Rimonabant has been shown to not only decrease the food intake and body weight, but also to increase serum adiponectin levels. This increase of the serum adiponectin levels has been hypothesized to be related to the rimonabant-induced amelioration of insulin resistance linked to obesity, although experimental evidence to support this hypothesis is lacking. To test this hypothesis experimentally, we generated adiponectin-knockout (adipo(-/-)) ob/ob mice. After 21 days of treatment with rimonabant at 30mg/kgBW, the body weight and food intake decreased to similar degrees in the ob/ob and adipo(-/-) ob/ob mice. Significant improvement of insulin resistance was observed in the ob/ob mice following rimonabant treatment, associated with significant up-regulation of the plasma adiponectin levels, in particular, of high-molecular-weight adiponectin. Amelioration of insulin resistance in the ob/ob mice was attributed to the decrease of glucose production and activation of AMP-activated protein kinase (AMPK) in the liver induced by rimonabant, but not to increased glucose uptake by the skeletal muscle. Interestingly, the rimonabant-treated adipo(-/-) ob/ob mice also exhibited significant amelioration of insulin resistance, although the degree of improvement was significantly lower as compared with that in the ob/ob mice. The effects of rimonabant on the liver metabolism, namely, decrease of glucose production and activation of AMPK, were also less pronounced in the adipo(-/-) ob/ob mice. Thus, it was concluded that rimonabant ameliorates insulin resistance via both adiponectin-dependent and adiponectin-independent pathways.

The prevalence of obesity has increased dramatically in recent years (1, 2). It is commonly associated with type 2 diabetes, coronary artery disease and hypertension, and the coexistence of these diseases in subjects has been termed the metabolic syndrome (3). There is a demand for effective and safe
Amelioration of Insulin Resistance by Rimonabant

anti-obesity agents that can produce and maintain weight loss and improve the metabolic syndrome.

The newly discovered endocannabinoid system, consisting of the cannabinoid type-1 (CB-1) receptor and endogenous lipid-derived ligands, contributes to the physiological regulation of energy balance, food intake and lipid and glucose metabolism, through both central orexigenic effects and peripheral metabolic effects (4-11). The endocannabinoid system is overactivated in genetic animal models of obesity (4, 6) and the selective CB-1 blocker, rimonabant, produces weight loss and ameliorates metabolic abnormalities in obese animals (12, 13). Patients with obesity and hyperglycemia associated with type 2 diabetes exhibit higher concentrations of endocannabinoids in the visceral fat and serum, respectively, than the corresponding controls (14). Rimonabant has been used clinically and been shown to produce substantial weight loss and reduction of waist circumference, and also improve insulin resistance and the profile of several metabolic and cardiovascular risk factors in diabetic as well as non-diabetic obese patients (15-18).

Adiponectin is an adipokine that is specifically and abundantly expressed in the adipose tissue and released into the circulation, that directly sensitizes the body to insulin (19, 20). Administration of recombinant adiponectin to rodents increases the glucose uptake and fat oxidation in muscle, reduces hepatic glucose production, and improves whole-body insulin sensitivity (21-23). Adiponectin-deficient (adipo(-/-)) mice exhibit insulin resistance and glucose intolerance (24, 25). Previous studies have shown that adiponectin stimulates fatty acid oxidation in the skeletal muscle and inhibits glucose production in the liver by activating AMP-activated protein kinase (AMPK) (26-29). We also reported that pioglitazone may induce amelioration of insulin resistance and diabetes via an adiponectin-dependent mechanism in the liver and adiponectin-independent mechanism in the skeletal muscle (30).

Rimonabant has been shown to increase the plasma adiponectin levels in animal models of obesity and diabetes, as well as in both diabetic and non-diabetic subjects (15, 31, 32). The results of the RIO-Lipids study provided evidence of a weight-loss-independent effect of rimonabant on the plasma adiponectin levels (15). Furthermore, the metabolic improvements induced by rimonabant could be attributed, at least in part, to a moderate, but significant increase in the plasma circulating adiponectin levels (15). However, whether the rimonabant-induced increase in the plasma levels of adiponectin might be causally involved in the effects of rimonabant, in particular, its insulin-sensitizing effects, has not been addressed experimentally.

To address this issue, in the present study, we used adipo(-/-) ob/ob mice (30) to investigate whether rimonabant might be capable of ameliorating insulin resistance in the absence of adiponectin. We found that rimonabant significantly decreased the body weight and food intake to similar degrees in the ob/ob and adipo(-/-) ob/ob mice. Furthermore, we found significant amelioration of the insulin resistance in the ob/ob mice, in association with significant up-regulation of the serum adiponectin levels after 21 days of treatment with rimonabant at 30 mg/kgBW. The amelioration of insulin resistance in the ob/ob mice was attributed to the decrease of glucose production and activation of AMPK in the liver, but not the increased glucose uptake by the skeletal muscle, induced by the drug. Interestingly, insulin resistance was also significantly, although only partially, improved in the adipo(-/-) ob/ob mice. Thus, the results suggest that rimonabant ameliorates insulin resistance via both adiponectin-dependent and adiponectin-independent pathways.

**EXPERIMENTAL PROCEDURES**

**Animals and Genotyping.** The mice were housed under a 12-h light-dark cycle and fed standard chow, CE-2 (CLEA Japan Inc., Tokyo, Japan); the composition of the chow was as follows: 25.6% (w/w) protein, 3.8% fiber, 6.9% ash, 50.5% carbohydrates, 4% fat, and 9.2% water. Ob/ob and adipo(-/-) ob/ob mice were generated by intercrossing adipo(+/+) ob/ob mice. All the mice were maintained on a C57Bl/6 background (30). All the experiments in this study were conducted on 16- to 20-week-old male littermates. The animal care and experimental procedures were approved by the Animal Care Committee of the University of Tokyo.

