Rimonabant, a Selective Cannabinoid CB1 Receptor Antagonist, Inhibits Atherosclerosis in LDL Receptor–Deficient Mice

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Objective—The objective of this study was to determine whether the potent selective cannabinoid receptor-1 antagonist rimonabant has antiatherosclerotic properties.

Methods and Results—Rimonabant (50 mg/kg/d in the diet) significantly reduced food intake (from 3.35±0.04 to 2.80±0.03 g/d), weight gain (from 14.6±0.7 g to −0.6±0.3 g), serum total cholesterol (from 8.39±0.54 to 5.32±0.18 g/L), and atherosclerotic lesion development in the aorta (from 1.7±0.22 to 0.21±0.037 mm²) and aortic sinus (from 101 000±7800 to 27 000±2900 μm²) of LDLR<sup>−/−</sup> mice fed a Western-type diet for 3 months. Rimonabant also reduced plasma levels of the proinflammatory cytokines MCP-1 and IL12 by 85% (P<0.05) and 76% (P<0.05), respectively. Pair-fed animals had reduced weight gain (6.2±0.6 g gain), but developed atherosclerotic lesions which were as large as those of untreated animals, showing that the antiatherosclerotic effect of rimonabant is not related to reduced food intake. Interestingly, rimonabant at a lower dose (30 mg/kg/d in the diet) reduced atherosclerosis development in the aortic sinus (from 121 000±20 000 to 62 000±11 000 μm², 49% reduction, P<0.05), without affecting serum total cholesterol (7.8±0.7 g/L versus 8.1±1.3 g/L in the control group). Rimonabant decreased lipopolysaccharide (LPS)- and IL1β-induced proinflammatory gene expression in mouse peritoneal macrophages in vitro as well as thiglycollate-induced recruitment of macrophages in vivo (10 mg/kg, po bolus).

Conclusions—These results show that rimonabant has antiatherosclerotic effects in LDLR<sup>−/−</sup> mice. These effects are partly unrelated to serum cholesterol modulation and could be related to an antiinflammatory effect. (Arterioscler Thromb Vase Biol. 2008;28:000-000)

Key Words: atherosclerosis • rimonabant • obesity • LDLR-deficient mice
matory condition in atherosclerotic lesions. Although the significant weight loss induced by rimonabant is therefore of potential interest in the field of atherosclerosis, rimonabant has also been shown to produce an improvement in other cardiovascular risk factors, eg, a decrease of the LDL/HDL cholesterol ratio and triglycerides in overweight patients, and also C reactive protein in dyslipidemic obese patients. The effects of rimonabant on these secondary end points could only partly be explained by weight loss, suggesting that rimonabant has additional metabolic or protective effects leading to a further decrease in risk factor levels.

Considering the beneficial effect of rimonabant on serum lipid parameters in patients as well as animal models, rimonabant would be expected to decrease atherosclerotic lesion development. However, atherosclerosis is a multifactorial disease, and besides dyslipidemia, inflammation is now recognized as one of the major factors influencing the course of the disease. Interestingly, rimonabant has been shown to reduce the levels of the proinflammatory cytokine TNFα in a model of obesity-associated hepatic steatosis, and is able to inhibit LPS-induced TNFα increase and adjuvant-induced arthritis in rats, suggesting that it might be able to directly or indirectly decrease the inflammatory component of atherosclerotic plaque progression.

To assess the potential effect of rimonabant on atherosclerosis, we have now evaluated the effect of this compound in several in vitro and in vivo models related to lesion formation. As a first step, the effect of rimonabant on lesion development was studied in LDLR−/− mice on high-fat diet. This mouse model of atherosclerotic lesion development is particularly interesting, because it has been shown previously that male LDLR−/− mice are more sensitive than apoE−/− mice to diet-induced obesity, and rimonabant is essentially targeted to obese and diabetic patients. To clarify the mechanism of action of rimonabant, we also determined whether rimonabant is able to modulate inflammatory gene expression in macrophages, which are one of the major cell types involved in atherosclerotic lesion progression. The effect of rimonabant on macrophage recruitment in vivo was also assessed.

**Materials and Methods**

For expanded Materials and Methods, please see the online data supplement, available online at http://atvb.ahajournals.org.

**Mice**

Male LDLR-deficient mice (LDLR−/−) on a C57BL/6 background were obtained from Charles River Company at the age of 3 months. Western diet (0.21% cholesterol; 20% fat) with or without rimonabant was from Research Diets. Mice were weighed every week and food ingestion was measured every day to restrict the food intake of the pair fed group. All procedures and care of animals were approved by the Animal Care and Use Committee of Sanofi-Aventis R & D.

