Blockade of cannabinoid CB1 receptors improves renal function, metabolic profile, and increased survival of obese Zucker rats

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Obesity is a major risk factor in the development of chronic renal failure. Rimonabant, a cannabinoid CB1 receptor antagonist, improves body weight and metabolic disorders; however, its effect on mortality and chronic renal failure associated with obesity is unknown. Obese Zucker rats received either rimonabant or vehicle for 12 months and were compared to a pair-fed but untreated group of obese rats. Mortality in the obese rats was significantly reduced by rimonabant along with a sustained decrease in body weight, transient reduction in food intake, and an increase in plasma adiponectin. This was associated with significant reduction in plasma total cholesterol, low-density lipoprotein cholesterol/high-density lipoprotein cholesterol ratio, triglycerides, glucose, norepinephrine, plasminogen activator inhibitor 1, and preservation of pancreatic weight and β-cell mass index. The cannabinoid antagonist attenuated the increase in proteinuria, urinary *N*-acetylglucosaminidase excretion, plasma creatinine, and urea nitrogen levels while improving creatinine clearance. Renal hypertrophy along with glomerular and tubulointerstitial lesions were reduced by rimonabant. Although the drug did not modify hemodynamics, it normalized the pressor response to angiotensin II. Our study suggests that in a rat model of chronic renal failure due to obesity, rimonabant preserves renal function and increases survival.

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KEYWORDS: rimonabant; endocannabinoids; type II diabetes; renal failure; metabolic risk factors; longevity

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Metabolic disorders and obesity are considered as major risk factors in the development of chronic kidney failure and ultimately end-stage renal diseases.[1] Obesity alone is an important predictor of shortened longevity and is associated with the progression of renal disease.[2] The greater incidence of renal failure in the obese population results from several factors including high glomerular filtration rate, altered renal hemodynamics, adipocyte-derived proinflammatory cytokines, and dyslipidemia.[3–6] For instance, a negative correlation has been established between triglycerides and creatinine clearance, and hypertriglyceridemia increases the risk of developing renal failure.[7] Improvement of lipid profile achieved by 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in dyslipidemic patients with chronic renal failure has been reported to ameliorate creatinine clearance.[8] Moreover, in animal models, hyperlipidemia resulting from either high-fat diet or genetic modification has been documented to promote chronic renal injury.[9–11]

Several reports have indicated that overactivation of the endocannabinoid system contributes to the pathogenesis of obesity and metabolic disorders through the stimulation of the cannabinoid CB1 receptor subtype.[12–15] This system plays a role in the physiological regulation of energy balance, food intake, and lipid and glucose metabolism through the cannabinoid CB1 receptors located both at the central and peripheral levels. Indeed, CB1 receptor-deficient mice display a lean phenotype and are resistant to diet-induced obesity.[16] Moreover, rimonabant, a selective CB1 receptor antagonist, produced a sustained weight loss in obese animals, which was associated with a transient reduction in food intake. Metabolic abnormalities linked with established obesity were corrected by rimonabant, including a near normalization of lipid profile, plasma insulin, and leptin levels.[15] These effects are mediated by the chronic blockade of CB1 receptors, as the antiobesity effect of rimonabant was no longer observed in CB1 knockout mice.[16] In line with these experimental results, rimonabant has demonstrated its efficacy in reducing body weight and cardiometabolic risk factors in obese patients, including significant improvements in waist circumference, high-density lipoprotein (HDL) cholesterol, triglycerides, and insulin resistance.[17–20]

Although the antiobesity effect of rimonabant is well documented, its effect on renal failure and mortality associated with obesity is unknown. Therefore, the specific aim of this study was to determine whether, in addition to improving metabolic function, long-term treatment with rimonabant reduces the development of chronic renal failure, and hence prolongs longevity in obese *fa/fa* Zucker rats. This strain is characterized by hyperphagia, obesity, dyslipidemia, and type II diabetes, resulting from a mutation of the leptin receptor-encoding gene, which impairs feeding behavior. As a consequence, these obese animals are prone to develop chronic renal failure and display a reduced lifespan relative to their lean controls.

RESULTS

Body weight and food intake

At 12 weeks of age, *fa/fa* Zucker rats displayed a greater body weight than age-matched lean *fa/+* Zucker rats (Figure 1a). Body weight for both strains continued to increase and reached a plateau after 6 months of treatment, after which it remained stable for the following 3 months. Chronic administration of rimonabant produced a dose-dependent and sustained decrease in body weight gain, which was statistically significant at 10 mg/kg/day. This antiobesity effect was associated with a transient reduction in food intake which attained a similar value to that measured in the lean *fa/+* controls (Figure 1b). Food intake returned to baseline after 8 weeks of treatment with rimonabant at 10 mg/kg/day. The pair-feeding protocol limited the body weight gain in *fa/fa* Zucker rats in a similar manner. After 9 months of treatment, the general health status of obese *fa/fa* Zucker rats (12-month old) began to deteriorate as they reduced their food intake and hence lost weight. At the end of the study (12-month treatment) *fa/fa* Zucker rats could no longer be considered as hyperphagic, since they exhibited a lower food consumption than *fa/+* Zucker rats. Chronic administration of rimonabant maintained overall food intake until the end of the study period and prevented the secondary body weight loss observed after 12 months in vehicle-treated *fa/fa* Zucker group.

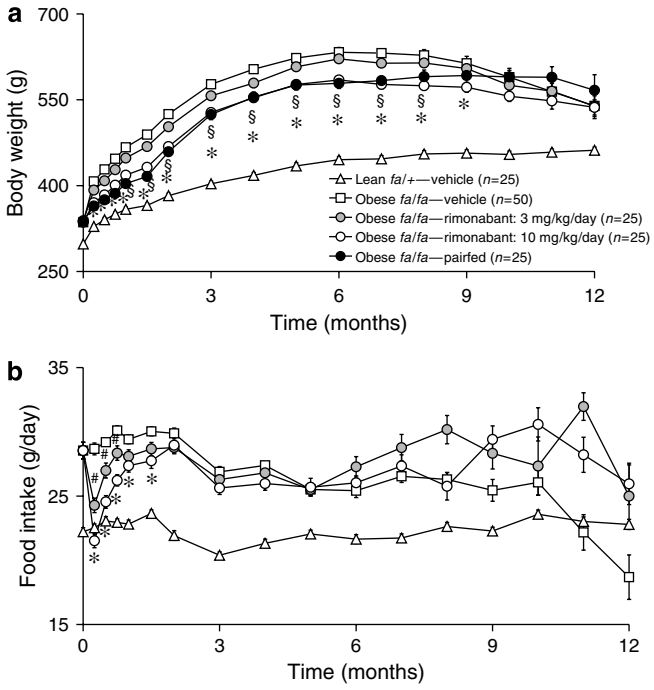

Figure 1 | Effects of rimonabant, 3 and 10 mg/kg/day p.o., and pair-feeding on the (a) body weight (b) and food intake of obese *fa/fa* Zucker rats in comparison with control lean *fa/+* Zucker rats over a 12-month treatment period. Values are expressed as means ± s.e.m. Statistical analysis was performed using two-way ANOVA followed by Newman–Keuls *post hoc* test. Body weight gain was significantly ($P<0.05$) reduced by rimonabant 10 mg/kg/day and pair-feeding from 2 weeks to 9 months and 8 months of treatment, respectively, in comparison with vehicle-treated group. Hyperphagia was suppressed transiently and dose-dependently by rimonabant. This reduction in food intake remained significant ($P<0.05$) up to 2 and 6 weeks after initiating the treatment with rimonabant at 3 and 10 mg/kg/day, respectively. *$P<0.05$ rimonabant 10 mg/kg vs obese *fa/fa*-vehicle; #$P<0.05$ rimonabant 3 mg/kg vs obese *fa/fa*-vehicle; §$P<0.05$ pair-feeding vs obese *fa/fa*-vehicle.

Survival

Nontreated obese *fa/fa* Zucker rats started to die after 9 months of monitoring with a mortality rate of 64% after 1 year, whereas mortality was limited to 4% in age-matched control lean Zucker rats (Figure 2). Rimonabant treatment postponed the onset of mortality significantly in a dose-dependent manner. After 1 year of treatment with rimonabant, the mortality rate was significantly reduced to 28% ($P<0.05$) and 20% ($P<0.05$) at 3 and 10 mg/kg/day, respectively. Although the mortality rate in the pair-feeding group was circumscribed to 33%, it did not reach statistical significance as the onset of mortality occurred earlier, after 5 months of food restriction.

