Rimonabant–mediated changes in intestinal lipid metabolism and improved renal vascular dysfunction in the JCR:LA-\textit{cp} rat model of prediabetic metabolic syndrome


Metabolic and Cardiovascular Diseases Laboratory, Alberta Institute for Human Nutrition, University of Alberta, Edmonton, Alberta, Canada.

Running head: RIMONABANT AND LIPID METABOLISM IN JCR:LA-\textit{cp} RATS

Address for reprints and other correspondence: J.C. Russell, Alberta Institute for Human Nutrition, 4-10 Agriculture Forestry Centre, University of Alberta, Edmonton, Alberta, T6G 2P5, Canada
email: Jim.Russell@ualberta.ca; Tel: (780) 484-5703 / (780) 945-9140; Fax: (780) 492-9270

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Abstract

Rimonabant (SR141716) is a specific antagonist of the cannabinoid CB₁ receptor. Activation of the receptor initiates multiple effects on central nervous system function, metabolism, and body weight. The hypothesis that rimonabant has protective effects against vascular disease associated with the metabolic syndrome was tested using JCR:LA-cp rats. JCR:LA-cp rats are obese if cp/cp, insulin-resistant, and exhibit associated micro- and macro-vascular disease with end stage myocardial and renal disease. Treatment of obese rats with rimonabant (10 mg/kg/d, 12 to 24 weeks of age) caused transient reduction in food intake for 2 weeks, without reduction in body weight. However, by 4 weeks, there was a modest, sustained, reduction in weight gain. Glycemic control improved marginally compared to controls, but at the expense of increased insulin concentration. In contrast, rimonabant normalized fasting plasma triglyceride, reduced plasma PAI-1 and acute phase protein haptoglobin in cp/cp rats. Further, these changes were accompanied by reduced post-prandial intestinal lymphatic secretion of apolipoprotein B48, cholesterol and haptoglobin. While macrovascular dysfunction and ischemic myocardial lesion frequency were unaffected by rimonabant treatment, both microalbuminuria and glomerular sclerosis were substantially reduced. In summary, rimonabant has a modest effect on body weight in freely eating obese rats and markedly reduces plasma triglyceride levels and microvascular disease, in part due to changes in intestinal metabolism, including lymphatic secretion of apoB48 and haptoglobin. We conclude that rimonabant improves renal disease and intestinal lipid over-secretion associated with an animal model of the metabolic syndrome that appears to be independent of hyperinsulinemia or macrovascular dysfunction.

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INTRODUCTION

Excessive weight gain, particularly in the form of abdominal (visceral) adipose tissue is a major public health problem in prosperous societies worldwide (16, 65). The resultant abdominal obesity has driven a developing epidemic of prediabetic insulin resistance and cluster of associated metabolic abnormalities that are termed the metabolic syndrome (9, 35). Insulin resistance leads to chronic hyperinsulinemia and has been implicated as a major determinant of early development of macro and micro–vasculopathy, including atherosclerosis, ischemic cardiovascular disease (CVD) and renal damage, which are strongly associated with the metabolic syndrome (9, 27). The contribution of the metabolic syndrome to the development of cardiovascular and renal disease makes the reduction of obesity and related insulin resistance a critical target for therapeutic interventions. Clinical approaches, to date, have included changes in diet, food intake, and physical activity, but these have proven relatively ineffective in the human population and may well be confounded by environmental, behavioral and genetic factors (8, 11, 31, 38). Significant efforts have been made to develop effective pharmaceutical treatments, with mixed results and/or the withdrawal of some of the most effective agents from further development and/or use (4, 6, 53). Basic research has been dependent on the use of animal models that mimic the metabolic and pathophysiological aspects of the metabolic syndrome (35). Desirable effects of putative pharmaceutical agents have been; reduction of food intake with associated weight loss, improvement in insulin sensitivity, reduction in plasma lipid concentrations, improved vascular function and anti–atherosclerotic/cardioprotective activity.

Rimonabant (SR141716, Acomplia™) is a highly selective antagonist of the cannabinoid-1 (CB₁) receptor, and has been shown to have pleitropic effects on metabolism, obesity and behavioral endpoints such as addictions (5, 12, 24, 30, 32, 33). Thus, rimonabant (and related compounds) offer a possible approach to prevention or treatment of the metabolic syndrome, prediabetic status and associated micro- and macro-vascular sequelae (3). More recently, the role of the CB₁ receptor in intestinal physiology, intestinal inflammation and conditions of obesity has become apparent (21, 22). In this study we have explored this possibility with emphasis on metabolic, lipid, macro and micro–vascular and endpoints using an established animal model, the JCR:LA-cp rat.
The JCR:LA-cp rat is a unique strain that has been used extensively in the study of the underlying mechanisms of the cardiovascular and renal disease associated with the metabolic syndrome (26, 28, 35, 37, 40, 43, 51, 60); including advanced intimal (atherosclerotic) lesions, macrovascular dysfunction, myocardial ischemic lesions and microvascular renal dysfunction (34, 49). The obese, disease–prone phenotype is due to the cp mutation that results in a stop codon in the extracellular domain of the leptin receptor (ObR) (65), and absence of all isoforms of the ObR. This results in significant hyperphagia, a rapid development of profound insulin resistance between the ages of 4 and 7 weeks and progressive development of a VLDL hypertriglyceridemia with delayed clearance of post-prandial chylomicrons (60, 61). More recently, the JCR:LA-cp rat has been established as a model for the over-secretion of intestinal chylomicrons providing new avenues to develop lipid lowering strategies for CVD risk (63).