**Determination of Atherosclerosis, Serum Lipoproteins, and Cytokines**

For atherosclerotic area determination, thoracic and abdominal segments of the aorta were opened longitudinally, pinned onto paraffin, and stained with Sudan black B. Serum lipoprotein and cytokine levels were measured by commercially available enzymatic or ELISA kits.

**Peritoneal Macrophage Isolation and Cell Culture**

Murine thioglycollate-elicited peritoneal macrophages were isolated as described (Bellosta et al, 1998). Macrophage mRNA levels were measured by quantitative polymerase chain reaction (PCR).

**Peritonitis Model of Macrophage Recruitment**

Female CB1R−/− and CB1R−/− mice on the C57Bl/6 background between 8 and 14 weeks of age were used (12 to 14 per group). Peritonitis was induced by a single intraperitoneal injection of 1 mL 4% thioglycollate.

**Statistical Analysis**

Statistical analysis of the in vitro experiment was performed using multivariate ANOVA tests followed by Scheffe post hoc test. In vivo experiments were analyzed using the nonparametric Kruskal-Wallis test or multivariate ANOVA tests followed by either Scheffe post hoc test or Dunnet post hoc test. Values of P<0.05 were considered as significant.

**Results**

**Effect of Rimonabant on Atherosclerosis in LDLR−/− Mice Fed a Western-Type Diet as Compared to Pair-Fed Mice**

As rimonabant would be expected to reduce food intake, a first experiment was designed to allow discrimination between effects of rimonabant resulting from a reduction of food intake and other effects of rimonabant. Therefore, 4 different groups of LDLR−/− mice were used: one group of mice was fed a standard diet (3% fat, no cholesterol), one group was fed a Western diet (0.21% cholesterol and 20% fat) ad libitum, a third group was fed a Western diet containing rimonabant (0.45 g/kg of food), and finally one group was fed Western diet but with access to food restricted to the mean amount of food consumed by the rimonabant-fed group of mice (pair fed group).

As shown in Figure 1A, during a 3 month period, the weight of LDLR-KO mice fed a Western diet increased significantly more than the weight of the standard diet group, even though the Western diet group ate less food than the standard diet group (Figure 1B). Actually, when food intake was expressed on energy (J) and not on a weight (g) basis, the daily calorie uptake was slightly higher for mice fed Western diet as compared to chow diet (65.9±0.8 versus 63.0±0.7 kJ/d, respectively).

Rimonabant had a strong effect on the body weight of treated mice. Indeed, their weight did not increase over time, and stayed below the weight of the standard diet group. As shown in Figure 1B, the food intake of the rimonabant-treated group was decreased in comparison to the Western diet group. The average food intake of the rimonabant group was 2.8 g per day, giving a calculated dosage of approximately 50 mg/kg. This effect of rimonabant on food intake was expected because it has already been reported in prior studies. Nevertheless, the weight loss in the rimonabant-treated group was more pronounced than in the pair fed group, showing that the effect on weight loss is not only attributable to a restriction in food intake.

LDLR-KO mice maintained on a standard chow diet had a higher level of cholesterol (total or LDL fraction) than control C57BL/6 mice, but their level of triglycerides was close to the value in control C57BL/6 mice (supplemental Table I). The
serum lipid levels were dramatically increased after a 3-month period of Western diet. Surprisingly, the pair-fed group which had a restricted access to food had lipid levels which were comparable to the levels of the Western diet group. Rimonabant (50 mg/kg) reduced lipid levels by 37% for total cholesterol, 50% for LDL cholesterol and 41% for triglycerides (supplemental Table I). On a reduced number of animals, the results on total and LDL cholesterol were confirmed by high-performance liquid chromatography (HPLC) analysis and showed an increase in HDL cholesterol (48%) for the rimonabant treated group (not shown). Whatever the diet, LDLR−/− mice after a 3-month period had normal glycemia and insulinemia. They did not develop any diabetes.

LDLR−/− mice did not develop significant atherosclerotic lesions when fed a standard diet for 3 months, although older animals are known to develop spontaneous lesions under these conditions. Conversely, fatty streak formation in the aorta was dramatically increased after a 3 month period of Western diet (supplemental Figure IA and Figure 2A). Similarly, the atherosclerotic plaque size was dramatically increased in the aortic sinus in the Western diet group compared with the standard diet group (supplemental Figure IC and Figure 2B). More interestingly, the levels of atherosclerotic lesions were identical in the aorta or in the aortic sinus whatever the Western diet group, fed ad libitum or pair-fed to the rimonabant group. A 3-month treatment with rimonabant nearly abolished the atherosclerotic development in the aorta (90% reduction compared with pair-fed group, P<0.001) or in the aortic sinus (70% reduction, P<0.01).

