measurement device (Model MK-5000; Muromachikikai, Tokyo, Japan). Each mouse was placed in a sealed chamber (560 ml volume) with an air flow rate of 500 ml/min at room temperature. The amount of oxygen consumed was converted to milliliters per minute by multiplying it with the flow rate.

Statistical analysis

Data were expressed as means ± SE. Differences between two groups were analyzed by Student's *t* test for unpaired comparisons. Individual comparisons among more than two groups were assessed with post-hoc Fisher's PLSD test. Differences were considered significant at $P<0.05$.

Results

Two-fold increase of PPARγ activity in the S112A mouse adipocytes

Transgenic mice with the PPARγ2 S112A mutation expressed under the control of the aP2 promoter were established to investigate the physiological role of increased PPARγ activity in mature adipocytes (Fig. 1A). A 13.5 kb wild-type allele and a 1.2 kb mutant allele in the transgenic mice were identified by Southern blot analysis (Fig. 1B). Eleven founder mice carrying 2 to 10 copies of the transgene were produced and four lines of transgenes carrying 2 copies expressed PPARγ2 in the adipose tissue. While a 3-fold higher PPARγ2 mRNA expression was found in the S112A mouse than in the wild-type mouse adipose tissue, there was no significant difference in the expression level of PPARγ1 (Fig. 1C). The mRNA levels of aP2, stearoyl-CoA desaturase (SCD)1 and C/EBPα, downstream target genes of PPARγ, were significantly upregulated in the WAT of the S112A mice as compared with that in the wild-type mice (Fig. 1D), confirming that enhanced PPARγ activity in the S112A mice.

S112A mice showed comparable WAT mass, insulin sensitivity and serum lipid levels to wild-type mice under the HFD condition

S112A mice showed similar body weight, epididymal WAT mass and food intake to wild-type mice on a normal diet (data not shown). Administration of a HFD for 20 weeks, while inducing a 2-fold increase in the PPARγ2 expression in the WAT of the S112A mice (Fig. 2A), had no significant effect on the body weight, linear growth (Fig. 2B) or epididymal WAT mass (wild-type: 1.58 ± 0.14 g; S112A: 1.78 ± 0.17 g ($n = 7$)) of these mice. Histological analyses revealed that the adipocyte size in the WAT was not significantly different between the wild-type and S112A mice under either the normal or HFD condition (Fig. 2C). No differences in insulin sensitivity or glucose tolerance were found between the wild-type and S112A mice under either the normal (data not shown) or HFD condition (Fig. 2D and E). The serum FFA, TG, adiponectin and leptin levels were comparable between the wild-type and S112A mice mice under both normal and HFD conditions (Fig. 2F–I). Oxygen consumption was also similar between the two genotypes (Fig. 2J).

Gene expressions in the WAT of the wild-type and S112A mice under the HFD condition

We next investigated the expression of the genes involved in lipid metabolism in the WAT of the S112A mice. The expressions of aP2, lipoprotein lipase (LPL), acyl-CoA oxidase (ACO), SCD1 and hormone-sensitive lipase (HSL), whose promoters contain a peroxisome proliferator response element (PPRE), were upregulated in the S112A mice (Fig. 3A and B). The expression levels of CD36 remained unchanged, even though the CD36 promoter also contains a PPRE (Fig. 3A).

Rosiglitazone increased the insulin sensitivity to a similar degree in both the mouse genotypes

We examined the effects of rosiglitazone on the insulin sensitivity and glucose tolerance in the wild-type and S112A mice. The body weights of the wild-type and S112A mice were comparable, and rosiglitazone treatment did not change the body weight of either genotype (Fig. 4A). The adipocyte size was reduced to a similar degree in the wild-type and S112A mice after rosiglitazone treatment (Fig. 4B). Rosiglitazone significantly increased the insulin sensitivity to a similar degree in both the wild-type and S112A mice (Fig. 4C). Moreover, wild-type and S112A mice treated with rosiglitazone showed similar decreases of the blood glucose and insulin levels in the glucose toler-

