-alpha levels*

Serum adiponectin level was significantly increased in mice treated with 30 mg/kg rimonabant. Serum TNF-alpha was found to be below the detectable limit

so we measured TNF alpha levels in adipose tissue. Rimonabant treatment caused a significant reduction in adipose TNF alpha levels compared with the control group even at 3 mg/kg (Table 4.1).

### *4.3.4 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 4.3A) whereas adiponectin mRNA levels were significantly increased at this dose (Figure 4.3B). 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 alpha and visfatin mRNA was significantly decreased after rimonabant treatment compared with the control group (Figure 4.3C and D).

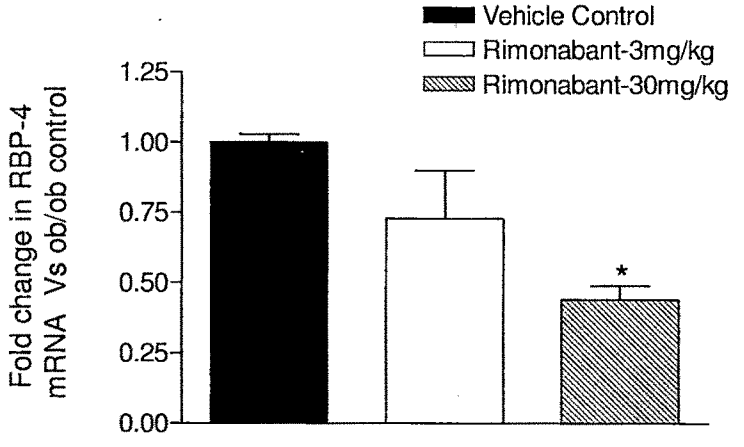


Fig. 4.3A

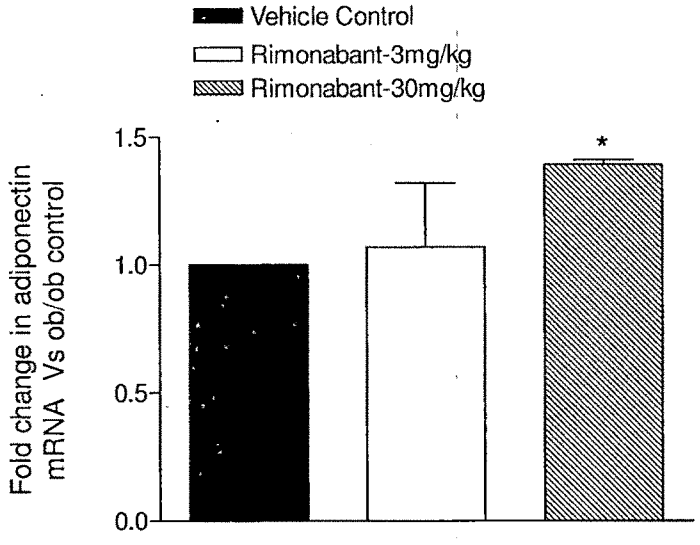


Fig. 4.3B

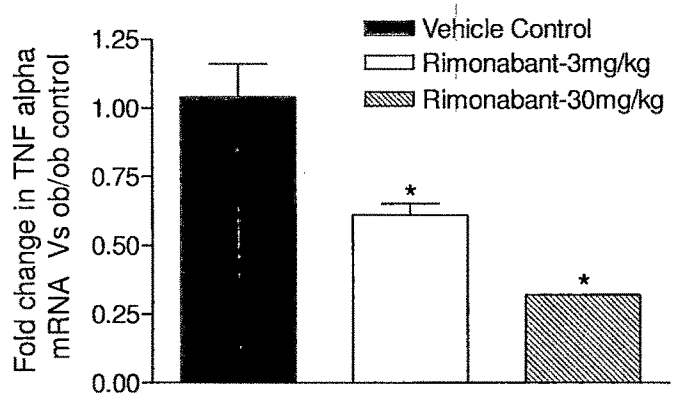


Fig. 4.3C

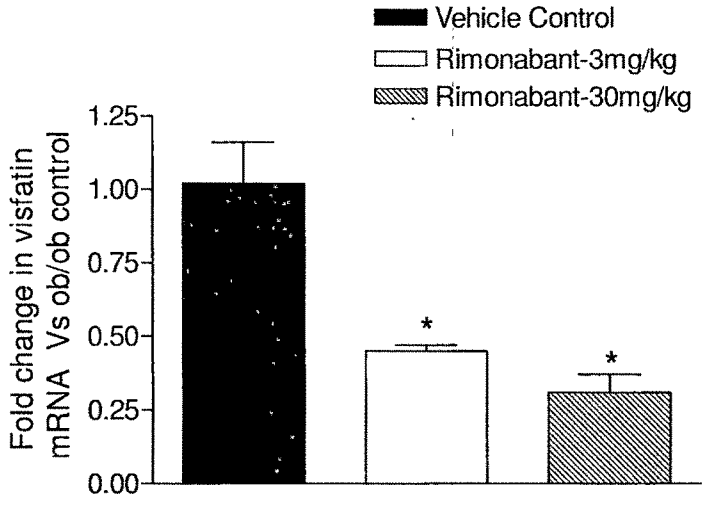


Fig. 4.3D

Fig 4.3: Analysis of the expression of RBP-4 (A), adiponectin (B), TNF-alpha (C) and visfatin (D) mRNA in WAT of ob/ob mice is using Quantitative RT-PCR. Fold change in treatment groups against the vehicle control is represented in the bar diagram. Each value represents mean ± S.E.M. ($n$ = 6 mice/group). * $P < 0.05$ compared with untreated group.

## 4.4 Discussion

Blockade of CB1 receptors not only modulate feeding behavior but also adipocyte biology and affect systemic glucose and lipid metabolism. Substantial data demonstrate role of endocannabinoids on insulin resistance and improvement in insulin sensitivity by CB1 blockade [Bermúdez-Siva et al., 2006]. However, the precise role of adipose derived cytokines in insulin sensitizing effect of rimonabant is still not clear. In this study we have investigated the effect of rimonabant on insulin and the WAT mRNA expression or serum levels of various adipokines in ob/ob mice, a model of obesity. Treatment for 2 weeks with rimonabant 30 mg/kg decreases body weight, fat pad, fasting plasma glucose, insulin and improved oral glucose tolerance, which indicates its insulin sensitizing

effect. Trillou *et al.* reported similar findings in a mouse model of diet-induced obesity [Trillou et al., 2003].