To clarify the mechanism of action of rimonabant, the serum levels of some adipokines and chemokines were also measured. Serum leptin increased after a 3-month period of Western diet from 8.7±1.1 ng/mL in the standard diet group to 45.8±6.5 ng/mL in the Western diet group fed ad libitum (supplemental Table II). In the group with a restricted access to food (pair-fed group) leptin levels were lower at 16.8±2.5 ng/mL. In the group treated with rimonabant, circulating leptin levels were dramatically reduced reaching a value of 0.53±0.05 ng/mL, much lower than the value obtained in the standard diet group.

In contrast, adiponectin levels were identical in untreated animals, whatever the composition of the diet or the food intake (supplemental Table II). Interestingly, in the rimonabant-treated group, the levels of adiponectin were significantly increased in comparison to the pair fed group, from 6.9±0.5 μg/mL to 8.1±0.5 μg/mL (P<0.05).

To assess the inflammatory state of the animals, MCP1, IL12p40/p70, and vascular cell adhesion molecule (VCAM)-1 levels were also determined. For these 3 proteins, the levels increased during the 3 month-period of Western diet, showing that this diet was able to induce an inflammatory state. The restriction of food intake in the pair-fed group had no influence on any of the 3 markers. Conversely, rimonabant treated animals had significantly reduced MCP-1, IL12p40/p70, and VCAM-1 levels (supplemental Table II).

Dose-Effect of Rimonabant on Atherosclerosis in LDLR−/− Mice Fed a Western-Type Diet

Considering the impressive effects of rimonabant, a second study was designed to test lower doses of rimonabant. Two groups of animals treated with different doses of Rimonabant (average dose of 10 mg/kg and 30 mg/kg in the diet) were compared to a Western diet group.

Although doses were lower, rimonabant again induced a decrease in body weight as compared to the untreated animals: final weights at the end of the 3-month treatment were reduced by 15% (P<0.001) and 20% (P<0.001) in the
10 mg/kg– and 30 mg/kg–treated animals, respectively (supplemental Table III). Again, triglyceride levels were also decreased; they were respectively 27% (ns) and 49% (P\textless 0.05) lower in the 10 mg/kg group and 30 mg/kg–treated groups as compared to the control group (supplemental Table III). In this study, triglyceride levels in the control group were higher than in the previous study because blood was withdrawn without fasting the animals.

Interestingly, total cholesterol and LDL cholesterol levels were not significantly different between the control group and the rimonabant 10 mg/kg–treated group. In the rimonabant 30 mg/kg–treated group, total cholesterol was decreased by 4% (ns) and LDL cholesterol by 14% (ns, supplemental Table III). As in the previous study, no changes in glycemia or insulinemia were observed between the groups.

In this study, the fatty streak lesions in the thoracic aorta covered a surface of 1.1 ± 0.37 mm², which is comparable to the area obtained for the untreated Western diet–fed group of the first study (1.60 ± 0.13 mm²; Figure 2C). A 67% to 68% reduction of lesion size was observed in atherosclerosis development for both the 10 mg/kg– and 30 mg/kg–treated groups as compared to the control group (supplemental Table III). As in the previous study, no changes in glycaemia or insulinemia were observed between the groups.

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Effect of Rimonabant on Inflammatory Gene Expression in Mouse Peritoneal Macrophages

Taking into consideration the effects of rimonabant on inflammatory marker levels in vivo, we also determined whether rimonabant is able to reduce inflammatory markers in macrophages, one of the main cell types involved in atherosclerosis–related inflammation. As expected, LPS (Figure 3A) and IL1\beta (Figure 3B) induced a strong increase in IL6, TNF\alpha, and MCP1 expression in mouse peritoneal macrophages. This effect was identical in macrophages from male and female animals (relative IL6, TNF\alpha, and MCP1 levels were 4860 ± 840 versus 4670 ± 1500, 98 ± 37 versus 78 ± 17, and 430 ± 84 versus 401 ± 41 in male versus female animals, respectively). Rimonabant (0.3 to 1 μmol/L) significantly reduced LPS as well as IL1\beta–induced IL6 and TNF\alpha mRNA expression, but did not significantly affect MCP1 mRNA levels in these cells. Similar effects were also observed in RAW 264.7 murine macrophages (supplemental Figure II). To determine whether these effects of rimonabant are attributable to CB1 receptor blockade, the same experiments were performed in parallel in peritoneal macrophages from CB1-receptor wild type (CB1\textsubscript{R/−/−}) and CB1-receptor deficient (CB1\textsubscript{R−/−}) mice. As shown in supplemental Figure III, rimonabant (1 μmol/L) reduced LPS–induced IL6 and TNF\alpha mRNA expression to a similar extent in CB1\textsubscript{R−/−} and CB1\textsubscript{R−/−} mice, suggesting that this effect of rimonabant was not related to CB1-receptor antagonism.
Effect of Rimonabant on Macrophage Recruitment in a Mouse Model of Thioglycollate-Induced Peritonitis

Resistance to thioglycollate injection induced macrophage recruitment into the peritoneal space has been correlated with atherosclerosis resistance in different mouse strains. Although the effect of thioglycollate was not different in CB1R-/- and CB1R-/- mice (Figure 4), rimonabant (10 mg/kg, po bolus) significantly reduced thioglycollate-induced macrophage recruitment in CB1R-/- mice (37% reduction, \( P < 0.01 \)), but was inactive in CB1R-/- mice.