**Rimonabant Treatment Study.** Rimonabant (SR141716) or vehicle (0.1% TWEEN80 in saline) was administrated to ob/ob and adipo(-/-) ob/ob mice at the dose of 30 mg/kg body weight by oral gavage, once daily for 21 consecutive days. Rimonabant was kindly provided by Sanofi-Aventis, Montpellier, France. We measured the body weights and food intake of the mice once daily for 21 consecutive days.

**Hyperinsulinemic-Euglycemic Clamp Study.** Clamp studies were carried out as described previously (30), with slight modifications. In brief, 2 days before the study, an infusion catheter was inserted into the right jugular vein under general anesthesia induced by sodium pentobarbital. Studies were performed on the mice under conscious and unstressed conditions after 8-h fasting. A primed continuous infusion of insulin (Humulin R, Lilly) was administered (25.0 milliunits/kg/min), and the blood glucose concentration, monitored every 5 min, was maintained at 100–130 mg/dl by administration of glucose (5 g of glucose per 10 ml enriched to ~20% with [6,6-2H2] glucose (Sigma)) for 120 min. Blood was sampled via
Amelioration of Insulin Resistance by Rimonabant
tail tip bleeds at 90, 105 and 120 min for
determination of the rate of glucose disappearance ($R_d$).
$R_d$ was calculated according to nonsteady-state
equations (30), and endogenous glucose production
and the exogenous glucose infusion rate (GIR) (30).

**Western-Blot Analysis.** Tissues were excised and
homogenized in ice-cold buffer A (25 mM Tris-HCl
(pH 7.4), 10 mM sodium orthovanadate, 10 mM
sodium pyrophosphate, 100 mM sodium fluoride, 10
mM EDTA, 10 mM EGTA, and 1 mM
phenylmethylsulfonyl fluoride). The sample buffer for
analysis under reducing conditions was composed of
3% SDS, 50 mM Tris-HCl (pH 6.8), 5% 2-
mercaptoethanol, and 10% glycerol. Samples were
mixed with 5 × sample buffer, heated at 95 °C for 5
min for heat denaturation, separated on
polyacrylamide gels, and then transferred to a
Hybond-P polyvinylidene difluoride transfer
membrane (Amersham Biosciences). Bands were
detected with ECL detection reagents (Amersham Biosciences).
To examine the Akt and AMPK phosphorylation and protein levels, lysates of liver and
muscle were analyzed using anti-phospho-Akt (Cell Signaling Technology, Inc., Beverly, MA), anti-Akt
(Cell Signaling Technology, Inc.) antibody, anti-
phospho-AMPK (Cell Signaling Technology, Inc., Beverly, MA) and anti-AMPK (Cell Signaling Technology, Inc.) antibodies. For the analysis under non-reducing conditions, 2-mercaptoethanol was
excluded from the sample buffer described above. To
examine the isoforms of adiponectin, the serum
samples were diluted 20-fold. Anti-mouse adiponectin
antiserum was obtained by immunizing rabbits with
the globular domain of mouse recombinant
adiponectin produced in E. coli (21).

**Tissue Sampling for Insulin Signaling Pathway Study.** Mice were anesthetized after 16 hours of
starvation, and 0.05 unit of human insulin (Humulin R,
Lilly) was injected into the inferior vena cava. After 5
minutes, the liver was removed, and the specimens
were used for protein extraction as described above.

**Plasma Adiponectin and Lipid Measurements.**
The mice were deprived access to food for 16 h before
the measurements. The plasma adiponectin levels were
determined with a mouse adiponectin enzyme-linked
immunosorbent assay kit (Otsuka Pharmaceutical Co.,
Ltd., Tokyo, Japan). Serum triglyceride and free fatty
acids (Wako Pure Chemical Industries Ltd., Osaka,
Japan) were assayed by enzymatic methods.

**Measurement of Adipocyte Size.** Epididymal
white adipose tissue and subcutaneous fat were
 Routinely processed for paraffin embedding, and 4-µm
sections of the liver fixed in phosphate-buffered 4% paraformaldehyde, according to a
previously described method (33), with slight
modification. In brief, the livers were washed once for 1
min with H2O. After an additional wash for 1 min
with 60% isopropanol, the livers were stained for
10 min at 37 °C with freshly diluted Oil Red O solution
(6 parts of Oil Red O stock solution and 4 parts of
H2O; the Oil Red O stock solution contained 0.5% Oil
Red O in isopropanol). After one wash for 2 min with
60% isopropanol and one wash for 1 min with H2O, the
livers were stained for 5 min with hematoxylin. The
stain was then washed off with running water and the
silanized slides were stained. Oil Red O staining was
quantified on digital images. Color images were
acquired with a Nikon digital camera and analyzed
using the Image J software. The % area of Oil Red O
staining was measured from 9-10 different sections per
mouse in each experimental group. Values were
expressed as % area.

**Analysis of O2 Consumption.** Oxygen
consumption was measured every 3 minutes for 24h in the
fasting mice using an O2/CO2 metabolism
measurement device (Model MK-5000; Muromachi-kikai, Tokyo, Japan). After rimonabant
treatment for 21 days, each mouse was placed in a
sealed chamber (560ml volume) with an air flow rate of
500ml/min at room temperature. The amount of
oxygen consumed was converted to milliliters per
minute by multiplying it with the flow rate.