Our hypothesis was that rimonabant treatment may have beneficial effects in the presence of the metabolic syndrome, beyond reduction of food intake, and confer protection against development of end stage renal and cardiovascular disease (3). The study endpoints were focused on insulin/glucose metabolism, intestinal and plasma lipid metabolism, cytokines and thrombosis, macro–vascular function and assessment of end stage lesions of the kidney and heart. The results indicate pleitropic effects of rimonabant, in the insulin resistant cp/cp rat, with a modest reduction in body weight coupled with reduction of hypertriglyceridemia, post-prandial lipemia and a proinflammatory status, with associated protection from renal damage and dysfunction.

MATERIALS AND METHODS

Animals

Male JCR:LA-cp rats, cp/cp (obese) and +/? (lean; a 2:1 mix of cp/+ and +/+), were bred and maintained in our established rat colony (40) and housed in an isolated HEPA filtered caging system (Tecniplast S.p.a., Buguggiate, Italy). Rats were housed individually at 11 weeks of age and placed on a reversed light cycle one week prior to the start of the experimental protocol, to facilitate metabolic studies during the active (dark) phase of their diurnal cycle. All food was Lab
Diet 5001 (PMI Nutrition International Inc., Brentwood, MO, USA). Rats were weighed and food intake determined twice per week throughout the experimental period and placed on the appropriate control or treated food from 12 to 24 weeks of age. All care and treatment of the rats was in accordance with the guidelines of the Canadian Council on Animal Care and was subject to prior institutional approval.

Drugs and Chemicals

Rimonabant was provided by Sanofi-Synthelabo Recherche, Ruel Malmaison, France and incorporated into powdered rat chow at a concentration, based on body weight and food consumption of the rats, to maintain a dose of 10 mg/kg/d, as recommended by Sanofi-Synthelabo Recherche. The food was moistened, pelleted by extrusion through a die and air dried as previously described (34, 50). Reagents and chemicals were obtained from Sigma Chemical (Oakville, ON, Canada).

Experimental Procedures

At 24 weeks of age, a standardized meal tolerance test, fat challenge test or postprandial lymph collection was executed. The rats were sacrificed in the fed state, at 25 weeks of age under isofluorane anesthesia. Blood was taken from the heart and plasma separated for the measurement of PAI-1, MCP-1, leptin and adiponectin. Urine was also collected from the bladder immediately before sacrifice. The right kidney and heart were taken for histology and the thoracic aorta for assessment of vascular function.

Meal Tolerance Test

The meal tolerance test followed a standardized protocol (44). In brief, rats were deprived of food for 16 hours over the light (inactive) period and the test was conducted in the early part of the dark period. Conscious unrestrained rats were subjected to 3 blood samplings during each session. Initially, animals were placed on a heated table to ensure vasodilation of the tail, and 0.5 ml of blood was taken from the tip of the tail (0 min). Rats were then replaced in their cages and
given a 5-gram pellet of rat chow (the test meal). Timing began when 50% of the test meal had been consumed, and samples of blood were taken at 30 and 60 min for the analysis of glucose and insulin. All rats ate the full test meal within 15 min of presentation.

**Postprandial Lipemia**

Following a 16 hr overnight fast, animals were subjected to an oral fat challenge, using a modification of the meal tolerance test described above. Briefly, a 5.0 g pellet made with 5001 laboratory chow was consumed by rats. The pellet consists of carbohydrate 49%, crude protein 24.0%, moisture 10%, minerals 6.5%, fiber 6.0% and fat 4.5 %, but further supplemented with 25% w/w dairy fat from double cream (raising the total fat content of the 5.0 g meal to approximately 30% w/w).

Blood samples were collected in tubes containing Na₂EDTA from the tail at t = 0 and 2, 3, 4 and 6 hrs following consumption of the pellet meal. Plasma and serum were immediately separated from whole blood by centrifugation (3000 rpm, 4° C, 10 min). Aliquots of plasma were stored at -80° C for biochemical analyses.

**Lymph Cannulation and Nascent Chylomicron Isolation**

Rats were anaesthetized with phenobarbitone (60mg/kg i.p.). The mesenteric lymph duct was cannulated and at the same time a gastric tube introduced into the upper duodenum as previously described (59). Following recovery from anaesthesia, rats were given a gastric infusion of Intralipid (KabiPharmacia, Sweden) 2% (v:v) in a 4% (w:v) glucose solution. Lymph was collected into tubes containing EDTA. Contaminating leukocytes were pelleted by short speed centrifugation and chylomicrons were isolated from lymph by density gradient ultracentrifugation (63).

**Analytical Methods**
Plasma assays. Glucose was determined using a glucose oxidase assay procedure (Diagnostic Chemicals Ltd., Charlottetown, PEI, Canada). Insulin was assayed by rat ELISA assay (Mercodia AB, Uppsala, Sweden). Plasma triglyceride (L-Type TG H), total cholesterol (Cholesterol E), and low density lipoprotein (LDL) Cholesterol (L-Type LDL-C) assays were obtained from Wako Pure Chemicals USA, Inc. (Richmond, VA, USA). High density lipoprotein (HDL) cholesterol was assayed using direct HDL assay (Diagnostic Chemicals). MCP-1 was measured by immunoassay (R&D Systems, Minneapolis MN, USA) and PAI-1 activity by an ELISA procedure (Diapharma Group Inc., West Chester, OH, USA). Leptin (Alpcos Diagnostics, NH, USA; Cat # 22-LEP-E06) and adiponectin (Alpcos Diagnostics; Cat # 44-ADPR-0434) were analyzed with commercially available enzymatic immunoassays for rodents. The acute phase protein of inflammation haptoglobin was measured using a colorimetric immunoassay (Tridelta Development, Wicklow, Ireland; Cat # TP801). Urine albumin and creatinine measurements were performed on a Beckman Coulter LX20i analyser using immuno turbidimetric and Jaffé methods as in previous studies (34, 50).