Discussion

The beneficial effects of rimonabant on weight, serum HDL/LDL cholesterol ratio, triglyceride levels, and serum inflammatory markers in clinical studies suggest that this compound might reduce atherosclerotic lesion development in obese patients. Western diet–fed LDLR-/- mice barely develop lesions on normal diet, but gain weight and develop extensive lesions on a high-fat Western-type diet, and would therefore be expected to at least partially mimic the patient population used in the clinical studies of rimonabant.

Interestingly, a 3-month treatment of LDLR-/- mice with rimonabant (10 to 50 mg/kg/d) incorporated into Western diet resulted in a dose-dependent inhibition of atherosclerosis development in the aorta and aortic sinus, together with a decrease in body weight, which was even stronger than the effects reported in previous studies in high-fat fed mice. However, the lesion size in pair-fed animals did not differ significantly from the lesion size in Western diet–fed untreated animals, demonstrating that the antiatherosclerotic effect of rimonabant was not related to inhibition of food intake.

Surprisingly, the relationship between atherosclerosis reduction and reduction of serum cholesterol and inflammation markers appeared to be nonlinear. Actually, serum triglycerides and leptin levels were already decreased at low doses (30 and 10 mg/kg/d, respectively), whereas serum cholesterol, IL12, and MCP-1 were only reduced, and adiponectin was only increased, at the highest dose of rimonabant (50 mg/kg/d).
This clearly suggests a complex mechanism of action for the antiatherosclerotic effect of rimonabant, with some mechanisms, like decreased leptin and perhaps triglyceride lowering, being present at low doses of rimonabant and others, like serum cholesterol changes and adiponectin, additionally playing a role at higher doses. However, the serum cholesterol decrease at a high dose of rimonabant was much stronger than the decrease observed in humans,\textsuperscript{13} suggesting that the importance of this effect might be exacerbated in mice as compared to patients.

Weight loss was already present at the lowest dose of rimonabant and must therefore be taken into consideration as one of the potential determinants of the antiatherosclerotic effect of rimonabant. Actually, although the link between obesity and cardiovascular disease is still unclear, emerging evidence indicates that factors produced by adipose tissue may directly influence vascular disease. Leptin, one of these factors, is produced primarily by adipocytes, and leptin levels are correlated to body mass index in rodents and humans.\textsuperscript{24}

Recently, an association between elevated leptin levels and cardiovascular events has been demonstrated in patients.\textsuperscript{25} Exogenous leptin has been shown to increase atherosclerosis in apoE\textsuperscript{−/−} mice,\textsuperscript{26} and the adipokine has been shown to modulate the development of atherosclerosis in LDLR\textsuperscript{−/−} mice through the immune response.\textsuperscript{27} As rimonabant at 30 mg/kg/d normalized leptin levels of Western diet–fed mice to the levels found in mice on normal chow diet, the effect of rimonabant on leptin levels could clearly contribute to the antiatherosclerotic effect of the lower doses of the compound. Interestingly, the reductions in leptin levels induced by rimonabant were more pronounced than expected from the weight reduction alone, especially at the higher dose. As inflammatory cytokines are known to induce leptin expression,\textsuperscript{28} and rimonabant at the highest dose reduced inflammatory cytokine levels, one possible mechanism for this additional effect of rimonabant could be related to a decrease of leptin expression after inhibition of inflammation in the animals.