**RNA Preparation and Taqman PCR.** Total
RNA was extracted from various tissues in vivo with
TRizol reagent (Invitrogen), in accordance with the
manufacturer’s instructions. After treatment with RQ1
RNase-Free DNase (Promega, Madison, WI) to
to remove genomic DNA, cDNA was synthesized with
MultiScribe Reverse-Transcriptase (Applied Biosystems, Foster City, CA). Total RNA was
prepared from 3T3L1 cells in vitro with an RNasey
Mini Kit (Qiagen Co., Düsseldorf, Germany), in
accordance with the manufacturer’s instructions.
mRNA levels were quantitatively analyzed by
fluorescence-based reverse transcriptase-PCR. The
reverse transcription mixture was amplified with
specific primers using an ABI Prism 7000 sequence
detector equipped with a thermocycler. The primers
were used for monocyte chemoattractant protein-1 (MCP-1),
resistin, phosphoenolpyruvate carboxykinase (PEPCK),
carnitine palmitoyltransferase-1A (CPT-1A), the
hepatic isoform of CPT-1, protein phosphatase 2C
(PP2C) and cyclophilin were purchased from Applied
Biosystems (Foster City, CA). The relative expression
levels were compared by normalization to the
area was measured in 200 or more cells per mouse in
each group, in accordance with a previously described
method (30), with slight modifications.

**Oil Red O Staining and Quantification.** Lipid
accumulation was assessed by Oil Red O staining in 6-
µm frozen sections of the liver fixed in phosphate-
buffered 4% paraformaldehyde, according to a
previously described method (33), with slight
modification. In brief, the livers were washed once for 1
min with H2O. After an additional wash for 1 min
with 60% isopropanol, the livers were stained for
10 min at 37 °C with freshly diluted Oil Red O solution
(6 parts of Oil Red O stock solution and 4 parts of
H2O; the Oil Red O stock solution contained 0.5% Oil
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Mini Kit (Qiagen Co., Düsseldorf, Germany), in
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mRNA levels were quantitatively analyzed by
fluorescence-based reverse transcriptase-PCR. The
reverse transcription mixture was amplified with
specific primers using an ABI Prism 7000 sequence
detector equipped with a thermocycler. The primers
were used for monocyte chemoattractant protein-1 (MCP-1),
resistin, phosphoenolpyruvate carboxykinase (PEPCK),
carnitine palmitoyltransferase-1A (CPT-1A), the
hepatic isoform of CPT-1, protein phosphatase 2C
(PP2C) and cyclophilin were purchased from Applied
Biosystems (Foster City, CA). The relative expression
levels were compared by normalization to the
Amelioration of Insulin Resistance by Rimonabant

expression levels of cyclophilin.

**Cell Culture and Differentiation of 3T3L1 Adipocytes and Rimonabant Treatment.** 3T3L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and 10% fetal bovine serum at 37 °C. Confluent cultures were induced to differentiate into adipocytes by incubation in DMEM containing 25 mM glucose, 10% fetal bovine serum, 0.25U/ml insulin, 0.25 µM dexamethasone, and 0.5 mM isobutyl-1-methylxanthine. After 2 days, the medium was changed to DMEM containing 25 mM glucose, 10% fetal bovine serum, and 0.025U/ml insulin. All studies were performed on adipocytes 10 days after the initiation of differentiation (Day 0). Rimonabant treatment (100 nM and 1 µM) was started on Day 0 and dimethyl sulfoxide (DMSO) was used as the vehicle. Prior to the start of the experiments, the differentiated adipocytes were serum-starved in DMEM containing 25 mM glucose for 16 h at 37 °C.

**RESULTS**

**Absence of adiponectin had no effect on rimonabant-induced suppression of body weight and daily food intake**

The body weight gain was similar between the untreated ob/ob and adipo(-/-) ob/ob mice (Fig. 1A), as reported previously (30). The food intake was also comparable between the untreated ob/ob and adipo(-/-) ob/ob mice (Fig. 1B). Rimonabant significantly decreased the body weight and food intake to similar degrees in the ob/ob and adipo(-/-) ob/ob mice (Fig. 1A, B). After 21 days of rimonabant treatment, both the ob/ob and adipo(-/-) ob/ob mice weighed 10% less than the corresponding untreated mice (Fig. 1A). Moreover, rimonabant treatment significantly decreased the white adipose tissue (WAT) mass in both subcutaneous and visceral (epididymal, mesenteric, and retroperitoneal) fat to similar degrees in the ob/ob and adipo(-/-) ob/ob mice (Fig. 1C). To determine whether the presence of adiponectin is required for the reduction of the average adipocyte size induced by rimonabant treatment, we histologically analyzed the epididymal fat pad and subcutaneous WAT after fixation and quantitation of the adipocyte size. The distribution of the adipocyte size in the rimonabant-treated ob/ob and adipo(-/-) ob/ob mice was similarly narrowed to that in the untreated ob/ob and adipo(-/-) ob/ob mice (Fig. 1D, 1E), and rimonabant treatment significantly reduced the average adipocyte size in the ob/ob and adipo(-/-) ob/ob mice to a similar degree (Fig. 1F). These findings indicate that the absence of adiponectin had no effect on either the rimonabant-induced decrease of the body weight or the food intake of the mice, and that rimonabant treatment can induce a reduction of adipocyte size in the absence of adiponectin or leptin or both.