To avoid a bias due to a difference of mortality between groups beyond 9 months of treatment, the effects of rimonabant on the metabolic abnormalities were investigated at 9 months, and those on end-stage renal disease at 12 months.

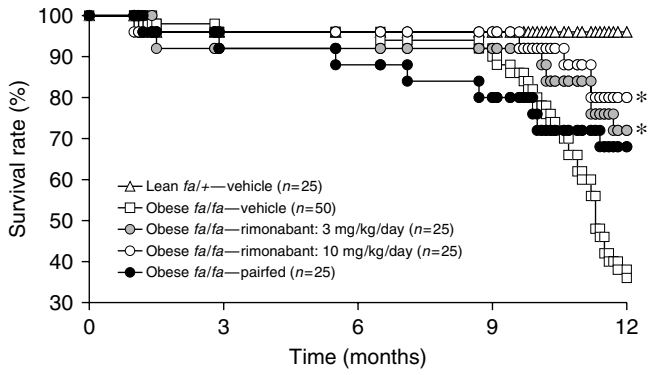

Figure 2 | Effects of rimonabant, 3 and 10 mg/kg/day p.o., and pair-feeding on the survival rate (%) of obese *fa/fa* Zucker rats in comparison with control lean fa/+ Zucker rats over a 12-month treatment period. Statistical analysis was performed with a log-rank test. *$P<0.05$ vs vehicle-treated obese *fa/fa* Zucker rats.

Metabolic disorders

Total plasma cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, and nonesterified fatty acids levels were significantly elevated in obese *fa/fa* Zucker rats in comparison with lean *fa/+* Zucker rats (Figure 3). After 9 months of treatment, rimonabant produced a dose-dependent reduction in these plasma lipid elevations and normalized LDL-cholesterol/HDL-cholesterol ratio. Overall, the pair-feeding protocol was also effective in reducing these plasma lipid abnormalities, but less so than rimonabant at 10 mg/kg/day.

At 9 months, plasma adiponectin levels were significantly lowered in obese *fa/fa* Zucker rats in comparison with the lean controls. Although rimonabant at 10 mg/kg/day slightly increased plasma adiponectin levels, it did not reach statistical significance. However, this increase was found to be significant after 6 months (Figure 4). Similar effects were obtained in the pair-fed group.

Untreated obese *fa/fa* Zucker rats displayed a slight hyperglycemia, which was significantly reduced only by rimonabant at 10 mg/kg/day (Table 1). Fasting plasma insulin and glucagon levels were moderately elevated in obese animals and were not significantly modified by rimonabant. In the pair-feeding group, plasma insulin levels tended to be more elevated and glucagonaemia significantly lowered. The increase in pancreas weight found in obese *fa/fa* Zucker rats at 12 months was prevented by rimonabant 10 mg/kg/day (Table 1). In addition, insulin immunostaining of pancreas cross-sections revealed a significant reduction in β-cell mass index and β-islet number in obese *fa/fa* Zucker rats, which were preserved by rimonabant at 10 mg/kg/day (Table 1 and Figure 5).

Regarding the plasma catecholamines levels, norepinephrine was significantly increased in obese *fa/fa* Zucker rats and was nearly normalized by rimonabant at 3 and 10 mg/kg/day, but not by the pair-feeding protocol (Figure 6). Plasma epinephrine levels did not differ between lean and obese animals, and remained unchanged after treatment (data not shown).

Hemodynamics

At 12 months, the surviving *fa/fa* Zucker-vehicle rats presented, under anesthesia, a slight increase in arterial pressure, which reached significance only for the systolic pressure (Table 2). No differences in heart rate, left ventricular pressure, and dP/dt_{max} were detected in obese

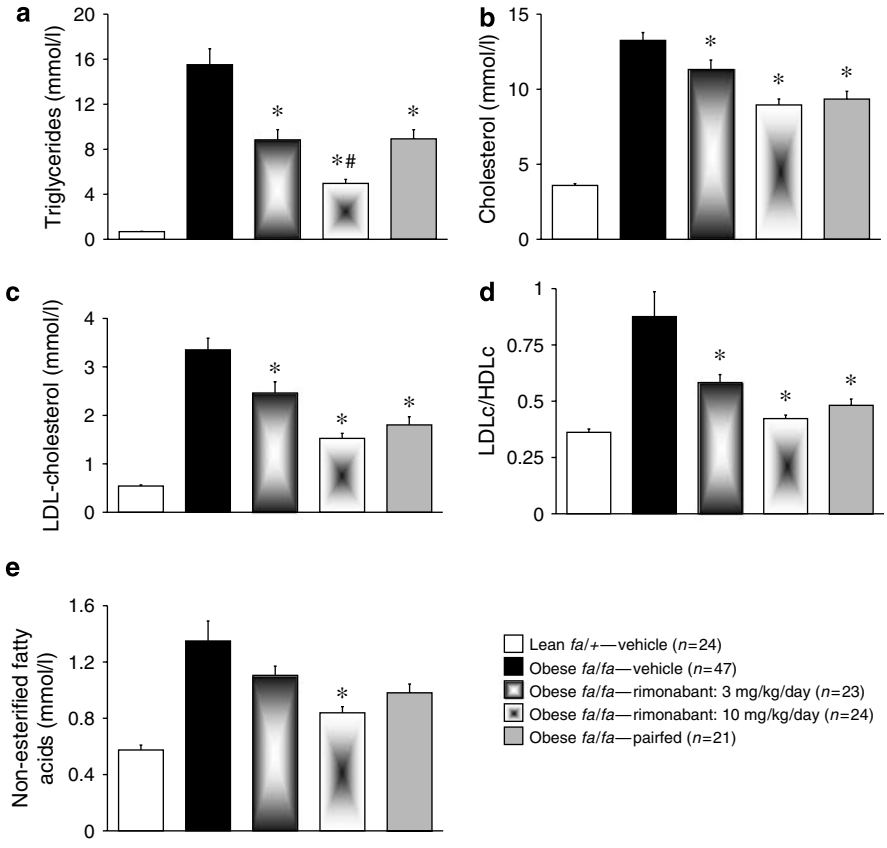

Figure 3 | Effects of rimonabant, 3 and 10 mg/kg/day p.o., and pair-feeding, on (a) plasma triglycerides, (b) cholesterol, (c) LDL-cholesterol, (d) LDL-cholesterol/HDL-cholesterol ratio, (e) non-esterified fatty acids in obese *fa/fa* Zucker rats in comparison with control lean *fa/+* Zucker rats after 9 months of chronic treatment. Values are expressed as means ± s.e.m. Statistical analysis was performed using Kruskal–Wallis test. *$P<0.05$ vs vehicle-treated obese *fa/fa* Zucker rats; no. $P<0.05$ rimonabant 10 mg/kg vs pair-feeding.

fa/fa Zucker rats compared to their lean controls. Hemodynamics was not affected by chronic administration of rimonabant or pair-feeding (Table 2). Intravenous injection of angiotensin II (30 and 100 ng/kg) resulted in a dose-dependent increase in mean arterial pressure, which was enhanced in obese *fa/fa* Zucker rats in comparison with the lean controls. This exaggerated pressor response to angiotensin II was normalized in the 10 mg rimonabant-treated group, but not in the pair-fed group (Figure 7). Cardiac mass expressed as function of body weight was not significantly increased in obese animals, and remained unaltered by the treatments (Table 2). Depressed renal blood flow observed in obese *fa/fa* Zucker rats was not significantly improved by rimonabant or pair-feeding.