Low doses of rimonabant have actually been shown to exert antiinflammatory effects in several different inflammation-related animal models,\textsuperscript{26–18} and this also suggested the idea to check whether the antiatherosclerotic effects of rimonabant could be consistent with an antiinflammatory effect at lower doses of the compound. The lower dose of rimonabant, which remained antiatherosclerotic (30 mg/kg/d), reduced circulating MCP1 levels by more than 20%, but this effect did not reach statistical significance. However, MCP1 levels in atherosclerotic animals were quite fluctuating, and we therefore used a direct induction of the stronger inflammation induced by LPS to study the potential antiinflammatory effects of rimonabant. The in vitro effects of rimonabant on LPS or IL1β-induced expression of IL6 and TNFα, but not MCP1, suggest that rimonabant, without being an outright antiinflammatory compound, might modulate inflammation through the expression of some proinflammatory factors. In this respect, TNFα is particularly interesting, because local loss of TNF receptor-1 in the arterial wall has recently been shown to be sufficient to decrease atherosclerosis progression,\textsuperscript{29} and the antiatherosclerotic effect of systemic TNFα inhibition is now well established.\textsuperscript{30,31} In addition to its proinflammatory effects on cells in the vascular wall, TNFα might also induce a proatherogenic serum lipid profile,\textsuperscript{32} suggesting that the inhibition of TNFα production might play a role in the antiatherosclerotic effects of rimonabant. The fact that the effect of rimonabant on IL6 and TNFα expression persisted in macrophages from CB1R\textsuperscript{−/−} mice clearly demonstrates that this effect is unrelated to CB1 receptor blockade, in contrast to the effect of rimonabant on LPS-induced TNFα induction in vivo, but in a similar way to the effect of rimonabant on indomethacin-induced intestinal ulcers.\textsuperscript{17} These data therefore strengthen the case for a modulation of inflammatory pathways by rimonabant, although the exact biochemical mechanism and in vivo relevance of this effect remain to be determined.

To determine whether these antiinflammatory effects of rimonabant could translate to in vivo effects at a similar dose, the effect of rimonabant on thioglycollate-induced macrophage recruitment was also assessed in normal and CB1 receptor–deficient mice. Interestingly, whereas rimonabant was able to significantly decrease macrophage recruitment, this effect was absent in CB1R\textsuperscript{−/−} mice, showing that this effect is related to CB1 receptor blockade by rimonabant. The level of thioglycollate-induced macrophage recruitment is correlated with atherosclerosis resistance in different mouse strains,\textsuperscript{22} and the inhibitory effects of rimonabant therefore suggest that part of the effects of rimonabant might be related to inhibition of macrophage recruitment to atherosclerotic lesions.

Up to now, rimonabant is approved in Europe, but not in the United States, because of the FDA’s concerns about potential neuropsychiatric side effects. Recently, the data from the first clinical trial (STRAVARIUS) designed to determine the antiatherosclerotic effect of rimonabant in patients by intravascular ultrasound (IVUS), became available.\textsuperscript{33} Although the effect of rimonabant on the primary end point (percent atheroma volume) did not reach statistical significance during the 18-month treatment period, rimonabant significantly decreased total atheroma volume, a secondary end point in this study. These first clinical data suggest that rimonabant indeed holds promise in the treatment of atherosclerosis, although longer treatment durations may be necessary to show this effect conclusively. The results from the ongoing CRESCENDO trial (5-year duration of treatment, NCT00263042), which directly measures cardiovascular outcomes, should clarify this point.\textsuperscript{34}

In summary, in LDLR\textsuperscript{−/−} mice fed a Western-type diet, rimonabant shows dose-dependent antiatherosclerotic effects which are absent in pair-fed animals. Whereas high doses increase the HDL/LDL cholesterol ratio and nearly abolish atherosclerotic lesion development, lower doses significantly decrease atherosclerosis without any effect on cholesterol levels but decrease circulating leptin levels. Rimonabant decreases the LPS-induced expression of IL6 and TNFα levels in mouse macrophages in vitro and thioglycollate-induced macrophage recruitment in vivo. It can therefore be concluded that rimonabant shows potent antiatherosclerotic effects in LDLR\textsuperscript{−/−} mice on Western diet and that these effects are not solely attributable to its effects on cholesterol.
levels, but probably related to a decrease of inflammation by rimonabant.

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F.D., V.V., A.M.M., P.S., and F.B. are employees of Sanofi-Aventis and hold significant stock of the company. R.J., P.D., N.H., A.G., and B.S. declare no competing interests beyond funding of their experiments through an unrestricted grant from Sanofi-Aventis.

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Expanded Materials and Methods

Mice and Diets:

Male LDLR deficient mice (LDLR⁻/⁻) on a C57BL/6 background were obtained from Charles River Company at the age of 3 months. Western diet (0.21% cholesterol; 20% fat) with or without rimonabant was from Research Diets (New Brunswick, NJ, USA). The dose of rimonabant of 50 mg/kg/day in the diet results in a circulating rimonabant exposure (as quantified by the area under the curve of rimonabant concentrations during 24 hours) which is roughly equivalent to the exposure observed at the dose of 10 mg/kg/day administered by oral gavage in previous studies on rimonabant-induced weight loss in mice (data not shown). Standard chow diet was from S.A.F.E. (Augy, France) and contained 3% fat and no added cholesterol (composition: grain 83.9%, vegetables (soybean, yeast) 8%, fish 4%, vitamins and minerals 4.1%). Mice were weighed every week and food ingestion was measured every day in order to restrict the food intake of the pair fed group. C57BL/6J mice, CB1-receptor wild type littermates (CB1R⁺/⁺) and CB1-receptor deficient (CB1R⁻/⁻) mice on the C57BL/6 background were bred by Sanofi-Aventis. 8-14 week-old female mice were used for ex-vivo isolation of peritoneal macrophages or the peritonitis model of macrophage recruitment. All procedures and care of animals were approved by the Animal Care and Use Committee of sanofi-aventis R & D.