**Rimonabant increased the energy expenditure and decreased the serum triglyceride and free fatty acid levels to a similar degree in the ob/ob and adipo(-/-) ob/ob mice.**

In addition to suppressing food intake, rimonabant has been demonstrated to increase the energy expenditure (10, 34), and the increase in energy expenditure has also been shown in CB-1 receptor-knockout mice (35). Since the involvement of adiponectin in this action of rimonabant remains unclear, we investigated the effects of rimonabant on energy expenditure. First, we measured the rectal temperature in the ob/ob and adipo(-/-) ob/ob mice. The temperature was essentially the same (Fig. 2A), and rimonabant treatment significantly increased the rectal temperature of the ob/ob and adipo(-/-) ob/ob mice to a similar degree (Fig. 2A). Secondly, we investigated the oxygen consumption after 21 days’ treatment with rimonabant and found that in the dark phase of the daily light cycle, rimonabant increased the energy expenditure to a similar degree in both the ob/ob and adipo(-/-) ob/ob mice (Fig. 2B). This effect of rimonabant on the energy expenditure in the ob/ob did not require the presence of adiponectin. We next investigated the effects of rimonabant treatment on the serum lipid levels. In addition to reducing the body weight, rimonabant has been demonstrated to reduce the serum triglyceride (TG) (15-18, 32) and free fatty acid (FFA) levels (32). However, the involvement of adiponectin in this action of rimonabant remains unclear. Both the serum TG and FFA levels were indistinguishable between the ob/ob and adipo(-/-) ob/ob mice (Fig. 2C, 2D), and rimonabant treatment significantly decreased the levels of both to similar degrees in the ob/ob and adipo(-/-) ob/ob mice (Fig. 2C, 2D). This effect of rimonabant on the serum lipids in the ob/ob mice did not require the presence of adiponectin. MCP-1 and resistin have been shown to be important mediators of insulin resistance linked to obesity (36-39). We analyzed the expression of MCP-1 and resistin in the epididymal WAT. The expressions of both MCP-1 and resistin were indistinguishable between the untreated and rimonabant-treated mice of either genotype (Fig. 2E, 2F).

**Rimonabant increased the plasma adiponectin levels in the ob/ob mice, in particular, of high-molecular-weight adiponectin**

Rimonabant treatment for 21 days significantly increased the plasma adiponectin levels in the ob/ob mice, whereas plasma adiponectin was not detectable in either the untreated or rimonabant-treated adipo(-/-) ob/ob mice (Fig. 3A). High-molecular-weight (HMW) adiponectin is known to be the most active and its serum levels have been reported to be decreased in obese individuals and murine models, which is associated with a decrease of the hepatic and muscle AMPK activity and fatty acid combustion, and
Amelioration of Insulin Resistance by Rimonabant

Therefore, we analyzed the plasma levels of this isoform of adiponectin by western blotting. Rimonabant treatment significantly increased the serum levels of HMW adiponectin in the ob/ob mice (Fig. 3B). On the other hand, the plasma levels of middle- molecular-weight (MMW) and low-molecular-weight (LMW) adiponectin were slightly, but not significantly, increased in the rimonabant-treated ob/ob mice (Fig. 3B). In regard to the adipot(-/-)ob/ob mice, plasma adiponectin was not detectable in either the untreated or rimonabant-treated mice (Fig. 3B). Rimonabant has been reported to increase adiponectin expression and secretion in 3T3F442A adipocyte (6, 40). We next investigated the direct effect of rimonabant on adiponectin secretion using murine adipocyte cell line, 3T3L1, and confirmed that treatment with 100nM and 1 μ M rimonabant actually increased the expression and secretion into the medium of adiponectin (Fig 3C, 3D).

Rimonabant improved hepatic insulin resistance in both the ob/ob and adipot(-/-)ob/ob mice, although the effect was significantly less pronounced in the adipot(-/-)ob/ob mice

We carried out hyperinsulinemic-euglycemic clamp studies in the ob/ob and adipot(-/-)ob/ob mice to investigate the effect of rimonabant on the insulin resistance in the liver and skeletal muscle. Without rimonabant treatment, the GIRs were comparable in the ob/ob and adipot(-/-)ob/ob mice (Fig. 4A). After 21 days of rimonabant treatment, the GIRs were significantly increased in both the ob/ob and adipot(-/-)ob/ob mice (Fig. 4A); however, the increase was significantly less pronounced in the adipot(-/-)ob/ob mice. Rimonabant treatment also produced a significant decrease of the EGP in both the ob/ob and adipot(-/-)ob/ob mice, but the effect was significantly less pronounced in the adipot(-/-)ob/ob mice (Fig. 4B). The rates of R_d were indistinguishable between the untreated ob/ob and adipot(-/-)ob/ob mice, and rimonabant treatment had no effect on this parameter in either genotype (Fig. 4C). We next studied the effects on insulin signaling and the downstream reactions in the liver (Fig. 4D, 4E). Insulin-stimulated Akt phosphorylation was significantly increased in rimonabant-treated ob/ob mice as compared with that in the untreated ob/ob mice (Fig. 4D), while insulin-stimulated Akt phosphorylation only tended to be increased in the rimonabant-treated adipot(-/-)ob/ob mice as compared with that in the corresponding untreated mice. The PEPCK expression levels in the liver were comparable in the untreated ob/ob and adipot(-/-)ob/ob mice (Fig. 4F). Rimonabant treatment significantly decreased the expression of PEPCK in both the ob/ob and adipot(-/-)ob/ob mice, but the effect was significantly less pronounced in the adipot(-/-)ob/ob mice (Fig. 4F). These findings indicate that rimonabant ameliorates hepatic, but not muscle, insulin resistance in mice with an ob/ob background, in both an adiponectin-dependent and adiponectin-independent manner.