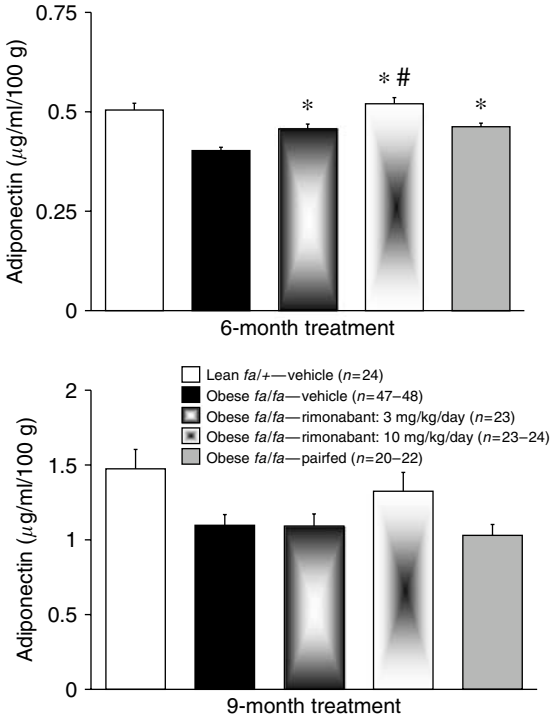

Figure 4 | Effects of rimonabant, 3 and 10 mg/kg/day p.o., and pair-feeding, on plasma adiponectin in obese *fa/fa* Zucker rats in comparison with control lean *fa/+* Zucker rats after 6 and 9 months of chronic treatment. Values are expressed as means ± s.e.m. Statistical analysis was performed using one-way ANOVA followed by a Newman–Keuls *post hoc* test. $^{*}P<0.05$ vs vehicle-treated obese *fa/fa* Zucker rats; $^{\#}P<0.05$ rimonabant 10 mg/kg vs pair-feeding.

Renal function

In comparison with lean *fa/+* Zucker rats, obese *fa/fa* Zucker rats developed a progressive and severe proteinuria with aging together with a significant increase in plasma creatinine and urea levels, and decrease in creatinine clearance (Figures 8 and 9). Chronic administration of rimonabant significantly delayed in a dose-dependent manner the increase in proteinuria (Figure 8). The pair-fed

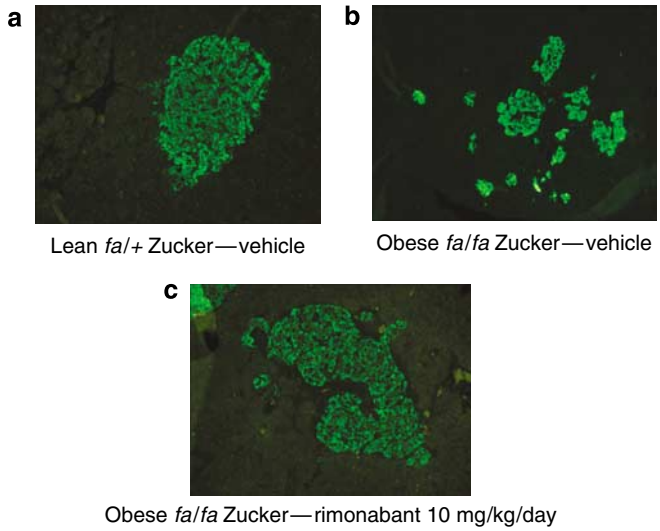

Figure 5 | Representative examples of pancreatic β-islet structure revealed by insulin immunostaining. (**a**) Normal β-islet in lean *fa/+* Zucker rats, (**b**) fragmentation of β-islet structure in obese *fa/fa* Zucker rats, and (**c**) preservation of β-islet integrity in rimonabant-treated (10 mg/kg/day) obese *fa/fa* Zucker rats. Original magnification × 200.

Table 1 | Effects of rimonabant (3 and 10 mg/kg/day) and pair-feeding on fasting plasma glucose, insulin, glucagon, pancreas weight, β-cell mass index, and β-islets number in obese *fa/fa* Zucker rats in comparison with control lean *fa/+* Zucker rats after 9 (for biochemistry) and 12 (for histology) months of chronic treatment

	Lean vehicle	Obese vehicle	Obese rimo 3 mg/kg/day	Obese rimo 10 mg/kg/day	Obese pair-fed
Glucose (mmol/l)	4.39 ± 0.15	10.09$^{\#}$ ± 0.92	7.80 ± 0.46	7.19* ± 0.31	8.27 ± 0.37
Insulin (ng/ml)	0.22 ± 0.03	0.59$^{\#}$ ± 0.07	0.72 ± 0.08	0.57 ± 0.09	0.82 ± 0.11
Glucagon (ng/ml)	0.62 ± 0.03	1.09$^{\#}$ ± 0.08	0.91 ± 0.08	0.91 ± 0.11	0.71* ± 0.06
Pancreas weight (g)	1.52 ± 0.05	2.02$^{\#}$ ± 0.09	1.70* ± 0.06	1.49* ± 0.07	1.71* ± 0.08
β-Cell mass index (FA per TA per PW)[a]	0.22 ± 0.04	0.06$^{\#}$ ± 0.01	ND	0.22* ± 0.05	ND
β-Islets number (IN per TA per PW)[a]	17 ± 3	8$^{\#}$ ± 1	ND	25* ± 8	ND
n	11–24	14–28	14–18	11–20	12–17

FA, fluorescent area; IN, β-Islets number, PW, pancreas weight; TA, tissue area.
$^{*}P<0.05$ vs obese vehicle.
$^{\#}P<0.05$ vs lean vehicle.
Statistical analysis for β-cell mass index and β-islet number were performed by Kruskal–Wallis test. Plasma glucose, insulin and glucagon levels, and pancreas weight were analyzed by one-way analysis of variance followed by a Newman–Keuls *post hoc* test.
[a]FA of insulin positive cells/TA/PW, β-islets number/TA/PW.

group also displayed a reduction in proteinuria but to a lesser extent than the group treated with rimonabant at 10 mg/kg/day. Urinary excretion rate of *N*-acetyl-glucosaminidase, a marker of tubular injury, was significantly increased in obese Zucker rats, and this elevation was markedly reduced by rimonabant treatment (Figure 8). Both long-term treatment with rimonabant and pair-feeding protocol prevented the elevation in plasma creatinine and urea levels, and improved the level of creatinine clearance significantly (Figure 9).

Reverse transcriptase-polymerase chain reaction experiments indicated that CB1 receptor mRNA is expressed at a low level with a C_t of about 33 in the kidney of obese *fa/fa* Zucker rats with no difference in comparison with lean control. This result was confirmed by immunohistochemistry analysis (Figure 10), which revealed a distinct immunoreactivity for CB1 receptors in glomeruli and tubular epithelial cells. In intrarenal arteries, CB1 receptor immunostaining was restricted to the endothelium and was totally absent in medial layers. Again no difference was observed in the pattern and in the intensity of CB1 receptor immunostaining between lean and obese Zucker rats (data not shown).

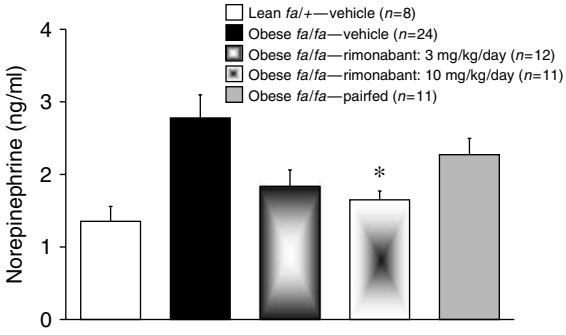

Figure 6 | Effects of rimonabant, 3 and 10 mg/kg/day p.o., and pair-feeding, on plasma norepinephrine in obese *fa/fa* Zucker rats in comparison with control lean *fa/+* Zucker rats after 9 months of chronic treatment. Values are expressed as means ± s.e.m. Statistical analysis was performed using Kruskal–Wallis test. *$P<0.05$ vs vehicle-treated obese *fa/fa* Zucker rats.

Histological analysis of kidney cross-sections revealed severe glomerular and tubulointerstitial lesions including glomerulosclerosis, tubular casts, dilatation, and atrophy, as well as interstitial fibrosis in vehicle-treated obese *fa/fa* Zucker rats in comparison with lean *fa/+* Zucker rats (Figures 11 and 12). Only rimonabant at 10 mg/kg/day decreased significantly the severity of both glomerular and tubulointerstitial lesions and reduced renal hypertrophy (Figure 13). The development of glomerular fibrosis that was detected in obese *fa/fa* Zucker rats was blunted both by rimonabant and pair-feeding (Figure 13). Concordantly, plasma plasminogen activator inhibitor 1 (PAI-1) levels that were significantly elevated in untreated-obese *fa/fa* rats were corrected by rimonabant treatment (Figure 13).