ApolipoproteinB-48 quantification.

ApoB48 concentration in plasma and lymph was measured using an adapted immune-western blot method as previously described (62). Briefly, total plasma or lymph apolipoproteins were separated by SDS-PAGE on a 3-8% tris-acetate polyacrylamide gel (Invitrogen, NuPage, CA, USA). The separated proteins were transferred onto a PVDF membrane (0.45 µm; ImmobilonP™, Millipore. MA USA). Membranes were incubated with a goat polyclonal antibody specific for apoB (Santa Cruz Biotech, CA) and a secondary antibody tagged with hydrogen peroxidase (Santa Cruz Biotech, CA) was used to visualize apoB by chemiluminescence (ECL-Advance, Amersham Biosciences, UK). The intensity of the probed bands was quantified using linear densitometric comparison with a known mass of the purified rodent apoB48 protein.

2-D Gel Electrophoresis and Protein Sequencing.

Qualitative analysis of smaller sized proteins in lymph from fasted and fed rats was
determined by 2D-gel electrophoresis as described in the Supplementary Data. Briefly, lymph samples were separated electrophoretically by pH and then in the second dimension by molecular weight. Proteins were visualized using silver staining and quantified by densitometry. Protein spots of interest were manually excised and subjected to tryptic digestion followed by liquid chromatography and tandem mass spectroscopic characterization and identification.

Vascular Function Studies

The vascular function of aortic rings, with intact endothelium, was assessed using established methods (48). In brief, thoracic aorta was excised, trimmed of adhering fat and connective tissue, and cut into 3-mm-long transverse rings which were mounted on stainless steel hooks under 1.5-g resting tension in 10 ml organ baths maintained at 37°C. Baths contained Krebs solution (in mmol/l, NaCl 116, KCl 5.4, CaCl2 1.2, MgCl2 2, Na2PO4 1.2, glucose 10, and NaHCO3 19), gassed with 95% O2 – 5% CO2. Tension was recorded isometrically with Grass FTO3C transducers (Grass Medical Instruments, Quincy, MA, USA) and displayed on a Digi-Med tissue force analyser (Model 210, Micro-Med, Louisville, KY, USA) linked to an IBM-compatible computer that acquired data digitally using DMSI 210/4 (Micro-Med) software.

The contractile response of endothelium-intact rings of aortae to phenylephrine (PE) was assessed through concentration-response curves for (1 nmol/l to 30 µmol/l). The basal nitric oxide– (NO) mediated relaxation of aortic rings (pre-contracted with PE to 80% of maximal contraction, as calculated from the initial concentration response curve) was assessed by determining the concentration response to the endothelial NO–releasing agent acetylcholine (Ach). One ring in 4 was treated with the inhibitor of nitric oxide synthase L-NAME (N^G-nitro-L-arginine methyl ester, 10 µmol/l) to confirm that the relaxation was NO dependent. The relaxant response of rings to the direct NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP) was also determined to confirm viability of the vascular smooth muscle.

Histology

Kidneys were cut through the hilum on the long axis, fixed in formalin, and subjected to conventional processing and sectioning, followed by H&E staining. The extent of glomerular
sclerosis was determined using a similar process to that of Schäfer et al (54) and guided by the interpretation of Ferrario and Rastaldi (14). Four fields of view of the right kidney of each rat were recorded at ×2 magnification on a digital camera system (Nikon E600 with DMX 1200 camera and ACT-1 software, Nikon Corporation, Tokyo, Japan). The images were visualized using Photoshop (V7.0, Adobe Systems Inc., San Jose, CA, USA), examined blind, and all glomeruli in each field (minimum of 40 per kidney) rated as normal or sclerotic. Results were expressed as the percent of glomeruli that exhibited sclerosis.

Hearts were cut transversely into 4 blocks, fixed in formalin, and subjected to conventional processing, embedded in a single paraffin block and sectioned, followed by H&E staining. The heart sections were examined blind by an experienced observer and the number of ischemic lesions identified in each of the sections summed for each heart. Lesions were categorized by 4 stages as previously described (40, 45, 51).

**Statistical Analysis**

Results are expressed as mean ± S.E.M. and were analysed using SigmaStat (Jandel Scientific, San Rafael, CA, USA) and plotted using SigmaPlot (Systat Software, Inc. San Jose, CA, USA) and Prism (Graphpad, San Diego, CA, USA). Results were compared using one–way analysis of variance (ANOVA) followed by multiple comparison tests. Body weight data from 16–24 weeks of age was analysed by two–way ANOVA. Concentration-response curves were analysed using the program ALLFIT (8), which fits the complete data set to the logistic equation and permits independent testing of differences between individual parameters. A value of $P<0.05$ was taken as being statistically significant.

**RESULTS**

**Food Intake and Body Weight**

Figure 1 shows food intake and body weights of $cp/cp$ rats, control and rimonabant–treated, over the period from 12 to 24 weeks of age, with data from control $+/\alpha$ rats shown for reference.
Rimonabant treatment caused a rapid and highly significant 30% decline in food consumption that within 2 weeks was no longer evident; food intake of rimonabant–treated rats rebounded to the range of \(cp/cp\) control rats for the remainder of the experiment. Body weight gain of the rimonabant–treated rats became significantly lower than that of the \(cp/cp\) control animals after 4 weeks of treatment and this persisted until the end of the study (\(P<0.0001\)).