Rimonabant increased the hepatic AMPK activities and CPT-1 expression levels in both ob/ob and adipot(-/-)ob/ob mice, but its effect was significantly less pronounced in the adipot(-/-)ob/ob mice

We carried out analysis of the liver metabolic activity after the clamp studies to investigate the effect of rimonabant on amelioration of insulin resistance. The AMPK activities were comparable in the untreated ob/ob and adipot(-/-)ob/ob mice (Fig. 5A). Rimonabant treatment for 21 days increased the AMPK activities in both the ob/ob and adipot(-/-)ob/ob mice, but its effect was significantly less pronounced in the adipot(-/-)ob/ob mice (Fig. 5A). The expression levels of CPT-1, the rate-limiting enzyme in fatty acid β-oxidation, were also comparable in the untreated ob/ob and adipot(-/-)ob/ob mice (Fig. 5B). Rimonabant treatment increased the CPT-1 expression in both ob/ob and adipot(-/-)ob/ob mice, but its effect was significantly less pronounced in the adipot(-/-)ob/ob mice (Fig. 5B). The expression levels of protein phosphatase 2C (PP2C) were indistinguishable between the untreated ob/ob and adipot(-/-)ob/ob mice, and rimonabant treatment had no effect on the PP2C expression in either genotype (Fig. 5C). As reported previously (26, 41), fatty acid oxidation is positively regulated by AMPK in the liver, therefore, we next carried out analysis of the hepatic TG content by Oil Red O staining. The % areas of Oil Red O staining in the liver were comparable in the untreated ob/ob and adipot(-/-)ob/ob mice (Fig. 5D). Rimonabant treatment significantly decreased the hepatic TG content in both the ob/ob and adipot(-/-)ob/ob mice, but its effect was significantly less pronounced in the adipot(-/-)ob/ob mice (Fig. 5D). We also investigated the AMPK activities in the muscle after the clamp studies. The AMPK activities in the muscle were indistinguishable between the untreated ob/ob and adipot(-/-)ob/ob mice, and rimonabant treatment had no effect on the muscle AMPK activity in either genotype (Fig. 5E). These findings indicate that rimonabant activates hepatic, but not muscle AMPK, in mice with an ob/ob background, in both an adiponectin-dependent and adiponectin-independent manner.

DISCUSSION

The selective CB-1 blocker rimonabant has been reported to produce weight loss and ameliorate insulin resistance and metabolic abnormalities in obese animals (12, 13), as also in patients with obesity (15-18). Rimonabant has also been reported to increase the plasma adiponectin levels in animal models of obesity and diabetes, as also in diabetic or nondiabetic subjects.
Amelioration of Insulin Resistance by Rimonabant (15, 31, 32). Adiponectin has been proposed to be a major insulin-sensitizing adipokine (19, 20) and is a plausible candidate as the adipokine mediating the rimonabant-induced amelioration of insulin resistance. Therefore, in this study, we used two obesity models, the ob/ob and adipo(-/-)ob/ob mice, to investigate whether the rimonabant-induced increase of plasma adiponectin might be causally involved in the insulin-sensitizing effects of the drug.

Rimonabant treatment decreased the body weight, food intake and weight of the WAT to similar degrees in the ob/ob and adipo(-/-)ob/ob mice. Furthermore, it also increased the energy expenditure and decreased the serum TG and FFA to similar degrees in the ob/ob and adipo(-/-)ob/ob mice. Thus, the involvement of adiponectin was not required for rimonabant to exert its effects.

Significant improvement of the insulin resistance was observed in the ob/ob mice following rimonabant treatment, in association with significant up-regulation of the plasma adiponectin levels, in particular, of HMW. Amelioration of insulin resistance in the ob/ob mice was considered to be attributable to improvement of the hepatic, but not muscle, insulin resistance. Interestingly, these improvements induced by rimonabant were significantly less pronounced in the adipo(-/-)ob/ob mice, indicating that adiponectin is involved in the rimonabant-mediated amelioration of hepatic insulin resistance. In fact, while significant decrease of the PEPCK expression levels was observed, the AMPK activity was significantly increased and the hepatic TG content was decreased in the ob/ob mice; all of these changes were significantly less pronounced in the adipo(-/-)ob/ob mice lacking adiponectin. We reported from a previous study that adiponectin, especially HMW adiponectin, stimulates AMPK activation in the liver (26, 42). These findings suggest that rimonabant treatment activates AMPK in the liver via increasing the secretion of HMW adiponectin, and then decreases the expression of PEPCK to inhibit glucose production and increase CPT-1 expression, thereby stimulating fatty acid oxidation in the liver.

On the other hand, rimonabant treatment also produced significant amelioration of hepatic insulin resistance in the absence of adiponectin. This amelioration was possibly attributable to the reduction of body weight (Fig. 1A), but not to suppression of MCP-1 and resistin expression (Fig. 2E, 2F). Alternatively, this amelioration was possibly due to the direct activation of AMPK by rimonabant in the liver. In fact, recent reports have shown that AMPK activity was significantly higher in the liver of hepatocyte-specific CB-1 receptor-knockout mice, even though the serum adiponectin levels in these animals remained unchanged (35, 43), suggesting that rimonabant treatment directly activates hepatic AMPK, even without the mediation of adiponectin, and decreases the expression of PEPCK to inhibit glucose production in the liver.

In addition, Osei-Hyiaman et al. have reported that CPT-1 activity in the liver was significantly increased when systemic CB-1 receptors were blocked pharmacologically in wild-type mice (35). Moreover, hepatic CPT-1 activity increased and hepatic TG content decreased when hepatic CB-1 receptors were blocked genetically (35). These data suggest that CB-1 receptor blockade stimulates CPT-1 activity and increases fatty acid combustion, to decrease the TG content in the liver. Consistent with this, rimonabant actually increased CPT-1 expression and decreased the TG content in the livers of ob/ob and adipo(-/-)ob/ob mice. However, these effects were markedly attenuated in the adipo(-/-)ob/ob mice, suggesting that increased CPT-1 expression and decreased hepatic TG content by rimonabant were also mediated by adiponectin-dependent as well as adiponectin-independent pathways.