To investigate the molecular mechanism of rimonabant induced insulin sensitivity, we examined RBP-4 expression in WAT. RBP4 is overexpressed in WAT of adipose specific GLUT4 knockout mice, which are insulin resistant, and under expressed in WAT of transgenic mice overexpressing GLUT4 in adipose tissues, which have enhanced insulin sensitivity, suggesting adipocyte-derived RBP4 may act as an insulin resistance factor [Yang et al., 2005]. RBP-4 expression and secretion is positively regulated by insulin [O¨st et al., 2007]. Higher RBP-4 level found in diabetic patients was normalized when treated with thiazolidinedione [Hammarstedt et al., 2008]. In the present study, two weeks treatment with rimonabant 30mg/kg was found to decrease the WAT mRNA expression of RBP-4 in ob/ob mice. This is the first report of rimonabant effect on RBP-4 expression in rodents. It is possible that decrease in RBP-4 expression by rimonabant may contribute to its insulin sensitizing effect.

To further explore the effects of rimonabant, we measured adiponectin mRNA in adipose tissue and their circulating levels. Our results showed that both adiponectin mRNA and circulating levels were up-regulated by rimonabant at 30mg/kg dose. Lower expression of adiponectin was reported in obese [Hu et al., 1996] and type-2 diabetic [Statnick et al., 2000] individuals. Physiological doses of adiponectin improve insulin resistance in mouse models of obesity and type-2 diabetes [Yamuchi et al., 2001]. In the present study, increased adipose tissue expression of adiponectin by rimonabant is in accordance with a previous study which also showed increased in adiponectin mRNA in WAT of Zucker fa/fa rat [Bensaid et al., 2003]. Elevated adiponectin expression correlated well with the levels in serum at 30mg/kg dose alone, which were in parallel to a fall in plasma glucose, insulin and improved glucose tolerance. Therefore, upregulation of adiponectin along with lowering of RBP-4 after rimonabant at 30mg/kg treatment may lead to the improvement in insulin sensitivity. Lim *et al.* in a recent study reported that exercise caused increase in adiponectin and decrease in RBP-4

levels and may lead to insulin sensitization in young and middle-aged women [Lim et al., 2008].