**Insulin and Glucose Metabolism**

Plasma insulin and glucose concentrations, fasting (0 min) and during the meal tolerance test, are shown in Fig. 2. At the end of the treatment period, rimonabant–treated rats had a modestly lower fasting and post-prandial plasma glucose concentration (\(P<0.01\) and \(P<0.05\) respectively vs untreated rats). However, this was accompanied by increased plasma insulin concentration (\(P<0.01\), fasting; \(P<0.05\), 30 and 60 min, post-prandial).

**Plasma Lipids**

As previously reported, HDL and total cholesterol concentrations were significantly lower in the \(+/−\) control rats compared to \(cp/cp\) control rats (Fig. 3). Rimonabant treatment had no effect on plasma total, LDL or HDL cholesterol concentrations of \(cp/cp\) rats. However, the plasma triglyceride concentration of rimonabant–treated rats was decreased by 70%, approaching, but not equal to, concentrations of \(+/−\) control rats.

The plasma apoB48 response to a fat challenge is shown in Fig. 4. At time 0 (fasting) concentrations of apoB48 in \(+/−\) control rats were lower than those of the \(cp/cp\) controls (\(P<0.0005\)). Interestingly, rimonabant treatment resulted in a significant reduction of fasting apoB48 concentration (50% vs untreated, \(P<0.05\)). The difference in apoB48 concentration persisted throughout the post-prandial period as shown by the area under the curve (AUC, right panel, \(P<0.005\)), suggesting improvements to intestinal secretion and/or clearance of triglyceride rich lipoproteins.

Post-prandial lymphatic apoB48, cholesterol and triglyceride concentrations are shown in Fig. 5. Rimonabant treatment resulted in a striking reduction of lymphatic apoB48 secretion (~50%,
Markers of Inflammation and Thrombosis

Fasting plasma concentration of the acute phase protein haptoglobin was not different by genotype or treatment. However, curiously, treatment with rimonabant reduced the area under the curve response for haptoglobin following a lipid challenge, suggesting a relationship with intestinal lipid secretion (Fig. 6). To verify the relationship of haptoglobin and intestinal derived lipid secretion, we performed 2D electrophoresis and MS/MS and confirmed a reduction in the mass of haptoglobin present in lymph (see Supplementary Data). Four spots of interest were identified: as; haptoglobin β chain (#85a, #146 and #157) and apolipoprotein E (#85b), all of which were reduced by rimonabant treatment.

Rimonabant treatment simultaneously reduced fed state plasma PAI-1 concentrations to not different from +/? rats (Fig. 7). Rimonabant treatment did not influence plasma MCP-1, adiponectin or leptin concentrations (Fig. 7).

Vascular Function

The contractile dose response of aortic rings to the noradrenergic agonist PE, and the relaxant response to ACh, is shown in Fig. 9. Aortae from cp/cp rats showed enhanced PE–mediated contractility compared to those from +/? controls. Aortae from rimonabant–treated rats showed no change in PE–mediated contractility, either in maximal response or in the EC50 for PE. Further, impaired ACh–mediated relaxation of PE pre-contracted aortic rings of cp/cp rats was not improved by rimonabant treatment (Fig. 9). There was no significant difference in the maximal relaxant response to SNP (~ 100%) or the EC50 between any of the groups (data not shown).

Renal Function and Glomerular Sclerosis

Urinary albumin excretion is markedly elevated in the cp/cp rats at 24 weeks of age, as shown
in Fig. 9, and this is accompanied by increased incidence and severity of glomerular sclerosis. Rimonabant treatment significantly reduced the albumin/creatinin ratio ($P<0.01$). Consistent with this, the fraction of glomeruli that were sclerotic was significantly reduced in the rimonabant–treated rats, as assessed by quantitative histological analysis.

Ischemic Myocardial Lesions

There were relatively few myocardial lesions in the hearts of the early middle aged rats with the exception of Stage 2 lesions, which are areas with strong inflammatory cell infiltration and cell lysis (see Fig. 2, Supplementary Data). There was no significant difference between the myocardial lesion frequency of $cp/cp$ control and rimonobant–treated $cp/cp$ rats ($P > 0.05$, Fig. 2, Supplementary data).

DISCUSSION

Rimonabant is a member of a unique class of agents that antagonize the CB$_1$ receptor, which is linked to many biochemical and physiological pathways, including those involving catecholamine and endogenous opioid synthesis in various organs (3). Cannabinoids are recognized to be widely distributed throughout the intestine, with regional variation and organ-specific actions (22). Our results provide a novel perspective on the metabolic and intestinal effects of rimonabant in an animal model that exhibits the metabolic syndrome and associated end stage complications.