Based on our findings, we propose that there are two distinct pathways by which rimonabant ameliorates insulin resistance. One, an adiponectin-dependent pathway and the other, an adiponectin-independent pathway (Fig. 6). Rimonabant increases the plasma levels of adiponectin, in particular, of HMW adiponectin, which induces AMPK activation and decreases gluconeogenesis in the liver, thereby ameliorating insulin resistance. On the other hand, in a manner independent of adiponectin, rimonabant directly induces AMPK activation and decreases gluconeogenesis in the liver, possibly via hepatic CB-1 receptor (35, 43), which also contributes to ameliorating insulin resistance. In addition, rimonabant decreases food intake and increases energy expenditure, which are related to reduction of body weight. This body weight loss may be also associated with ameliorating insulin resistance via adiponectin-dependent and adiponectin-independent pathways (Fig. 6).

Rimonabant is metabolized in the liver by cytochrome P-450 CYP3A4 and amidohydrolase, and excreted into the bile (44, 45). The oral bioavailability of rimonabant is low to moderate; this is due to the extensive first-pass metabolism of the drug (46). Therefore, in this study, the concentration in the liver of the orally administered rimonabant might be higher than that in other tissues such as the muscle, because of the first-pass effect of the liver. Although intraperitoneally administrated rimonabant was reported in a previous study to significantly increase the glucose uptake in the soleus muscle of ob/ob mice (10), no improvement of the insulin resistance in the muscle was observed in our study. One of the reasons for this difference may be the lower concentration of rimonabant in the muscle due to the first-pass effect of the liver.
Amelioration of Insulin Resistance by Rimonabant

In the four double-blind trials (RIO-Lipids (15), RIO-Europe (16), RIO-North America (17) and RIO-Diabetes (18), the most frequent adverse events among individuals treated with rimonabant were nausea, dizziness, diarrhea and insomnia, each occurring at a 1-9% greater frequency than that in the placebo group. In the RIO-Lipids, RIO-Europe and RIO-North America, the drug had to be discontinued due to the development of psychiatric disorders (mainly depression) in 6-7% of rimonabant-treated individuals, an absolute increase of 2-5% over the frequency in the placebo group (44). Substance dependence with rimonabant has not been reported. The absence of appearance of clinical signs in toxicology studies with a recovery period indicates that rimonabant does not possess the potential to produce withdrawal syndrome (46).

Many reports have shown the efficacy of cannabinoid agonists in chronic pain (47). In a rodent model of inflammatory pain, anandamide, one of the endogenous cannabinoids, suppressed the development and maintenance of thermal hyperalgesia (48). This analgesic effect was diminished by concurrent administration of the CB-1 antagonist, rimonabant, and anandamide. Even though rimonabant alters the sensitivity to pain (48), it does not necessarily induce pain itself. On the contrary, rimonabant has recently been shown to prevent indomethacin-induced intestinal injury by decreasing the levels of the pro-inflammatory cytokine, tumor necrosis factor alpha (TNFα), in rodents (49), indicating its potential anti-inflammatory activity in acute and chronic diseases. In neurogenic inflammatory pain, including arthritis and neuropathy, many cytokines, especially TNFα, play a key role in the generation and maintenance of hyperalgesia (50). On the basis of these findings, Costa indicated that the anti-TNFα effect of rimonabant might contribute to its anti-inflammatory activity and consequently to the relief of pain (51). However, further investigation and accumulation of further evidence on the effect of rimonabant on pain are needed. At least, in the four clinical trials mentioned above, side effects associated with pain, such as hyperalgesia or hypoalgesia, were not reported. Furthermore, it has been suggested that while females might perceive pain differently from males (52, 53), the anti-obesity effects of rimonabant appeared to be similar in males and females (46).

In conclusion, this study demonstrated for the first time that rimonabant ameliorates insulin resistance via both adiponectin-dependent and adiponectin-independent pathways.
REFERENCES

Amelioration of Insulin Resistance by Rimonabant


ACKNOWLEDGMENTS

We thank Ritsuko Hoshino, Katsuyoshi Kumagai, Sayaka Sasamoto, Kayo Nishitani, Namiko Kasuga and Hiroshi Chiyonobu for their excellent technical assistance and animal care.

This work was supported by a grant for CREST from Japan Science and Technology Corporation, a grant for Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research, a grant for TSBMI from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant-in-Aid for Scientific Research in Priority Areas (A) (16209030) and (A) (18209033) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T. K.), and a Grant-in-Aid for Scientific Research in Priority Areas (C) (19591037) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to N. K.). We certify that none of the authors of this manuscript have any financial interests to declare in relation to this work.

The abbreviations used are; AMPK, AMP-activated protein kinase; CB-1, cannabinoid type-1; GIR, glucose infusion rate(s); EGP, endogenous glucose production; $R_d$, rate of glucose disappearance; MCP-1, monocyte chemoattractant protein-1; PEPCK, phosphoenolpyruvate carboxykinase; CPT-1, carnitine palmitoyltransferase-1; PP2C, protein phosphatase 2C; WAT, white adipose tissue; TG, triglyceride; FFA, free fatty acid; HMW, high-molecular-weight; MMW, middle-molecular-weight; LMW, low-molecular-weight; TNFα, tumor necrosis factor-alpha.
Amelioration of Insulin Resistance by Rimonabant