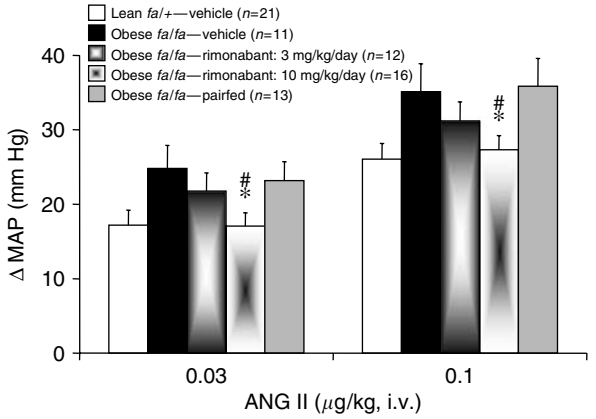

Figure 7 | Effects of rimonabant, 3 and 10 mg/kg/day p.o., and pair-feeding, on pressor response to angiotensin II injected intravenously at 30 and 100 ng/kg in anesthetized obese *fa/fa* Zucker rats in comparison with control lean *fa/+* Zucker rats after 12 months of chronic treatment. Values are expressed as means ± s.e.m. Statistical analysis was performed using two-way ANOVA followed by a Newman–Keuls *post hoc* test. *$P<0.05$ vs vehicle-treated obese *fa/fa* Zucker rats; #$P<0.05$ rimonabant 10 mg/kg vs pair-feeding.

Table 2 | Effects of rimonabant (3 and 10 mg/kg/day) and pair-feeding on hemodynamics in anesthetized obese *fa/fa* Zucker rats in comparison with control lean *fa/+* Zucker rats after 12 months of chronic treatment

	Lean vehicle	Obese vehicle	Obese rimo 3 mg/kg/day	Obese rimo 10 mg/kg/day	Obese pair-fed
MAP (mm Hg)	97 ± 4	110 ± 9	114 ± 8	106 ± 7	114 ± 7
HR (bpm)	374 ± 7	388 ± 15	380 ± 14	396 ± 10	373 ± 12
SAP (mm Hg)	140 ± 5	171 ± 10#	172 ± 7	156 ± 8	169 ± 10
LVP (mm Hg)	142 ± 5	160 ± 11	172 ± 5	163 ± 6	163 ± 10
dP/dt max (mm Hg/s)	11 337 ± 619	11 625 ± 1261	13 555 ± 528	11 831 ± 583	11 247 ± 761
dP/dt min (mm Hg/s)	−7515 ± 323	−6553 ± 301	−8432 ± 333	−7536 ± 597	−7333 ± 711
Cardiac index (ml/min/kg)	313 ± 22	255 ± 28	284 ± 24	231 ± 12	291 ± 29
RBF (ml/min/g)	3.0 ± 0.2	1.3 ± 0.2#	1.3 ± 0.1	1.4 ± 0.2	1.5 ± 0.3
HW/BW (g/100 g)	0.46 ± 0.01	0.52 ± 0.03	0.51 ± 0.03	0.49 ± 0.02	0.47 ± 0.03
n	21–24	11–13	12–15	16–18	13–14

HR, heart rate; HW/BW, heart weight/body weight ratio; LVP, left ventricular pressure; MAP, mean arterial pressure; RBF, renal blood flow; SAP, systolic arterial pressure. Hemodynamics was analyzed by one-way ANOVA followed by a Newman–Keuls *post hoc* test.
#$P<0.05$ vs lean vehicle.

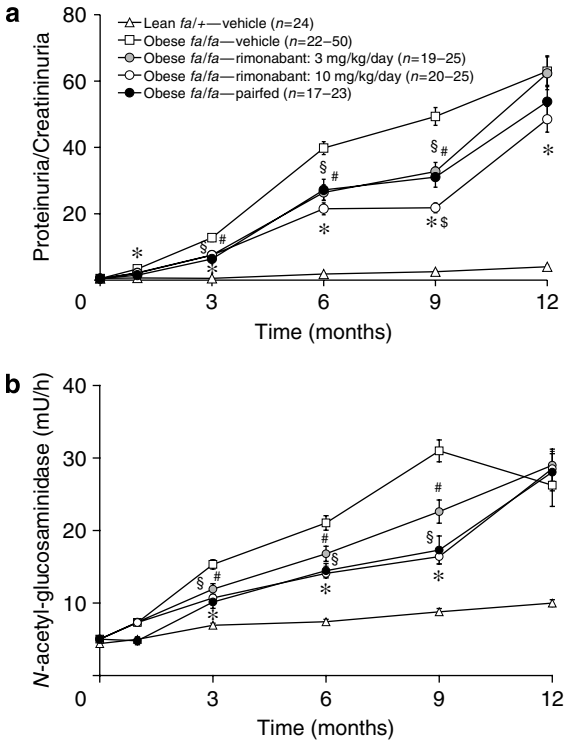

Figure 8 | Effects of rimonabant, 3 and 10 mg/kg/day p.o., and pair-feeding, on the (a) proteinuria/creatininuria ratio (b) *N*-acetyl-glucosaminidase excretion rate in obese *fa/fa* Zucker rats in comparison with control lean *fa/+* Zucker rats after 1, 3, 6, 9, and 12 months of chronic treatment. Values are expressed as means ± s.e.m. Statistical analysis was performed using Kruskal–Wallis test. The reduction in proteinuria/creatininuria ratio produced by rimonabant was significant ($P<0.05$) up to 9 and 12 months of treatment at 3 and 10 mg/kg/day, respectively. Prevention in the elevation of *N*-acetyl-glucosaminidase excretion by rimonabant remained significant up to 9 months for both doses. Pair-feeding was effective up to 9 months on proteinuria/creatininuria ratio and on *N*-acetyl-glucosaminidase excretion rate. $^{*}P<0.05$ rimonabant 10 mg/kg vs obese *fa/fa*-vehicle; $^{\#}P<0.05$ rimonabant 3 mg/kg vs obese *fa/fa*-vehicle; $^{\S}P<0.05$ pair-feeding vs obese *fa/fa*-vehicle; $^{\$}P<0.05$ rimonabant 10 mg/kg vs pair-feeding.

DISCUSSION

Obesity is considered to be a major public health problem as it is recognized to predispose to diabetes, cardiovascular, and end-stage renal disease, and to be associated with a higher mortality rate. Obese patients are prone to glomerulopathy including focal segmental glomerusclerosis and glomerular hypertrophy.[21] In line with these observations, patients with pre-existing renal disease appeared to be vulnerable specially to the harmful consequences of obesity, which could lead to an accelerated loss of renal function.[22] Accordingly, hypocaloric diet-induced body weight loss promoted a significant reduction in proteinuria in obese patients with chronic nephropathies.[23] Several factors may contribute to the pathogenesis of obesity-related renal failure including glomerular hyperfiltration, hyperlipidemia, insulin resistance, adipocyte-derived hormones (i.e. leptin), and systemic inflammation.[23,24]

Recently, both experimental and clinical studies have underlined the critical role of the endocannabinoid system in the development of obesity. Chronic blockade of CB1 receptors by rimonabant in preclinical studies promoted a sustained reduction in body weight along with a significant improvement in the metabolic abnormalities related to obesity.[12,15] However, the long-term benefits of rimonabant on renal function and survival in obese animals remained unknown and are addressed in this study.