Rimonabant and Food Intake/Body Weight Reduction

Rimonabant–treated $cp/cp$ rats, showed an initial reduction in food intake (23, 33, 59) consistent with previous reports in animals and humans. Unlike the case of diet–induced obesity in mice, (33) there was no corresponding reduction in body weight in rimonabant–treated $cp/cp$ rats during this initial period of treatment. Interestingly, Janiak et al (23) found that $fa/fa$ Zucker rats treated with rimonabant at 10 mg/kg/d showed a similar pattern of reduced food intake, but
required 8 weeks to return to baseline intake. In the present study, body weight of rimonabant–treated \( cp/cp \) rats was 13% lower than that of controls at the end of the treatment period. The \( cp/cp \) rat is resistant to reduction in body weight in the presence of changed caloric intake due to altered composition of food intake, such that if pair fed to \( +/- \) control rats shows only a 21% lower body weight than freely fed \( cp/cp \) animals at 36 weeks. For comparison the body weight of matched \( +/- \) rats is 44% lower than \( cp/cp \) animals(39). In this context, the reduction in body weight of the rimonabant-treated \( cp/cp \) rats, in the absence of long term reduction in food intake, is of real significance. In comparison, 6-month old \( fa/fa \) Zucker rats in the Janiak et al (23) study showed a 7% reduction in body weight when treated with rimonabant at the equivalent dose. The origin of these differences between rat strains is not clear, but may be related to the more severe insulin resistance and hyperinsulinemia of the \( cp/cp \) rat and polygenetic differences between strains carrying the \( fa \) and \( cp \) mutations (52).

While data on humans is inconsistent, two recently published large clinical trials show a similar lag in reduction of body weight and waist circumference following initiation of rimonabant treatment (30, 32). In these clinical studies, reduction in body weight was not accompanied by long term reduction in food intake, suggesting a fundamental change in metabolism, unrelated to food intake and possibly reflected in increased oxidative activity. It has been suggested, by Pagotto et al. (30), that \( CB_1 \) antagonists may have an anti-obesity effect through direct action on adipocytes. In support of this concept, rimonabant has been shown to increase oxygen consumption and glucose uptake by murine skeletal muscle (24).

**Rimonabant and Glucose/Insulin Metabolism**

Treatment of \( cp/cp \) rats with rimonabant was associated with elevated plasma insulin concentration and normalized glucose levels. These changes may reflect an improvement in glucose control and are consistent with the physiological adaptation of the \( cp/cp \) rat to maintain euglycemia (37). These results do not indicate an increase in insulin sensitivity *per se*, as seen in this model with other agents, such as S15261 or D-fenfluramine (4, 41, 50). In contrast to our results, it has recently been reported that rimonabant inhibits basal insulin secretion by pancreatic islets of \( fa/fa \) Zucker and ZDF rats (15). This was an *in vitro* study using islets isolated from 7-11
week old (juvenile) rats, which at that age are not highly insulin resistant and never exhibit the marked metabolic dysfunction seen in the the \textit{cp/cp} rat. Our results were obtained in fully adult animals exhibiting developed metabolic syndrome. Given evidence that hyperinsulinemia plays a significant role in vascular disease (1, 26, 28, 37, 49, 50), the increased plasma insulin concentrations in rimonabant–treated \textit{cp/cp} rats must be considered carefully.

\textit{Rimonabant and Triglyceride Metabolism}

Hyper–triglyceridemia of the \textit{cp/cp} rat has been shown to be, at least in part, due to over-secretion of hepatic triglyceride–rich VLDL particles (46, 60). Reduction of plasma triglyceride by rimonabant treatment, of over 70\%, is striking and greater than that previously reported (23, 33). It maybe possible that rimonabant may act on both hepatic (29) and intestinal lipoprotein secretion in the JCR:LA-\textit{cp} rat. Poirier \textit{et al.}(31) observed a 15\% decrease in plasma triglyceride in high fat fed mice treated with rimonabant, while Janiak \textit{et al.} (23) reported a 50\% decrease in plasma TG in treated \textit{fa/fa} rats (at 9 months of age). The results suggest that the beneficial metabolic effects of rimonabant may be more pronounced in the presence of severe hyperinsulinemia and/or known additional contributions of lipogenesis from the intestine. Since there was no change in total circulating cholesterol concentration \textit{per se} in treated \textit{cp/cp} rats, the results suggest a marked reduction in triglyceride associated with VLDL, triglyceride–rich and/or chylomicron fractions.

\textit{Rimonabant and Cholesterol Metabolism}

Changes in cholesterol metabolism with rimonabant treatment have been reported in animal models and humans, but these are not entirely consistent (10, 23, 33). In the diet induced obese mouse, rimonabant caused only modest reductions in triglyceride and total cholesterol with an increase in HDL-C : LDL-C ratio (33). In the \textit{fa/fa} Zucker rat, there was a slight decrease in total cholesterol and modest decrease in LDL cholesterol (23). Our results show no change in any of the plasma cholesterol fractions. For comparison, a large clinical study has shown no change in total cholesterol and modest to small change in LDL and HDL concentrations, together with a
20% decrease in triglycerides (10). These changes, overall, resemble those seen in the \( cp/cp \) rat, that we consider to be a more extreme model for the metabolic syndrome than either the diet induced obese mouse or the \( fa/\overline{a} \) rat. Given new insights into the ability of the liver and intestine to cooperatively maintain plasma cholesterol homeostasis, it is possible that, in the JCR:LA-cp rat, rimonabant may act selectively on intestinal cholesterol metabolism \textit{per se} and not on the liver.

From a cardiovascular risk point of view, reduction in plasma triglyceride is a potentially very significant finding, particularly with respect to the role of postprandial lipemia and risk of CVD (36). In addition, recent studies in our laboratory have shown intestinal apoB lipoproteins, chylomicrons and postprandial lipemia may be major contributors to vascular disease in the \( cp/cp \) rat (25, 34, 61, 62, 63).