FIGURE LEGENDS

Fig. 1. Absence of adiponectin had no effect on rimonabant-induced suppression of body weight and daily food intake. (A, B), Body weights (A) and food intake (B) of ob/ob (left panels) and adipo(-/-)ob/ob mice (right panels) not treated (open squares) and treated (filled squares) with rimonabant (n=12-14 per group). Values are means±S.E. of data obtained from the analysis of ob/ob and adipo(-/-)ob/ob mice. *P<0.05, **P<0.01. (C), Weight of the total visceral white adipose tissue (left panel) and subcutaneous WAT (right panel) of ob/ob and adipo(-/-) ob/ob mice not treated(open bars) and treated (filled bars) with rimonabant (n=9-14 per group). Values are means±S.E. of data obtained from the analysis of ob/ob and adipo(-/-)ob/ob mice. **P<0.01. (D, E), Histogram of adipocyte size from epididymal WAT (D) and subcutaneous WAT (E) of ob/ob (left panels) and adipo(-/-)ob/ob mice(right panels) not treated (open bars) and treated (filled bars) with rimonabant (n=5-8 pre group). Values are means±S.E. of data obtained from the analysis of ob/ob and adipo(-/-)ob/ob mice. **P<0.01. (F), Average size of adipocyte from epididymal WAT (left panel) and subcutaneous WAT (right panel) of ob/ob and adipo(-/-)ob/ob mice not treated (open bars) and treated (filled bars) with rimonabant (n=5-8 pre group). Values are means±S.E. of data obtained from the analysis of ob/ob and adipo(-/-)ob/ob mice. ***P<0.005.

Fig. 2. Rimonabant increased the energy expenditure and decreased the serum triglyceride and free fatty acid levels to a similar degree in the ob/ob and adipo(-/-)ob/ob mice. (A, B), Rectal temperature (A) and O₂ consumption (B) in ob/ob and adipo(-/-)ob/ob mice not treated (open bars) and treated (filled bars) with rimonabant (n=6-10 per group). Values are means±S.E. of data obtained from the analysis of ob/ob mice and adipo(-/-)ob/ob mice. *P<0.05. **P<0.01. (C, D), Serum triglyceride (TG) (C) and free fatty acid (FFA) (D) levels in ob/ob and adipo(-/-)ob/ob mice not treated (open bars) and treated (filled bars) with rimonabant. (C, n=11-14 per group, D, n=4-5 per group, respectively). Values are means±S.E. of data obtained from the analysis of ob/ob mice and adipo(-/-)ob/ob mice. *P<0.05. **P<0.01. (E, F), MCP-1 (E) and resistin (F) expression levels in the epididymal WAT of ob/ob and adipo(-/-)ob/ob mice not treated (open bars) and treated (filled bars) with rimonabant (n=7-8 per group). Values are means±S.E. of data obtained from the analysis of ob/ob mice and adipo(-/-)ob/ob mice. Values are means±S.E. n.s., not significant.

Fig. 3. Rimonabant increased the plasma adiponectin levels, in particular, of high-molecular-weight adiponectin, in the ob/ob mice. (A), Plasma adiponectin levels in ob/ob and adipo(-/-)ob/ob mice not treated (open bar) and treated (filled bar) with rimonabant (n=7-14 per group). Values are means±S.E. of data obtained from the analysis of ob/ob mice and adipo(-/-)ob/ob mice. **P<0.01. N.D., not detectable. (B), The different isoforms of plasma adiponectin of ob/ob and adipo(-/-)ob/ob mice not treated (open bars) and treated (filled bars) with rimonabant were analyzed by Western blotting and quantitated by densitometry. The relative ratio of each molecular-weight category of adiponectin was normalized to that in the control ob/ob mice not treated with rimonabant (n=4-8 per group). Results are representative of three independent experiments. Values are means±S.E. of data obtained from the analysis of ob/ob mice and adipo(-/-)ob/ob mice. *P<0.05. n.s., not significant. (C, D), Effects of rimonabant on adiponectin mRNA expression (C) and adiponectin secretion in the conditioned medium (D) of mouse 3T3L1 adipocytes (n=4-9 per group). Controls (open bars), DMSO as the vehicle (filled bars), 100nM rimonabant (gray bars), and 1µM rimonabant (latticebars) Values are means±S.E. of data obtained from the analysis of 3T3L1 adipocytes. *P<0.05, **P<0.01.

Fig. 4. Rimonabant improved hepatic insulin resistance in both ob/ob and adipo(-/-)ob/ob mice, although the effect was significantly less pronounced in the adipo(-/-)ob/ob mice. (A-C), Glucose infusion rates (GIR) (A), endogenous glucose production (EGP) (B), and rates of glucose disappearance (Rd) (C) in ob/ob and adipo(-/-)ob/ob mice not treated(open bars) and treated (filled bars) with rimonabant in the clamp study (n=5-7 per group). Values are means±SE of data obtained from the analysis of ob/ob mice and adipo(-/-)ob/ob mice. *P<0.05, **P<0.01, ***P<0.005. (D, E), Phosphorylations of Akt in the livers of ob/ob (D) and adipo(-/-)ob/ob mice (E) not treated (open bars) and treated (filled bars) with rimonabant after the injection of insulin (n=4-5 per group). Results are representative of three independent experiments. Values are means±S.E. of data obtained from the analysis of ob/ob mice and adipo(-/-)ob/ob mice. *P<0.05. (F), Phosphoenolpyruvate carboxykinase (PEPCK) expression levels in the livers of ob/ob and adipo(-/-)ob/ob mice not treated (open bars) and treated (filled bars) with rimonabant after the clamp studies (n=6-7 per group). The relative expressions after normalization to the expression level of cyclophilin were compared. Values are means±S.E. of data obtained from the analysis of ob/ob mice and adipo(-/-)ob/ob mice. *P<0.05., **P<0.01.