As expected, *fa/fa* Zucker rats displayed a marked obesity and hyperphagia in comparison with age-matched *fa/+* Zucker rats. In this strain, as in other genetically obese animals, defective leptin signaling is associated with an increased endocannabinoid tone, which contributes to overeating and obesity.[25] Although rimonabant at 3 mg/kg/day caused a transient reduction in food intake, it did not reduce significantly the body weight gain. The effect of rimonabant on body weight was significant at 10 mg/kg/day with a rapid onset (1 week) after initiating the treatment, and remained sustained until the general status of control *fa/fa* Zucker rats began to deteriorate with body weight loss and reduction in appetite. These results were in agreement with previous reports, indicating that the effect of rimonabant on body weight was coupled with a temporary hypophagia.[12,15] In the vehicle-treated obese group, the secondary decline in body weight and food intake occurred concomitantly with the onset of death. Indeed, obese *fa/fa* Zucker rats started to die rapidly after 9 months of follow-up with a mortality rate of 64% at 12 months in comparison with 4% monitored in the control lean *fa/+* Zucker rats. The shortened longevity of obese *fa/fa* Zucker rats was caused by the development of a severe renal failure, this result being in line with the literature. At 12 months, they exhibited a significant renal hypertrophy, a marked elevation in plasma creatinine and urea levels, and a decrease in creatinine clearance. Proteinuria, a marker of glomerular injury, rose dramatically over the time course of this study. Histological analysis indicated that untreated obese rats displayed glomerular and tubulointerstitial injuries, including glomerular enlargement, glomerulosclerosis, collapsing of the glomerular tuft, and at the tubule level, cast formation, dilatation, atrophy, and inflammatory infiltrates. Accordingly, the urinary excretion rate for *N*-acetyl-glucosaminidase, a marker of tubular injury, was significantly increased in obese *fa/fa* Zucker rats relative to lean littermates. Long-term treatment with rimonabant, at both doses tested, prolonged markedly the life span of *fa/fa* Zucker rats. A beneficial effect on mortality was evident even at 3 mg/kg/day, a dose that did not significantly modify the development of obesity in this strain. This beneficial effect occurred in parallel with a delay in the progression of renal failure. The increase in proteinuria was prevented by rimonabant together with an improved creatinine clearance and a reduction in glomerular injury and renal hypertrophy. In addition, rimonabant limited the severity of tubulointerstitial lesions, a result in line with the reduction in excretion rate for *N*-acetyl-glucosaminidase.

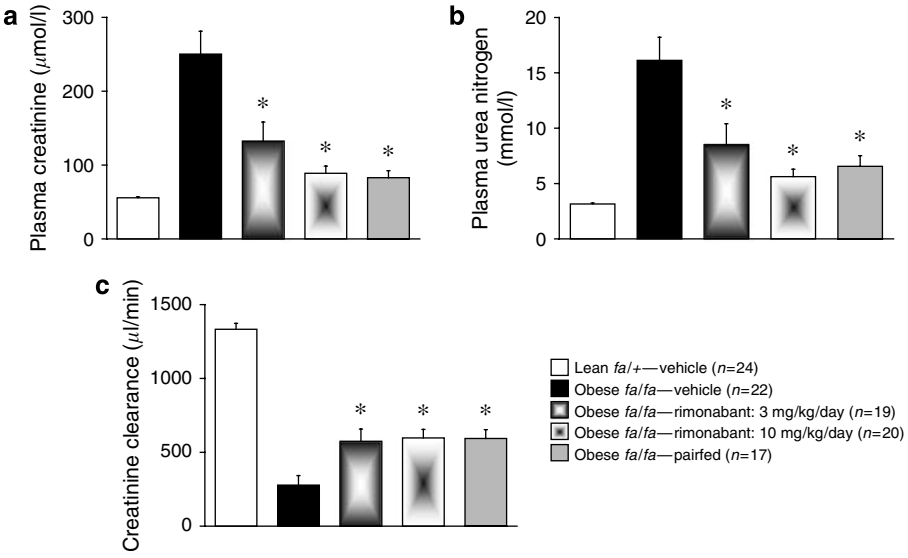

Figure 9 | Effects of rimonabant, 3 and 10 mg/kg/day p.o., and pair-feeding, on the (a) plasma creatinine, (b) plasma urea nitrogen, (c) creatinine clearance in obese *fa/fa* Zucker rats in comparison with control lean *fa/+* Zucker rats after 12 months of chronic treatment. Values are expressed as means ± s.e.m. Statistical analysis was performed using Kruskal–Wallis test. *$P < 0.05$ vs vehicle-treated obese *fa/fa* Zucker rats.

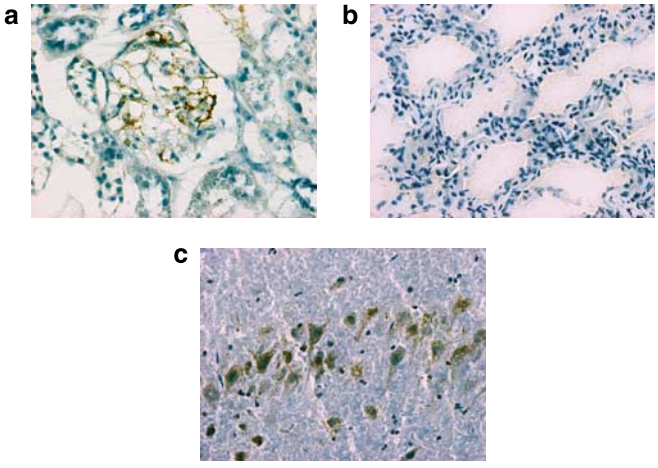

Figure 10 | Representative examples of CB1 receptor immunostaining in obese *fa/fa* Zucker rats. (**a**) Glomerulus showing a positive focal immunostaining for CB1 receptors. (**b**) Tubules of the renal medulla did not display any CB1 receptor immunoreactivity. (**c**) Positive control for CB1 receptor immunostaining found in the brain.

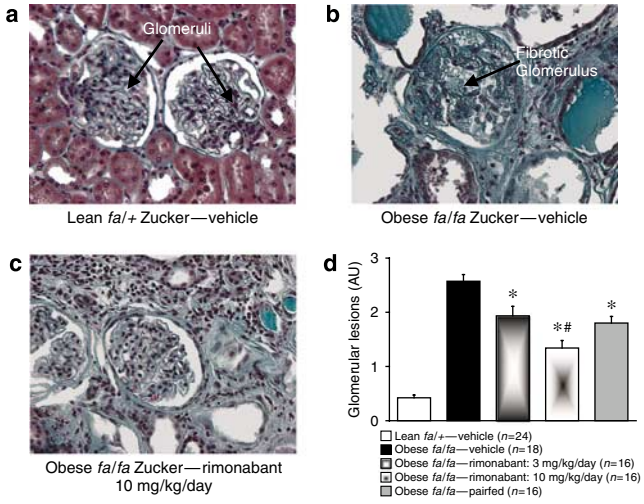

Figure 11 | Representative examples of glomerular structure. (**a**) Normal glomeruli in lean *fa/+* Zucker rats, (**b**) severe diffuse glomerulosclerosis in obese *fa/fa* Zucker rats, and (**c**) less severe glomerulosclerosis with focal pattern in rimonabant-treated (10 mg/kg/d) obese *fa/fa* Zucker rats. Masson's trichrome. Original magnification × 400. (**d**) The effects of rimonabant, 3 and 10 mg/kg/day p.o., and pair-feeding, on glomerular lesions in obese *fa/fa* Zucker rats in comparison with control lean *fa/+* Zucker rats after 12 months of chronic treatment. Values are expressed as means ± s.e.m. Statistical analysis was performed using one-way ANOVA followed by Newman–Keuls *post hoc* test. *$P < 0.05$ vs vehicle-treated obese *fa/fa* Zucker rats; #$P < 0.05$ rimonabant 10 mg/kg vs pair-feeding.

Reverse transcriptase-polymerase chain reaction and immunohistochemistry experiments performed in this study revealed a low expression of renal CB1 receptors, suggesting that a putative overactivation of the endocannabinoid system at the kidney level would result, if any, either in an increase in circulating endocannabinoids and/or in an enhanced receptor signaling in obese Zucker rats. Although the role of renal CB1 receptors is still unknown, we cannot exclude that their activation might contribute to the progression of renal failure in obese Zucker rats.