\textit{Rimonabant and Intestinal Lipid Metabolism}

Rimonabant treatment of \( cp/cp \) rats significantly reduced the plasma level of apoB48-containing particles in the fasting state (49%), as well as following an oral fat challenge (Fig. 4). As shown in Fig 5, the elevated post-prandial intestinal secretion of both apoB48 and cholesterol, reflecting the contribution to plasma chylomicron concentration by the intestine, was significantly reduced by rimonabant, with no reduction in triglyceride secretion. The marked reduction in post-prandial apoB (58%) and cholesterol (45%) content of the lymph of rimonabant–treated rats (Fig. 5) indicates a reduced production of chylomicron particles. However, the lack of change in lymphatic triglyceride content \textit{per se}, indicates production of fewer larger chylomicron particles and is suggestive of increased receptor-mediated clearance of intestinal derived particles from the plasma compartment. Rimonabant may potentially have an impact on the intestine by inhibiting transport of anandamide, (64) a CB\textsubscript{1} receptor ligand, in enterocytes. We speculate that this could down regulate the formation of chylomicrons or increase clearance of triglyceride in circulation, or both.

\textit{Rimonabant and Pro-inflammation}
Haptoglobin is an acute phase protein that reflects inflammatory response (7), which is an inherent component of micro and macro–vascular disease in the \( cp/cp \) rat (52). We have previously reported that dietary fatty acid composition alters haptoglobin levels of the \( cp/cp \) male rat following an oral fat challenge (16). The reduction of post-prandial plasma haptoglobin levels observed in rimonabant–treated rats (Fig. 6) is consistent with a reduced inflammatory status and may contribute to the reduced renal micro–vascular dysfunction (Fig. 9). Notably, we demonstrate that haptoglobin can be derived from mesenteric lymph and is associated with intestinal lipid secretion. Moreover, Rimonabant appears to influence not only the number and cholesterol content of intestinal derived lipoprotein particles, but also the corresponding secretion of haptoglobin into plasma. While other acute phase proteins such as serum amyloid A (SAA) have been shown to readily associate with lipoprotein fractions, little is known about the lipoprotein binding capacity for haptoglobin. Haptoglobin maybe present in mesenteric lymphatics associated with gut associated lymphoid tissue response or indeed directly with intestinal enterocytes (58), however this understanding is not yet clear.

The intestine, in addition to the liver, expresses apoE mRNA and the secretion is dependent on a number of poorly understood variables. The mechanism of the reduced apoE secretion into the lymph of rimonabant–treated \( cp/cp \) rats, under conditions of a fat challenge (Fig. 6) is not obvious, but is consistent with the known effects of the endocannabinoid system in the gut (3, 22).

MCP-1 is a principal chemotactic factor in migration of monocytes/macrophages and mediates chronic inflammation (13). Our data show higher plasma MCP-1 levels in the \( cp/cp \) rat compared to lean controls, which is consistent with our earlier observations of widespread activation and endothelial adherence of macrophages in atherosclerosis–prone adult \( cp/cp \) rats (43, 51). Schepers \textit{et al} (55) and Takebayashi \textit{et al}. (57) have shown an association between plasma MCP-1 and urinary albumin excretion in type 2 diabetic patients, consistent with our findings in the \( cp/cp \) rat. In contrast to Takebayashi \textit{et al}. (57), we have observed a reduced albumin/creatinine ratio and incidence of glomerular sclerosis with rimonabant treatment. However, the fact that plasma MCP-1 concentration does not improve in rimonabant–treated \( cp/cp \) rats suggests this pathway is not related to glomerular damage and end stage renal disease.

Elevated plasma levels of both leptin and the adipocyte peptide adiponectin in the \( cp/cp \) rat,
(Fig. 7) are reduced by several interventions that have beneficial effects on the insulin resistant status of the rats (34, 48). The absence of any effect by rimonabant is consistent with different mechanisms of end stage disease involving insulin/glucose metabolism and lipid metabolism.

Clinically, the metabolic syndrome is associated with a two-fold elevation of plasma PAI-1 levels (2), prior to the development of type 2 diabetes. The \( cp/cp \) rat has been shown to have elevated circulating levels of PAI-1 (56), consistent with the finding of both macro and micro arterial thrombi (43, 51). Reduction of PAI-1 levels in rimonabant–treated \( cp/cp \) rats, may indicate an improvement in the pro-thrombotic status and is consistent with reduced glomerular sclerosis. We know that glomerular sclerosis is associated with accumulation of extracellular matrix and this is related to increased concentration of PAI-1 (19, 20). Thus, in the presence of an inflammatory status (such as atherosclerosis), the reduction of PAI-1 activity seen in the rimonabant–treated \( cp/cp \) rat represents an important therapeutic marker (20) of reduced renal dysfunction. Consistent with this notion, Di Marzo and Szallasi (12) have also reported that rimonabant can ameliorate the pro-thrombotic and inflammatory status of the \( fa/fa \) Zucker rat.