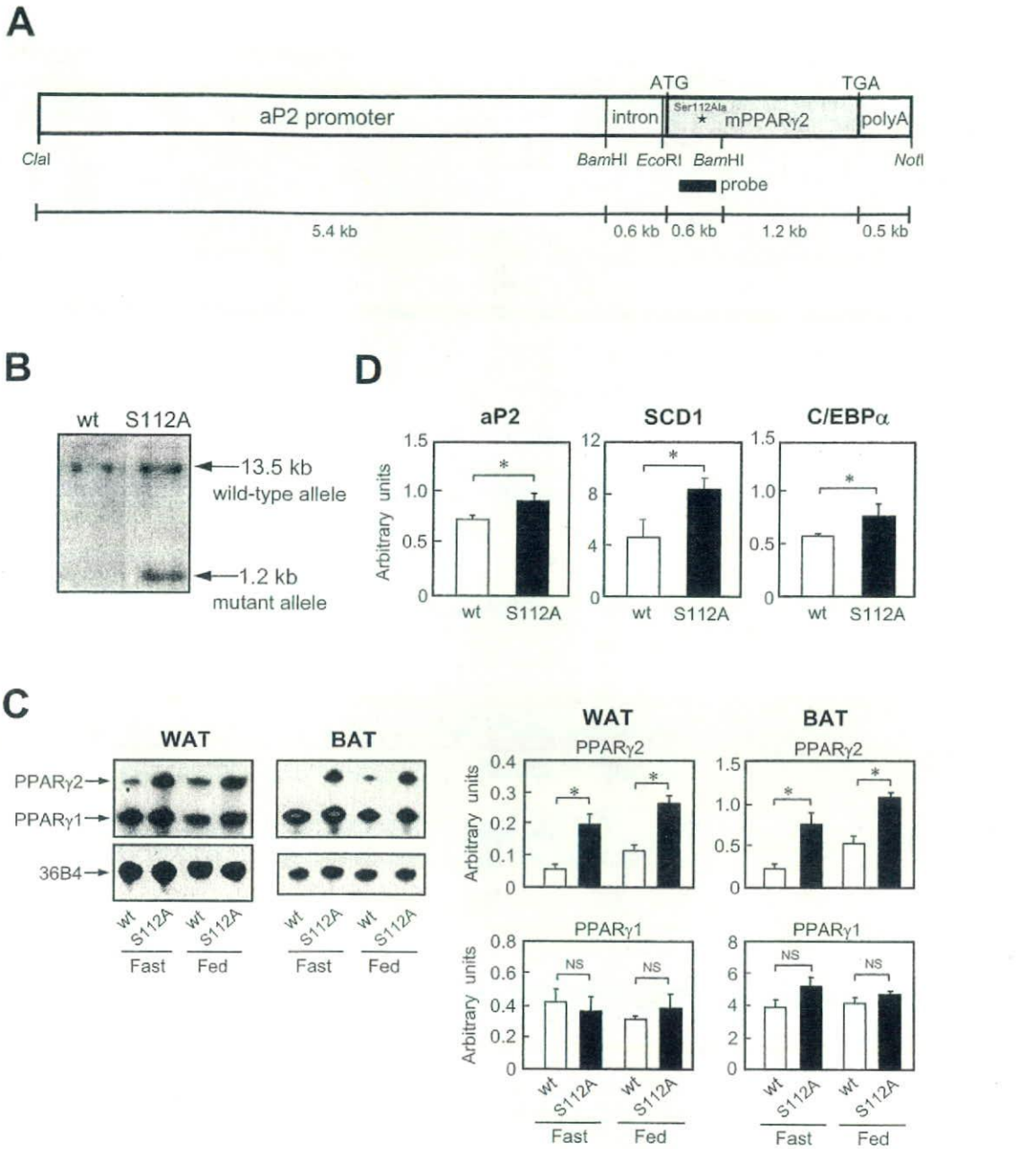

Fig. 1. Generation of transgenic mice expressing PPARγ2 with the S112A mutation. A, Schematic of the transgene and the probe used for Southern blot analysis. B, Southern blot analysis of *Bam*H1-digested mouse genomic DNA from wild-type (wt) and S112A mice hybridized with the probe, a 0.4 kb cDNA fragment. C, Expression of PPARγ mRNAs under the fed and 24 h fasting conditions as determined by RPA. PPARγ1 and PPARγ2 mRNAs are shown as protected bands of 185 and 273 bp, respectively. 36B4 RNA (220 bp protected band) was used as the internal control. D, TaqMan RT-PCR analyses of aP2, SCD1 and C/EBPα mRNAs after 24 h' fasting. Values are expressed as means ± S.E. ($n = 3$) *P<0.05. NS, no significant difference.

ance test (Fig. 4D). Rosiglitazone treatment significantly reduced the serum levels of FFA, but not TG, to a similar degree (Fig. 4E and F) in both the mouse genotypes. The serum adiponectin levels increased (Fig. 4G) and leptin levels decreased to a similar degree in both the mouse genotypes after rosiglitazone treatment (Fig. 4H). These data suggest that rosiglitazone increased the insulin sensitivity to a similar degree in the two genotypes.