The mechanism of the renoprotection afforded by rimonabant might be also indirect in origin and could result from the correction of the metabolic disorders. Lipid abnormalities including high plasma cholesterol and triglycerides levels have been considered to contribute to the progression of renal failure in clinical studies. In this respect, statins have been shown to correct hypercholesterolemia and to improve renal function in diabetic patients with

dyslipidemia.[26,27] Dyslipidemia has also been reported to elicit a deterioration of the renal function in several animal models including B6.ROP Os/+ mice, a model with reduced renal mass,[10] obese *fa/fa* Zucker rats,[11,28] and apolipoprotein E knockout mice.[9] At the glomerular level, mesangial cells bind and take up native and oxidized LDL, triggering cellular proliferation, cytokine production, and matrix deposition.[29–31] As reported previously in diet-induced obese mice,[15] long-term treatment with rimonabant improved markedly the lipid disorders in obese *fa/fa* Zucker rats with a near normalization of the lipid profile, and in particular total correction of the LDL/HDL ratio. Similarly, in overweight or obese patients, chronic blockade of CB1 receptors by rimonabant combined with a hypocaloric diet produced a sustained decrease in body weight, waist circumference, and plasma triglycerides levels, together with an increase in HDL-cholesterol levels and a shift toward less atherogenic LDL particles.[17,18] Therefore, the beneficial effects of rimonabant on lipid metabolism may participate in the preservation of the renal function and hence the prolonged life span observed in obese *fa/fa* Zucker rats.

In addition, since chronic blockade of angiotensin AT1 receptors have been shown to correct metabolic disorders, to prevent the development of renal failure, and consequently to prolong survival of obese *fa/fa* Zucker rats,[32,33] we investigated whether long-term treatment with rimonabant could modify angiotensin II-mediated responses in this strain. Interestingly, we found that the pressor response to exogenous angiotensin II was enhanced in obese animals relative to lean controls, and was normalized by rimonabant treatment. This increased pressor response to angiotensin II has been previously documented in the obese Zucker rats.[34,35] The nature of the cross-talk between the CB1 receptor and the AT1 receptor is so far unclear, but could also be possibly indirect in origin. For example, expression of vascular AT1 receptors increased in parallel with LDL levels in hypercholesteromic patients.[36] Therefore, one might speculate that rimonabant, by correcting hypercholesterol-

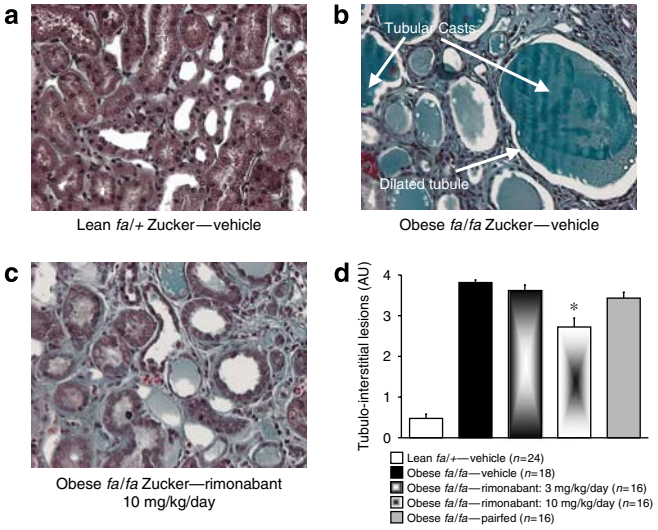

Figure 12 | Representative examples of tubulointerstitial structure. (**a**) Normal tubules and absence of fibrosis and inflammation in the interstitium in lean *fa/+* Zucker rats, (**b**) tubular lesions characterized by tubular dilatation and casts (arrows) associated with interstitial fibrosis and inflammation in obese *fa/fa* Zucker rats, and (**c**) less intense tubulointerstitial lesions in rimonabant-treated (10 mg/kg/day) obese *fa/fa* Zucker rats. Masson's trichrome. Original magnification ×400. (**d**) The effects of rimonabant, 3 and 10 mg/kg/day p.o., and pair-feeding, on tubulointerstitial lesions in obese *fa/fa* Zucker rats in comparison with control lean *fa/+* Zucker rats after 12 months of chronic treatment. Values are expressed as means±s.e.m. Statistical analysis was performed using Kruskal–Wallis test. *$P<0.05$ vs vehicle-treated obese *fa/fa* Zucker rats.

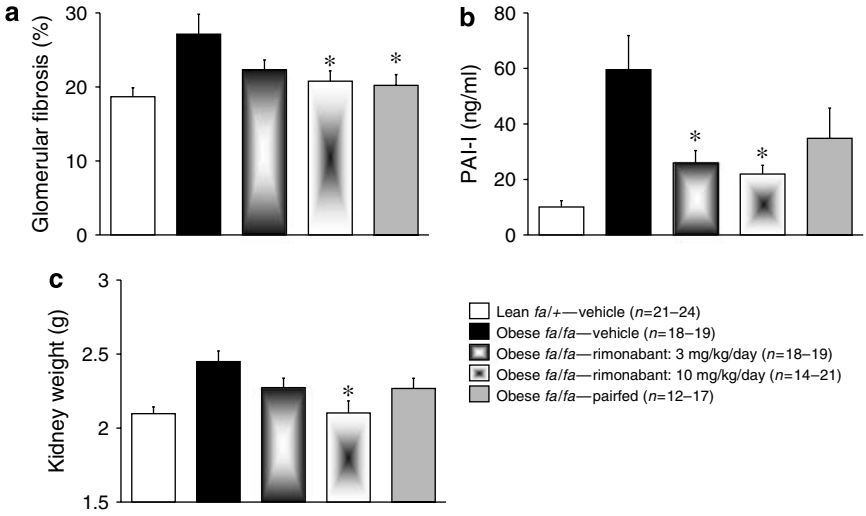

Figure 13 | Effects of rimonabant, 3 and 10 mg/kg/day p.o., and pair-feeding, on the (a) glomerular fibrosis (%), (b) plasma PAI-I levels, (c) kidney weight in obese *fa/fa* Zucker rats in comparison with control lean *fa/+* Zucker rats after 12 months of chronic treatment. Values are expressed as means±s.e.m. Statistical analysis for glomerular fibrosis and plasma PAI-1 levels was performed by Kruskal–Wallis test. Kidney weight was analyzed by one-way ANOVA followed by a Newman–Keuls *post hoc* test. *$P<0.05$ vs vehicle-treated obese *fa/fa* Zucker rats.

emia, could reduce vascular expression of AT1 receptors and hence the pressor response to angiotensin II.

The renoprotection provided by rimonabant is unlikely to be related to hemodynamic changes as neither renal blood flow, arterial pressure, nor cardiac hemodynamics were modified by a 12-month treatment with rimonabant. In agreement with previous reports,[37–39] obese Zucker rats displayed a moderate systolic hypertension and an elevated sympathetic nervous activity as illustrated by a significant increase in plasma norepinephrine levels relative to lean controls. Unlike pair-feeding, rimonabant was very effective in correcting this elevation in circulating norepinephrine levels. This increase in catecholamines was probably compensatory in nature and might occur in response to a decrease fatty acid oxidation capacity, which deteriorates with aging and contributes to triglyceride accumulation in skeletal muscle and liver of Zucker rats.[40,41] Restoration of fatty acid oxidation and reduction of fatty acid synthesis by rimonabant[42,43] could potentially be responsible for the normalization of circulating norepinephrine levels observed in this study.

PAI-1 is a key player in the fibrogenic process leading to glomerulosclerosis, and elevated PAI-1 levels have been documented in patients with obesity, type II diabetes, or hypertension, and correlated with albuminuria.[44,45] Genetically obese rodents (*ob/ob* mice, *fa/fa* Zucker rats) displayed elevated plasma PAI-1 levels associated with high tissue expression in adipose tissue, vessels, heart, liver, and kidney.[32,46,47] PAI-1 inhibits fibrinolysis, but also plasmin-mediated matrix metalloproteinase activation, leading to extracellular matrix accumulation and fibrosis. Upregulation of PAI-1 synthesis has been reported in human diabetic nephropathy and focal segmental glomerulosclerosis (for review see Rerolle *et al.*[48]). Oxidized LDL induced PAI-1 promoter activity through autocrine activation of TGF-β signaling in human mesangial cells.[49] Elevation in plasma PAI-1 levels has been shown to be correlated with the degree of liver steatosis and adiposity.[47] Increased plasma-free fatty acid levels could possibly increase circulating PAI-1 in obese *fa/fa* Zucker rats since a free fatty acid response element has been identified and localized in the PAI-1 promoter.[50] Thus, chronic hyperlipidemia could promote the progressive increase in plasma PAI-1 levels, hence contributing, at least in part, to the development of renal failure. Therefore, the mechanism by which rimonabant lowered plasma PAI-1 levels might be indirect and could result from the correction of dyslipidemia (decrease in LDL-cholesterol and free fatty acids) and from the reduction in fat mass, as weight loss in obese patients leads to a notable decrease in circulating PAI-1.[51] This effect on plasma PAI-1 levels fitted with the reduction in glomerular fibrosis observed in the rimonabant-treated group.