Over expression of TNF-alpha is associated with increased adiposity and implicated in causing insulin resistance through inhibition of insulin receptor tyrosine kinase activity in adipose tissue [Hotamisligil et al., 1995; Hotamisligil et al., 1993]. In our earlier study we also observed significantly higher TNF-alpha mRNA expression in obese diabetic db/db mice as compared to the lean C57BL/6J mice. In the present study, two weeks treatment with rimonabant was found to lower the TNF-alpha expression and their protein levels in the visceral adipose tissue even at 3mg/kg dose. Similar reductions in LPS induced serum TNF-alpha by rimonabant has been reported [Croci et al., 2003]. TNF-alpha inhibits insulin action in many ways by impairing phosphorylation of serine residues on insulin receptor substrate-1 (IRS-1), activity of insulin receptor, and decreases expression of IRS and GLUT-4 [Hotamisligil et al., 1993; Hotamisligil et al., 1996]. Two fold increase in insulin-stimulated tyrosine phosphorylation of the insulin receptor in the adipose tissue of TNF-alpha knockout mice, suggest that insulin receptor signaling is an important target for TNF-alpha [Hotamisligil et al., 1999]. Inhibition in TNF-alpha by rimonabant may therefore improve the insulin signaling. Further, antiatherosclerotic effect of systemic TNF alpha inhibition is now well established [Branen et al., 2004; Canault et al., 2004] In addition to its proinflammatory effects on cells in the vascular wall, TNF alpha might also induce a proatherogenic serum lipid profile [Popa et al., 2007] suggesting that the inhibition of TNF alpha production might play a role in the anti-atherosclerotic effects of rimonabant.

Visfatin is another adipocytokine known to alleviate insulin resistance, exhibited as an insulin mimetic effect [Fukuhara et al., 2005]. However, Pagano *et al* could not find positive correlation between visfatin and insulin sensitivity [Pagano et al., 2006]. The current study for the first time demonstrates 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 condition like atherosclerosis

and inflammatory bowel disease [Dahl et al., 2007; Moschen et al., 2007]. In addition, visfatin expression is detected in synovial fibroblasts of patients with rheumatoid arthritis and visfatin itself activates the NFkB and related cytokines in cultured synovial fibroblasts [Brentano et al., 2007]. The parallel suppression of visfatin and TNF-alpha by rimonabant even at low dose emphasizes its anti-inflammatory properties. Our data supports previous finding of reduction in serum levels of inflammation markers such as RANTES, MCP-1 after long-term treatment with rimonabant in Zucker fa/fa rats [Schafer et al., 2008]. Further, Dol-Gleizes et al (2009) reported the anti-atherosclerotic effect of rimonabant could be related to an anti-inflammatory effect rather than the alteration in serum cholesterol suggest that modulation of pro-inflammatory adipokines (visfatin, TNF alpha and RBP-4) and anti-inflammatory (adiponectin) in WAT tissue may reduce systemic pro-atherogenic condition.

Global CB1 blockade enhances insulin sensitivity or glucose utilization; however CNS CB1 blockade did not improve the insulin sensitivity indicating involvement of peripheral CB1 receptors [Nogueiras et al., 2008; Son et al., 2010]. This is further supported by increased adipocyte glucose uptake in vitro following CB1 receptor stimulation [Gasperi et al., 2007; Pagano et al., 2007]. The improvement in insulin resistance by rimonabant due to its direct effect on adipocytes causing modulation of adipokines may be speculated. However, further in vitro studies are required to explain this phenomenon. These findings may give a new insight to the development of peripheral CB1 antagonists as target for cardiometabolic disease including atherosclerosis.In conclusion, we suggest that the mechanism for the insulin-sensitizing effects of rimonabant may involve increase in adiponectin with concomitant decrease in RBP-4 and TNF-alpha expression in ob/ob mice. Further, our results indicate that low dose of rimonabant exerts an anti-inflammatory effect which may improves the proinflammatory state of adipose tissues in various target organs.