Fig. 5. Rimonabant increased the hepatic AMPK activities and CPT-1 expression levels in both ob/ob mice and adipo(-/-)ob/ob mice, but the effects were significantly less pronounced in the adipo(-/-)ob/ob mice. (A),
Phosphorylations of AMPK in the livers of ob/ob and adipos (-/-)ob/ob mice not treated (open bars) and treated (filled bars) with rimonabant after the clamp studies (n=4-5 per group). Results are representative of three independent experiments. Values are means±S.E. of data obtained from the analysis of ob/ob mice and adipos (-/-)ob/ob mice. *P<0.05, **P<0.01. (B, C), Carnitine palmitoyltransferase-1 (CPT-1) (B) and protein phosphatase 2C (PP2C) (C) expression levels in the liver of ob/ob and adipos (-/-)ob/ob mice not treated (open bars) and treated (filled bars) with rimonabant after the clamp studies (n=4-9 per group). Relative expressions after normalization to the expression level of cyclophilin were compared. Values are means±S.E. of data obtained from the analysis of ob/ob mice and adipos (-/-)ob/ob mice. *P<0.05, **P<0.01. (D), Oil Red O staining in the livers of ob/ob and adipos (-/-)ob/ob mice not treated (open bars) and treated (filled bars) with rimonabant (n=6-10 per group). Representative liver histology as viewed on a computer monitor is shown. Original magnification, x 100. Values are means±S.E. of data obtained from the analysis of ob/ob mice and adipos (-/-)ob/ob mice. *P<0.05, **P<0.01. (E), Phosphorylation levels of AMPK in the muscle of ob/ob and adipos (-/-)ob/ob mice not treated (open bars) and treated (filled bars) with rimonabant after the clamp studies (n=5 per group). Results are representative of three independent experiments. Values are means±S.E. n.s., not significant.

**Fig. 6. Rimonabant ameliorates insulin resistance via both adiponectin-dependent and adiponectin-independent pathways.** There are two distinct pathways by which rimonabant ameliorates insulin resistance. One, an adiponectin-dependent pathway and the other, an adiponectin-independent pathway. Rimonabant increases the plasma levels of adiponectin, in particular, of HMW adiponectin, which induces AMPK activation and decreases gluconeogenesis in the liver, thereby ameliorating insulin resistance. On the other hand, in a manner independent of adiponectin, rimonabant directly induces AMPK activation and decreases gluconeogenesis in the liver, possibly via hepatic CB-1 receptor, which also contributes to ameliorating insulin resistance. In addition, rimonabant decreases food intake and increases energy expenditure, which are related to reduction of body weight. This body weight loss may be also associated with ameliorating insulin resistance via adiponectin-dependent and adiponectin-independent pathways.
**Fig. 2**

**A** Rectal temperature

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>ob/ob</td>
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<tr>
<td>adip (-/-)ob/ob</td>
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**B** 

<table>
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<tr>
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<tr>
<td>adip (-/-)ob/ob</td>
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**C** Serum triglyceride level

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<td>adip (-/-)ob/ob</td>
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**D** Serum free fatty acid level

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<tr>
<td>adip (-/-)ob/ob</td>
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**E** MCP-1 expression

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<td>adip (-/-)ob/ob</td>
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**F** Resistin expression

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<tr>
<td>adip (-/-)ob/ob</td>
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</table>
Fig. 3

A. Plasma adiponectin level

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<tr>
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</thead>
<tbody>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>adipo(-/-)ob/ob</td>
<td></td>
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</table>

N.D. = Not detected

B. Adiponectin Isoform

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<td></td>
</tr>
<tr>
<td>adipo(-/-)ob/ob</td>
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</table>

HMW adiponectin (ob/ob mice)

MMW adiponectin (ob/ob mice)

LMW adiponectin (ob/ob mice)

C. Adiponectin expression

<table>
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<tr>
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arbitrary unit

D. Adiponectin level

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<th>Control</th>
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µg/ml
**Fig. 4**

**A**

**Glucose infusion rate (GIR)**

![Graph showing glucose infusion rate (GIR) for control and Rimona-bant treated ob/ob and adipo(-/-) ob/ob mice.](image)

Control | Rimona-bant
--- | ---
ob/ob | ob/ob

**B**

**Endogenous glucose production (EGP)**

![Graph showing endogenous glucose production (EGP) for control and Rimona-bant treated ob/ob and adipo(-/-) ob/ob mice.](image)

Control | Rimona-bant
--- | ---
ob/ob | ob/ob

**C**

**Rate of glucose disappearance (Rd)**

![Graph showing rate of glucose disappearance (Rd) for control and Rimona-bant treated ob/ob and adipo(-/-) ob/ob mice.](image)

Control | Rimona-bant
--- | ---
ob/ob | ob/ob

**D**

**Insulin pAkt/Akt**

![Graph showing insulin pAkt/Akt for control and Rimona-bant treated ob/ob and adipo(-/-) ob/ob mice.](image)

Insulin | pAkt/Akt
--- | ---
- | +
- | +

**E**

**adipo(-/-)ob/ob mice**

![Graph showing pAkt/Akt for control and Rimona-bant treated adipo(-/-) ob/ob mice.](image)

**F**

**PEPCK expression**

![Graph showing PEPCK expression for control and Rimona-bant treated ob/ob and adipo(-/-) ob/ob mice.](image)
Fig. 5

A. **pAMPK**

Rimonabant

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B. **CPT-1 expression**

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C. **PP2C expression**

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D. **Oil Red O staining**

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E. **pAMPK/AMPK**

Rimonabant

<table>
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Fig. 6

Brain

Food Intake ↓
Energy Expenditure ↑

Body weight ↓

Rimonabant

Adipose tissue

Adiponectin ↑

Insulin resistance ↓

Liver

AMPK ↑
Gluconeogenesis ↓
Fatty acid oxidation ↑
Rimonabant ameliorates insulin resistance via both adiponectin-dependent and adiponectin-independent pathways

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J. Biol. Chem. published online November 13, 2008

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