**Rimonabant and Vascular Damage**

Rimonabant reduced the level of albuminuria and incidence of glomerular damage, indications of micro-vascular protection. These results are consistent with a hypothesis that the renal damage is related to elevated VLDL and/or chylomicron concentrations (17). However, contrary to the effects of rimonabant on micro–vasculature, we did not observe any concomitant benefit in macro–vascular function; either on the hyper contractile response to PE or the impaired endothelium–mediated relaxation (Fig. 8). This is, perhaps, consistent with the increase in hyperinsulinemia, which appears to be a critical mediator of macro–vascular dysfunction in this model (27, 40, 47). Moreover, rimonabant treatment did not decrease the myocardial damage (See Supplementary Data), possibly due the relatively young age of the rats in this study compared to those used in earlier studies of myocardial lesions (40, 42, 43). The absence of any reduction in insulin levels may also underlie both the absence of reduction in macro vascular dysfunction or ischemic myocardial lesions.
Conclusions

Unlike other pharmaceutical interventions studied in the *cp/cp* rat, rimonabant has dimorphic effects that do not equally affect micro and macro-vascular dysfunction. For instance, ACE inhibitors, endopeptidase inhibitors, insulin sensitizers and ethanol reduce hyperinsulinemia, hypertriglyceridemia, vascular dysfunction and myocardial lesions, with several simultaneously reducing renal micro vascular dysfunction and damage (45, 49, 52). In contrast, rimonabant reduces triglyceride levels and renal vascular disease, but does not reduce insulin levels, macro–vascular dysfunction or the incidence of myocardial lesions, supporting the notion of two complimentary etiological processes: one, that involves macro–vascular disease and appears to be related to hyperinsulinemia; and a second, that involves renal micro–vascular complications and which is plasma triglyceride, and possibly PAI-1, dependent. Further, we show that rimonabant appears to have critical regulatory effects on the enterocyte and the associated lipid absorption pathways. We speculate that the contribution of the intestine to over-production of lipids and/or the pro-inflammatory acute phase response may prove to important in the etiology of micro-vascular complications. Given the major role of renal failure in the complications of type 2 diabetes, further study of the underlying mechanisms is clearly indicated.

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Disclosures

The authors report no dualities of interest other than grant support for the study.


FIGURE LEGENDS

Figure 1. Food intake and body weight of JCR:LA-cp rats during treatment from 12 to 24 weeks of age. ○, +/? control; ●, cp/cp control; □, cp/cp rimonabant–treated. Values are mean ±SEM, 10 rats per group. **, *$P*<0.005$, ***, *$P*<0.001$; rimonabant–treated vs cp/cp control. †; body weight of the rimonabant–treated rats was significantly reduced compared to the cp/cp control rats over the period from 16 to 24 weeks, as indicated by arrow (*$P*<0.0001$). Food intake data are plotted at the mid point of the time interval of measurement.

Figure 2. Plasma insulin and glucose concentrations in the meal tolerance test. Values are mean ±SEM, 10 rats per group. *, *$P*<0.05$; **, *$P*<0.001$; vs cp/cp control. Plasma insulin levels of +/? controls are markedly lower than the cp/cp controls and no statistical tests were performed on data from +/? rats.

Figure 3. Fasting plasma lipid concentrations of rimonabant–treated JCR:LA-cp rats. Values are mean ±SEM 10 rats per group. **, *$P*<0.0001$, vs cp/cp control.

Figure 4. Plasma apoB48 concentrations following a fat challenge (left panel) and area under the curve (AUC) (right panel). ○, +/? control; ●, cp/cp control; □, cp/cp rimonabant–treated. Values are mean ±SEM, 3 rats per group. *, *$P*<0.05$; **, *$P*<0.01$, vs cp/cp control.

Figure 5. Lymph apoB48, cholesterol and triglyceride concentrations during a 30 min collection, post Intralipid infusion. Values are mean ±SEM, 3 rats per group, *, *$P*<0.05$; **, *$P*<0.01$; ***; *$P*<0.001, vs cp/cp control.

Figure 6. Plasma haptoglobin concentrations, fasting (upper left panel) and area under the curve (AUC) (upper right panel) following a fat challenge. Lymphatic concentrations of haptoglobin β chain fragments of cp/cp rats, control and rimonabant–treated are shown in the lower panel, along with apoE levels, both during saline infusion of the intestine and during a fat infusion. Values are mean ±SEM, 10 rats per group in upper panels, 2 and 3 rats per group in lower panel, **, *$P*<0.01$; ***; *$P*<0.001, vs cp/cp control in upper panels and *, *$P*<0.05$ in lower panel.
(rimonabant–treated and fat challenged vs all other groups).

Figure 7. Fed state concentration of PAI-1, MCP-1, leptin and adiponectin in plasma of control and rimonabant–treated cp/cp rats. Values are mean ±SEM, 7-10 animals per group for PAI-1, leptin and adiponectin, and 4-6 animals for MCP-1. *, p<0.05; **, p<0.01; vs cp/cp control.

Figure 8. Effect of rimonabant treatment on PE–mediated contractile (panel A) and Ach–mediated relaxant (panel B) aortic vascular function in cp/cp rats. Values are mean ± SEM, 10 rats per group. The dose response curves were calculated using the logistic equation and the ALLFIT program. Statistical analysis showed significantly greater lower PE-mediated contractility (P<0.001), and greater relaxation in response to ACh (P<0.01) in the +/-? aortae compared to the cp/cp aortae. There were no differences in either contractility or relaxation (either in maximal response or in EC50) between the rimonabant–treated and control cp/cp aortae.