The reduction in dyslipidemia produced by rimonabant treatment might be ascribed to induction of lipolysis in adipose tissue in parallel with an increased energy expenditure.[42] CB1 receptors are expressed in adipocytes and regulate directly the plasma levels of adiponectin,[14] an adipocyte-derived hormone that promotes fatty acid β-oxidation, and lipoprotein lipase activity.[52] Plasma adiponectin levels are depressed in obesity and have been reported to be increased by rimonabant, both in experimental and clinical settings.[14,15,18] In this study, although rimonabant produced a dose-dependent increase in plasma adiponectin concentrations at 6 months, this effect did not reach statistical significance after 9 months of treatment. Interestingly, we found that plasma adiponectin levels doubled between 6 and 9 months in untreated obese *fa/fa* Zucker rats, which could be related to the progression of chronic renal failure. Indeed, plasma adiponectin levels are elevated in patients with chronic renal disease due to a reduction in glomerular filtration rate.[53] This elevation, which coincided with a deterioration of the renal function and hence the onset of mortality, could be potentially responsible for the attenuation of rimonabant-induced adiponectin synthesis.

Besides adipose tissue, it is probable that rimonabant also targets the liver to ameliorate lipid abnormalities in obese animals. CB1 receptors are expressed by hepatocytes and upon activation they promote fatty acid synthesis through the induction of the expression of the lipogenic transcription factor SREBP-1c and its downstream target enzymes, acetyl coenzyme-A carboxylase-1 and fatty acid synthase.[43] Moreover, high-fat diet not only upregulated CB1 receptors in the liver, but increased the hepatic levels of the endocannabinoid anandamide due to a reduction in activity of fatty acid amidohydrolase, the enzyme responsible for its catabolism.[43]

At 13 months of age, glycemia and hyperinsulinemia were moderately elevated in obese Zucker rats. These results were in agreement with previous reports in old Zucker rats,[33,54] although considerable variability in glucose homeostasis has been indicated for this animal model depending on the origin of the strain, the age, the regimen, and the fasting conditions. In contrast with experimental and clinical studies demonstrating the beneficial effects of rimonabant treatment on insulin resistance,[12,15,17] we did not observe a reduction in fasting plasma insulin levels by rimonabant (3 or 10 mg/kg/day) or pair-feeding in old obese Zucker rats. This lack of efficacy could be potentially attributed to a hyperinsulinemia, which was too mild to be improved in this experimental setting or to an inadequate time frame of measurement. Indeed, fasting insulin levels are classically reported to be increased by at least a 10-fold factor in young adult Zucker rats.[55,56] Both CB1 and CB2 receptors are expressed in the islets of Langerhans.[57] CB1 receptors are present mainly in glucagon-secreting α-cells, whereas CB2 receptors are localized in both α- and β-cells. According to Juan-Pico *et al.*,[57] activation of CB2 receptors and, to a less extent, CB1 receptors led to a decrease in insulin secretion. Nonetheless, rimonabant treatment was very effective in preventing the increase in pancreas weight and preserving the β-cell mass index. To our knowledge, this is the first report of a beneficial effect of chronic rimonabant treatment on endocrine pancreatic structure. The decrease in β-cell mass index

results from an alteration in the replication rate and/or the death rate of β-cells. Since elevated free fatty acids have been shown to promote β-cell apoptosis in obese Zucker rats,[58,59] reduction in plasma free fatty acids levels by rimonabant might contribute indirectly to its positive effects on pancreas and β-cell mass index. Recently, a protective effect of CB1 antagonists on pancreas has been described by Matsuda *et al.*[60] using a model of pancreatitis.

The comparative analysis shown in Table 3 summarizes the overall biological responses observed in the different experimental groups. Table 3 underlines that the beneficial effects of rimonabant were dose-dependent and that the 10 mg/kg/day dose improved all key parameters. Furthermore, rimonabant 10 mg/kg/day was overall superior to the pair-feeding protocol. This global analysis indicates that the sustained beneficial effects of rimonabant do not solely rely on its effects on food intake. In comparison with the pair-fed group, rimonabant 10 mg/kg/day shows greater effects on survival rate, plasma triglycerides, nonesterified fatty acids, adiponectin, glucose, PAI-1 and norepinephrine levels, the exacerbated response to angiotensin II, proteinuria, renal lesions, and hypertrophy. Our results, obtained after 12 months of treatment, contrast with the interpretation of short-term favorable effects (4–14 days) of rimonabant, which have been attributed principally to a decrease in food intake.[61] However, it is certainly possible that rimonabant-induced changes in food intake and body weight are significant factors in our study, and it would be interesting to evaluate whether rimonabant could be beneficial in renal failure unrelated to obesity.

In conclusion, we demonstrate that long-term treatment with the CB1 receptor antagonist, rimonabant, in a rat model of chronic renal failure related to metabolic abnormalities, preserves renal function, reduces renal lesions, lowers body weight, corrects lipid profile, and, ultimately, increases survival.

MATERIALS AND METHODS

Animals

Male 12-week-old obese *fa/fa* Zucker rats and lean *fa/+* Zucker rats were purchased from Charles River Laboratories (St Germain sur l'Arbresle, France). All animals were housed individually, with food and water freely available, in an air-conditioned room ($22 \pm 2^{\circ}$C) and maintained under a 12-h light/dark cycle (light on 0700 hours). Animals were fed *ad libitum* a standard laboratory chow (Dietex M20, Saint Gracien, France). This study was performed in accordance with the European Community Standards on the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Sanofi-Aventis Research & Development.

Experimental protocol

Twelve-week-old male obese *fa/fa* Zucker rats were treated with either rimonabant (3 and 10 mg/kg/day per os (p.o.), $n = 25$/group) or its vehicle ($n = 50$) for 12 months, and compared with a pair-fed group ($n = 25$) receiving the same daily food intake as the group treated with rimonabant at 10 mg/kg/day. Rimonabant was incorporated with rodent diet (Dietex M20). Lean *fa/+* Zucker rats treated with the vehicle ($n = 25$) were included as a control to evaluate the progression of the pathological state in obese *fa/fa* Zucker rats. Body weight, food intake, and survival rate were monitored weekly in each group.

Table 3 | Comparative analysis summarizing the effects of rimonabant at 3 and 10 mg/kg/day vs pair-feeding on obesity, survival rate, metabolic, and renal parameters in obese *fa/fa* Zucker rats

Parameters	Rimonabant 3 mg/kg/day	Rimonabant 10 mg/kg/day	Pair-fed
Body weight reduction	No effect	+	+
Survival	+	+	No effect
Triglycerides lowering effect	+	+,#	+
Cholesterol lowering effect	+	+	+
LDL-cholesterol lowering effect	+	+	+
LDL-cholesterol/HDL-cholesterol lowering effect	+	+	+
NEFA lowering effect	No effect	+	No effect
Adiponectin 6 month increase	+	+,#	+
Glucose lowering effect	No effect	+	No effect
Pancreas weight reduction	+	+	+
Norepinephrine lowering effect	No effect	+	No effect
ANG II ΔMAP reduction	No effect	+,#	No effect
Proteinuria reduction	+	+,#	+
Urinary NAG reduction	+	+	+
Plasma creatinine reduction	+	+	+
Plasma urea reduction	+	+	+
Creatinine clearance improvement	+	+	+
Glomerular lesions reduction	+	+,#	+
Tubular lesions reduction	No effect	+	No effect
Glomerular fibrosis reduction	No effect	+	+
Plasma PAI-1 reduction	+	+	No effect
Kidney weight reduction	No effect	+	No effect

ANG II, angiotensin II; LDL, low-density lipoprotein; MAP, mean arterial pressure; NAG, N-acetyl-glucosaminidase; NEFA; nonesterified fatty acids; PAI-1, plasminogen activator inhibitor 1. No effect, no statistical significant effect; +statistical significant effect ($P < 0.05$) vs obese vehicle; #statistical significant effect ($P < 0.05$) vs obese pair-fed.