Determination of atherosclerosis:

Atherosclerotic lesion development and progression were evaluated after 3 months of treatment. After blood sampling, the mice were perfused with PBS via a cannula placed in the left ventricle of the heart. The heart and entire aorta were excised, the aorta was removed from the heart, dissected free of adherent fat tissue, and cut into 2 parts at the level of the diaphragm. The
resulting thoracic and abdominal aorta segments were then opened longitudinally, pinned onto paraffin and stained with Sudan Black B: vessel segments were washed with distilled water, bathed with ethylene glycol/absolute ethanol (v/v) for 5 minutes and stained with Sudan Black B (MERCK Diagnostica) for 10 minutes. After a further wash with ethylene glycol/absolute ethanol (v/v), followed by distilled water, the stained vessels were mounted with aqueous mounting medium (FLUOPREP, BioMerieux).

After staining, aortas were photographed under a microscope. The intimal surface occupied by atherosclerotic lesions in the thoracic or abdominal regions was measured morphometrically (Morpho Expert, Explora Nova, La Rochelle, France). Atherosclerosis was also quantified in aortic root cross sections as described previously.¹

**Determination of serum cholesterol, lipoprotein fraction, and triglycerides:**

Blood was collected in Microtainer SST (BD) for 2 hours before being centrifuged at 6000 g during 10 minutes. Serum was removed and immediately frozen at -20°C. Serum cholesterol, LDL and triglycerides levels were measured by enzymatic methods using a clinical chemistry analyzer (PENTRA 400, HORIBA_ABX) and enzymatic kits commercialized by the same manufacturer and specific to the lipids assayed: Cholesterol (ABX Pentra Cholesterol CP), LDL (ABX Pentra LDL Direct CP) and Triglycerides (ABX Pentra TG CP).

**Determination of adipokines and cytokines in serum:**

Different levels of leptin, adiponectin, MCP1 and VCAM-1 were determined by ELISA kits, following the manufacturer's instruction (R&D Systems, Quantikine Elisa kits). Serum levels of IL12 p40/p70 were determined using a Multiplex Bead Immunoassays commercialized by Biosource using LUMINEX 100 technology.
**RNA analysis:**

RNA extraction was performed using TRIzol reagent and reverse transcription was performed according to the manufacturer’s protocol (Invitrogen Life technologies, Cergy-Pontoise, France). RNA levels were measured by quantitative PCR using brilliant SYBR Green QPCR Master Mix on the MX4000 detection system (Stratagene). The amplifying primers were: murine TNFα (FOR, 5’-ATCCAGTTTGGTGCAGGAGC-3’ and REV, 5’-CGTCTGTCGAAATGGGCATC-3’), murine IL-6 (FOR, 5’-CCAGTTGCCTTCTTGGGACTG-3’ and REV, 5’-CAGGTCTGTTGGAGATGGTATCC-3’), murine MCP1 (FOR, 5’-GCAACTCTCAGTGAAGCC-3’ and REV, 5’-GCTGGTGAATGAGTAGCAGC-3’), and as control, murine GAPDH (FOR 5’-TGATGACATCAAGAAGGTGGTGAAG-3’ and REV, 5’-TCCTTGGAGCCATGTGGGCCAT-3’).

Crossing threshold (Ct) values were determined for target genes and normalised to the Ct of GAPDH using the following equation: relative values = \(2^{-\left(\text{Ct target gene - Ct GAPDH}\right)}\). Results are expressed as means +/- SD \((n=3)\) relative to the level of controls or LPS-treated controls. All experiments were repeated at least 3 times.

**Peritoneal macrophage isolation and cell culture:**

Peritoneal macrophages were obtained by peritoneal lavage with PBS 3 days after intraperitoneal injection of 1 ml 4% thioglycollate (Sigma Aldrich, St Quentin, France). Cells were then washed twice with serum-free RPMI (GIBCO BRL), plated and allowed to adhere to dishes for 2 hours. Plates were then washed 3 times with RPMI to remove nonadherent cells and incubated overnight in RPMI containing fetal calf serum (FCS, 10%). Then, cells were incubated in RPMI containing 0.5% FCS for 24 hours before treatment. Cells were treated with the indicated reagents, E. Coli 0111B4 LPS (Sigma Aldrich, St Quentin, France), IL1β (TEBU, France), and rimonabant (sanofi-aventis, France).
Peritonitis macrophage recruitment model:

Female CB1R\(^{+/+}\) and CB1R\(^{-/-}\) mice on the C57Bl/6 background between 8 and 14 weeks of age were used (12-14/group). Mice were randomized according to their body weight. Peritonitis was induced by a single intraperitoneal injection of 1 ml 4% thioglycollate. Rimonabant (10 mg/kg) or vehicle (CMC 0.5%) were given by oral administration to mice on day 0: 1 hours before thioglycollate injection and every day during 3 days. At day 3 (4 gavages), the mice were sacrificed by cervical dislocation and peritoneal lavage fluid (5 ml of PBS) was collected. The macrophages were counted using a haemocytometer. Viability, as determined by trypan blue exclusion, was consistently greater than 95%. Macrophage purity, as determined by Wright's-stained cytospin preparations, was greater than 90-95%.

References

**Expanded Figure Legends**

**Fig. 1.** Effect of rimonabant on body weight (A) and food intake (B) in LDLR−/− mice. Body weight (A) was measured weekly and food intake (B) was measured daily in LDLR−/− mice fed either standard chow diet (▼), Western type diet (○), or Western type diet containing rimonabant (50 mg/kg/d, ●) or pair fed Western type diet restricted to the amount eaten by the rimonabant-treated animals (∇). Data are expressed as mean±SEM (n=10-17).

**Fig. 2.** Effect of rimonabant on atherosclerotic lesion size in the aorta (top) and aortic sinus (B).

(Top): Effect of rimonabant (50 mg/kg/d) as compared to pair-feeding on the atherosclerotic lesion size in the aorta (A) and aortic sinus (B) of LDLR−/− mice.

(Bottom): Effect of increasing doses of rimonabant (10 mg/kg/d and 30 mg/kg/d) on the atherosclerotic lesion size in the aorta (C) and aortic sinus (D) of LDLR−/− mice. Data are expressed as mean±SEM. Lesion size in the aorta (A, C) as well as the aortic sinus (B, D) was determined by image analysis.

**Fig. 3.** Effect of rimonabant on inflammatory gene expression induced by LPS and IL-1β in peritoneal macrophages.

Peritoneal macrophages were pre-treated with rimonabant at indicated concentrations for 2 hours followed by 3 hours of LPS (100 ng/ml) (panel A) or IL-1β (10 ng/ml) (panel B). Cells were then harvested for IL-6, TNFα and MCP1 mRNA analysis by Q-PCR.
Fig. 4. Effect of rimonabant on thioglycollate-induced macrophage recruitment in CB1R^{+/+} and CB1R^{-/-} mice.

CB1R^{+/+} and CB1R^{-/-} mice were pre-treated with rimonabant (10 mg/kg) or vehicle (methylcellulose) for 1 hour and injected by thioglycollate 4%. Mice were then treated with rimonabant or vehicle once a day during 3 days. Mice were sacrificed and peritoneal lavage fluid was recovered and macrophage number was determined using a Mallassez cell. Results were represented as mean±SEM (n=18) mice. (**) p<0.01, Mann and Whitney test).
## Online Tables

### Online Table I. Effect of rimonabant and pair-feeding on serum lipid, glucose and insulin levels

<table>
<thead>
<tr>
<th></th>
<th>C57Bl/6</th>
<th>Standard Diet</th>
<th>Western Diet (WD)</th>
<th>WD-Pair Fed</th>
<th>WD rimonabant (50 mg/kg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>triglycerides</strong> g/l</td>
<td>0.69 ± 0.06</td>
<td>0.60 ±0.12***</td>
<td>1.43 ± 0.22</td>
<td>1.96 ± 0.25</td>
<td>0.85 ± 0.11**</td>
</tr>
<tr>
<td><strong>total cholesterol</strong> g/l</td>
<td>0.79 ± 0.02</td>
<td>2.87 ± 0.10***</td>
<td>8.39 ± 0.54</td>
<td>9.43 ± 0.63</td>
<td>5.32 ± 0.18***</td>
</tr>
<tr>
<td><strong>LDL-cholesterol</strong> g/l</td>
<td>0.039 ± 0.002</td>
<td>0.83 ± 0.06***</td>
<td>2.94 ± 0.23</td>
<td>3.41 ± 0.26</td>
<td>1.46 ± 0.07***</td>
</tr>
<tr>
<td><strong>glucose</strong> g/l</td>
<td>2.97 ± 0.18</td>
<td>2.79 ± 0.22***</td>
<td>1.97 ± 0.15</td>
<td>1.59 ± 0.17</td>
<td>1.76 ± 0.11</td>
</tr>
<tr>
<td><strong>insulin</strong> pg/ml</td>
<td>304 ± 11</td>
<td>299 ± 11</td>
<td>364 ± 31</td>
<td>305 ± 14</td>
<td>264 ± 6*</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM (n=9-17). Blood samples were taken at the end of a 12-week treatment period. Statistical analysis was carried out by analysis of variance (*: p<0.05, **: p<0.01, ***: p<0.001 vs Western-diet pair-fed untreated animals)
Online Table II: Effect of rimonabant and pair-feeding on serum cytokine levels.