Figure 9. Urinary albumin/creatinin ratio, as an index of renal vascular function (panel A), and fractional glomerular sclerosis (panel B) of control and rimonabant–treated rats. Values are mean ± SEM, 10 rats per group. *, p<0.05; **, p<0.01, ***, p<0.001; vs cp/cp control.
Supplementary Data

Methods

2-D Gel Electrophoresis and Protein Sequencing

Qualitative analysis of smaller sized proteins in lymph from fasted and fed IR rats was determined by 2-D Gel Electrophoresis. Lymph samples were prepared using the 2-D Clean-Up Kit (GE Healthcare, Baie d’Urfé, Québec) and 2D Quant Kit (GE Healthcare) as specified in the manufacturer’s instructions. The lymph proteins were solubilized in rehydration buffer (7M urea, 2M thiourea, 20 mM DTT, 0.8 % (vol/vol) IPG buffer pH 3-10, bromophenol blue) and loaded by cup-loading onto rehydrated 24 cm Immobiline DryStrips IPG strips pH 3-10 linear (GE Healthcare). The first dimension isoelectric focusing protocol included a gradual increase in voltage to a total of 70,000 Volt-h over a 24 h period (IPGPhor, GE Healthcare) and immediately frozen at -80 ºC after isoelectric focusing. Prior to the second dimension separation, strips were equilibrated in 50 mM Tris-HCl, 6 M urea, 30% (vol/vol) glycerol, 2% (wt/vol) SDS and bromophenol blue, for 15 min with 1% (wt/vol) DTT, and then alkylated using 2.5 % (wt/vol) iodoacetamide for 15 min. The 24 cm IPG strips were placed on 10% (wt/vol) polyacrylamide 26 cm-wide slab gels and initially separated at 5W/gel for 30 min, followed by 17W/gel for 3.5 to 4 h with buffer temperature set to 20 ºC in a ETTAN DALT-six gel electrophoresis system (GE Healthcare). To help minimize technical variation, six gels were cast at once in the multi-gel caster and six gels were run at once in the second dimension system where possible. The resulting gels were then fixed overnight and proteins visualized using Sypro Ruby fluorescent stain (Bio-Rad Laboratories, Hercules, CA). The gels were destained briefly and scanned using the Typhoon Trio laser scanner (GE Healthcare).

For each gel, the protein spots were quantified using Progenesis SameSpots software (Nonlinear Dynamics, Durham, NC). The individual spot volume measurements for each protein were corrected for the total spot volume on each gel, and were also normalized across gels using same reference gel. Statistical differences in treatment spot volumes were analyzed using the software package included in the Progenesis SameSpots software, using a multivariate ANOVA comparison across treatments.
LC-MS/MS Identification of Lymph Proteins

Protein spots of interest were manually excised and sent to a mass spectrometry facility for further processing and identification (Centre Genomique du Quebec, Sainte-Foy, Canada). All of the procedures for sample preparation, tryptic digestion, mass spectrometry and database searches by the Centre Genomique du Quebec are described in detail in [1].

Results

Lymph Proteins

A typical 2D electrophoretic gel is shown in Fig. 1, with the region in which the protein spots of interest were located outlined. Spots #85a, #146 and #147 were identified as haptoglobin β chain fragments and spot #85b as apolipoprotein E. The spot density in lymph collected from cp/cp rats, control and rimonabant–treated, is shown in Fig. 6 of the full text. The three haptoglobin fragments and apoE levels were significantly reduced in lymph of rimonabant–treated rats, but only when the rats were subjected to the intra intestinal infusion of the lipid challenge.

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Myocardial Lesions

Myocardial lesion frequency in control, +/- and cp/cp rats together with rimonabant–treated cp/cp rats are shown in Fig. 2. There were relatively few myocardial lesions in the hearts of the early middle aged rats with the exception of Stage 2 lesions. Stage 1 lesions are areas of early ischemia or necrosis; Stage 2 lesions are areas with strong inflammatory cell infiltration and cell lysis; Stage 3 lesions are foci of chronic inflammatory cells without cell dropout; and Stage 4 lesions are mature scarred lesions. Stage 2 lesions were frequent in the cp/cp hearts compared to +/- hearts (P<0.005). There were no significant differences in lesion frequency between cp/cp controls and rimonabant–treated cp/cp rats (P > 0.05).

References

Figure Legends

Figure 1. Panel A, 2D electrophoretic gel of lymph from a \textit{cp/cp} rat. The box in the gel outlines the area of interest with the spots that were quantitated (shown expanded in panel B). Data from the quantitation is shown in Fig. 6 of the full text.

Figure 2. Frequency of ischemic myocardial lesions in rimonabant–treated and control rats. Lesion stages as described in the Methods. Values are mean $\pm$ SEM, 10 rats in each group. The $+/?\,$ hearts had significantly fewer Stage 2 lesions than the \textit{cp/cp} control hearts ($P<0.005$). The apparent differences between \textit{cp/cp} control and rimonabant–treated rats were not statistically significant ($P>0.05$).
A

**Insulin (mU/l)**

- +/- Control
- cp/cp Control
- cp/cp Rimonabant

B

**Glucose (mg/100 ml)**

- Time (min): 0, 30, 60

*Significant differences: *p < 0.05, **p < 0.01, ***p < 0.001*
[Graph with data points and error bars showing plasma apoB48 levels over time for cp/cp Control, cp/cp Rimonabant, and +/-? Control groups.]

**AUC arbitrary units**

- cp/cp Control
- cp/cp Rimonabant
- +/-? Control

**Time hours**

- 0
- 2
- 4
- 6

**Plasma apoB48 ug/ml**

- 0
- 40
- 80
- 120
- 160
- 200
- 400
- 600
- 800

Levels are represented with various symbols and error bars, indicating statistical significance as follows:

- ***: Highly significant difference
- **: Significant difference
- *: Trend towards significance

The graph illustrates the effect of Rimonabant on plasma apoB48 levels compared to controls over time.
AUC (arbitrary units)

Plasma Haptoglobin (ng/ml)

Spot density (log normal)

Spot #85a haptoglobin
Spot #146 haptoglobin
Spot #147 haptoglobin
Spot #85b apoE