Metabolic profile

After 9 months of chronic treatment, blood samples were collected under fasted conditions (24 h) for biochemical analysis. Blood collection was performed under isoflurane anesthesia on EDTA, and plasma were separated by centrifugation (3000 *g* for 10 min at 4°C) and stored frozen (−20°C) until further processed. Plasma samples were analyzed for glucose, total cholesterol, LDL, HDL, triglycerides using a Cobas Mira Biochemical Analyzer (Roche, Basel, Switzerland), insulin (ELISA, Crystal Chem Inc., Chicago, IL, USA), nonesterified fatty acids (colorimetric assay, WAKO Chemicals, Neuss, Germany), glucagon (ELISA, WAKO Chemicals), adiponectin (ELISA, B-Bridge International Inc., Mountain View, CA, USA), and catecholamines (HPLC, C_{18} column, extraction kit and mobile phase from Chromsystems, München, Germany).

Renal function

Proteinuria was assessed every 3 months from urine samples taken under fasted conditions (24 h). For this purpose, each animal was placed individually in a metabolic cage for a 24-h urine collection. After total urinary volume had been measured, the urine was aliquoted, frozen (−20°C) and stored until processed for the determination of proteins levels (Biomérieux, Marcy L'Etoile, France). In addition, at the end of the 12-month treatment period, creatinine and urea nitrogen levels were quantified in both plasma and urinary samples (ABX Diagnostics kits, Montpellier, France), and creatinine clearance calculated. Urinary *N*-acetyl-glucosaminidase level (colorimetric assay; Roche), a marker of tubular injury, and plasma PAI-1 (ELISA, Immuclone; American Diagnostic Inc., Stamford, CT, USA) were also measured.

Hemodynamics

At the end of the treatment period, rats were anesthetized (isoflurane 1.5%), and the right carotid artery and the left femoral vein were catheterized for arterial pressure measurement and drug administration, respectively. Hemodynamic recording was performed using a data acquisition system (IOX software, EMKA Technologies, Paris, France). To determine whether AT_1 receptor activity was altered by the chronic blockade of CB1 receptors, the pressor response to intravenous bolus injection of angiotensin II (30 and 100 ng/kg) was measured in the different groups.

Then the carotid catheter was advanced into the left ventricle to monitor left ventricular pressure and end-diastolic left ventricular pressure, and to calculate heart rate, maximal and minimal first derivatives of developed pressure (dP/dt_{max} and dP/dt_{min}). To measure regional blood flow and cardiac index, an additional catheter was inserted into the left femoral artery. Animals were administered a bolus injection of 400 000 fluorescent microspheres (20×10^6/ml, 15 μm; Invitrogen, Cergy Ponroise, France) into the left ventricle, followed by heparinized saline infusion at 2 ml/min during 15 s. Simultaneously, femoral arterial blood withdrawal was started at 1 ml/min during 45 s.

Animals were then euthanized and heart, kidneys, and pancreas were rinsed by intra-arterial infusion of saline before being weighed, frozen for microspheres processing, or fixed until further histological analysis. Microspheres were extracted by filtration after potassium hydroxide digestion, dissolved in 2-ethoxyethyl acetate, and fluorescence intensity was measured by spectrofluorimetry (Safire II, Tecan, France). Renal blood flow, cardiac output, and cardiac index were calculated according to Richer *et al.*[62]

Histology

Renal histology and molecular biology. Kidneys were longitudinally sectioned through the hilum and fixed in Bouin's solution. Paraffin-embedded renal tissue sections were stained with Masson's trichrome for semi-quantitative analysis. In each kidney, the vessels, the glomeruli, the tubules, and the interstitium were systematically analyzed. The following parameters were assessed: (1) in the glomeruli—glomerulosclerosis, glomerular tuft collapsing, and podocyte hyperplasia or droplet accumulation; (2) in the tubules—casts, dilatation, and atrophy; (3) in the interstitium—fibrosis and inflammatory infiltrates. A scoring system was used as described previously.[63,64] Briefly, a score 0 was given to a normal glomerulus, a score 1 to any lesions involving less than 1/4 of the tuft, a score 2 for less than 1/2, a score 3 for less than 3/4, and a score 4 for more than 3/4. In each rat, 100 glomeruli were scored at original magnification ×400. The glomerular lesion index was calculated as follows: ((0 × the number of normal glomeruli) + (1 × the number of score 1 glomeruli) + (2 × the number of score 2 glomeruli) + (3 × the number of score 3 glomeruli) + (4 × the number of score 4 glomeruli))/100. For the cortical tubulointerstitial component, a score 0 was given to a normal microscopic field, a score 1 for any lesions involving less than 25% of the microscopic field, a score 2 for less than 50%, a score 3 for less than 75%, and score 4 for more than 75%. For each rat, 20 nonadjacent cortical microscopic fields were assessed at original magnification ×100. The tubulointerstitial lesion index was calculated as above. Glomerular fibrosis was determined after picrosirius red staining by quantitative analysis (MorphoExpert, Explora Nova, France) on 50 glomeruli.

Total RNA was isolated from kidney samples of obese and lean Zucker rats using RNeasy kit (Qiagen, Hilden, Germany). Purity was assessed with a capillary electrophoresis Caliper Laboratory Chip system (Agilent 2001 Bioanalyzer; Agilent Technologies, Waldbronn, Germany). Quantitative duplex real-time polymerase chain reaction was performed using QuantiTect Probe reverse transcriptase-polymerase chain reaction 1-Step kit (Quiagen) and TaqMan Gene Expression Assays (Applied Biosystems, Dormstadt, Germany) for CNR1 and AGTR1 (both FAM/MGB probes) and for GAPDH (VIC/MGB probe) as a control. Each sample was assessed in triplicate. For relative quantification, the $\Delta\Delta C_T$ method was applied.[65]

Immunohistochemistry for CB1 receptor was performed on renal frozen 5 μm cross-sections from both obese *fa/fa* Zucker rats and lean *fa/+* Zucker controls using avidin–biotin peroxidase method. Sections were incubated with a rabbit anti-mouse/rat CB1 receptor primary antibody (no. IMG-71170, Imgenex, San Diego, CA, USA) diluted at 1/100, and then with biotinylated donkey anti-rabbit IgG secondary antibody (1/200) (no. 711-065-152, Jackson, Bar Harbor, Maine, USA). Following incubation with avidin–peroxidase complex, immunostaining was revealed with a diaminobenzidine and peroxide solution (no. 760124, DAB-MAP kit, Ventana, Illkirch, France). The specificity of the immunostaining was evaluated by performing negative control experiments in which the primary antibody was substituted with a nonrelevant antibody. Brain-frozen cross-sections from obese *fa/fa* Zucker rats were used as positive control for CB1 receptor immunostaining.

Pancreas histology. β-Islet morphology was assessed after insulin immunostaining of pancreas 10 μm cross-sections using an anti-mouse monoclonal insulin antibody (1/1000 in 1%BSA–PBS—0.1%Triton, ab6995, Abcam, TEBU, France), and revealed with a goat anti-mouse IgG conjugated with FITC (1/100) as a secondary antibody. The relative cross-sectional area of β-cells was determined

by dividing insulin positive β-cell area by the total pancreas cross-sectional area. The β-cells mass index was expressed as the ratio between the relative cross-sectional area of β-cells and the pancreas weight. Pancreatic β-islet number was determined after Heidenhain's Azan staining.

Statistical analysis

Results are expressed as means $\pm$ s.e.m. Survival rates were compared by means of a log-rank test. On the basis of the normality of the distribution and the homogeneity of variance (Levene test), one-way analysis of variance (ANOVA) or two-way ANOVA was performed using SAS version 8.2 software. This primary analysis was followed by a Newman–Keuls *post hoc* test when appropriate. In case of non-homogeneity of variance, Kruskal–Wallis test was applied. Differences between groups were considered significant if $P < 0.05$.

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