<table>
<thead>
<tr>
<th></th>
<th>Control C57Bl/6</th>
<th>Standard Diet WD</th>
<th>Western Diet (WD)</th>
<th>WD-Pair Fed</th>
<th>WD rimonabant (50 mg/kg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin ng/ml</td>
<td>10.39 ± 1.35</td>
<td>8.69 ± 1.11**</td>
<td>45.84 ± 6.52***</td>
<td>16.76 ± 2.53</td>
<td>0.53 ± 0.21***</td>
</tr>
<tr>
<td>adiponectin µg/ml</td>
<td>n.d.</td>
<td>6.78 ± 0.20</td>
<td>6.91 ± 0.21</td>
<td>6.88 ± 0.45</td>
<td>8.10 ± 0.46*</td>
</tr>
<tr>
<td>MCP-1 pg/ml</td>
<td>27.8±5.3</td>
<td>28.1 ± 3.2***</td>
<td>216.3 ± 37.3</td>
<td>236.5 ± 46.1</td>
<td>32.9 ± 7.1***</td>
</tr>
<tr>
<td>IL12p40/p70 pg/ml</td>
<td>n.d.</td>
<td>233 ± 38</td>
<td>244 ± 42</td>
<td>58 ± 8**</td>
<td></td>
</tr>
<tr>
<td>VCAM-1 ng/ml</td>
<td>940 ± 26</td>
<td>728 ± 45**</td>
<td>1063 ± 116</td>
<td>1062 ± 42</td>
<td>806 ± 44*</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM (n=7-10, except IL12 assay where n=4-5, n.d.: not determined). Blood samples were taken at the end of a 12-week treatment period. Statistical analysis was carried out by analysis of variance (*: p<0.05, **: p<0.01, ***: p<0.001 vs Western-diet pair-fed untreated animals)
### Online Table III: Effect of different doses of rimonabant on serum lipid and cytokine levels

<table>
<thead>
<tr>
<th></th>
<th>Western Diet</th>
<th>WD rimonabant 10 mg/kg</th>
<th>WD rimonabant 30 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final Weight †</td>
<td>g</td>
<td>38.0±1.1</td>
<td>30.7±1.5***</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>g/l</td>
<td>8.11±1.25</td>
<td>8.77±0.70</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>g/l</td>
<td>2.83±0.42</td>
<td>2.87±0.25</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>g/l</td>
<td>4.06±0.80</td>
<td>2.96±0.30</td>
</tr>
<tr>
<td>Leptin</td>
<td>ng/ml</td>
<td>34.27±5.3</td>
<td>14.89±3.5*</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>µg/ml</td>
<td>7.87±0.31</td>
<td>7.51±0.49</td>
</tr>
<tr>
<td>MCP1</td>
<td>pg/ml</td>
<td>132±18</td>
<td>170±22</td>
</tr>
</tbody>
</table>

† at the end of the 3 month study

Data are expressed as mean±SEM (n=8-10). Blood samples were taken at the end of a 12-week treatment period. Statistical analysis was carried out by analysis of variance (*: p<0.05, **: p<0.01, ***: p<0.001 vs Western-diet fed untreated animals)
Effect of rimonabrant on atherosclerotic lesions in the thoracic aorta (A, B) and the aortic sinus (C, D). The inhibitory effect of rimonabrant is shown in representative photomicrographs of the Sudan Black B stained thoracic aorta (top) and Masson’s Trichrome stained aortic sinus (bottom) of pair-fed animals (A, C) and rimonabrant (50 mg/kg) treated animals (B, D).
Online Fig. II

Effect of rimonabant on inflammatory gene expression induced by LPS in RAW 264.7 cells.
RAW 264.7 cells were pre-treated with rimonabant at the indicated concentrations for 2 hrs followed by 12 hrs of LPS stimulation (1μg/ml). Cells were then harvested and IL-6, TNFα and MCP1 mRNA analyzed by Q-PCR.
Effect of rimonabant on LPS-induced inflammatory gene expression in CB1R^{+/+} and CB1R^{-/-} peritoneal macrophages.

Peritoneal macrophages were pre-treated with rimonabant (1 μM) for 2 hours followed by 3 hours of LPS (100 ng/ml). Cells were then harvested for IL-6 and TNFα mRNA analysis by Q-PCR.