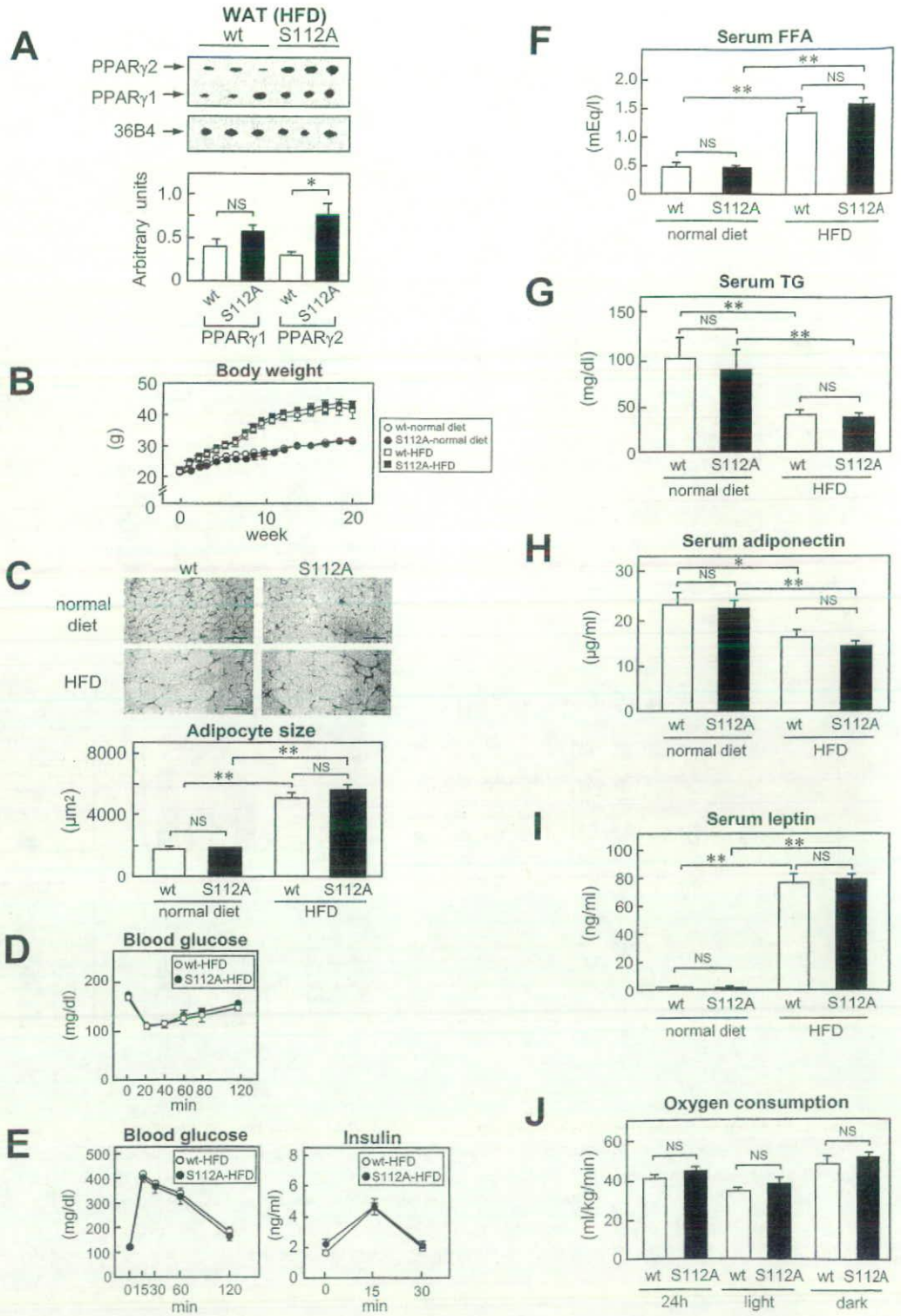

Fig. 2. Comparable body weights, insulin sensitivity and serum lipid levels in both mouse genotypes under the HFD condition. Eight-week-old mice were fed a HFD for 15–20 weeks. A, Expression of PPARγ1 and PPARγ2 mRNAs in the WAT under the fed conditions as determined by RPA ($n = 3$). B, Body weight gain after administration of a HFD for 20 weeks ($n = 50$) and administration of a normal diet ($n = 38$). C, Histology of WAT. Bars indicate 100 μm. D, Insulin tolerance test. E, Glucose tolerance test. F–J: serum FFA (F), TG (G), adiponectin (H), leptin (I) and oxygen consumption levels (J). Values are expressed as means ± S.E. ($n = 3$–6) $^*P<0.05$, $^{**}P<0.01$. NS, no significant difference.

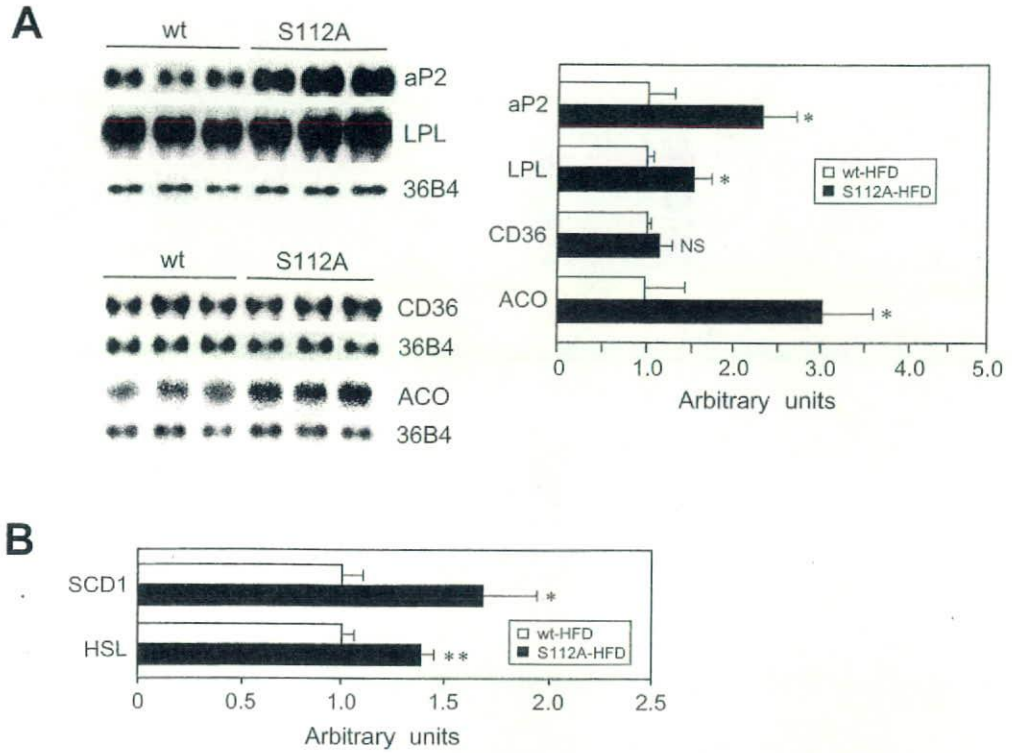

Fig. 3. Gene expressions in the WAT of the wild-type and S112A mice after administration of a HFD for 20 weeks (A, B). Northern blot analysis (A), TaqMan RT-PCR analysis (B). Values are expressed as means ± S.E. ($n = 6$–7) *P<0.05, **P<0.01. NS, no significant difference.

Discussion

S112A mice with enhanced PPARγ activity in mature adipocytes showed comparable insulin sensitivity, glucose tolerance and body weight to wild-type mice, both under normal and HFD conditions. While a 50% reduction of PPARγ activity has been reported to exert protection from HFD-induced obesity and insulin resistance [3, 4], increased PPARγ activity in mature adipocytes had no effects on these parameters.

Whereas S112A knock-in mice of Lazar *et al.* [10] showed comparable body weights to wild-type mice, just like our S112A mice, they exhibited increased insulin sensitivity, unlike our S112A mice. What is the reason for this difference between the S112A mice and S112A knock-in mice? Possibly because the aP2 promoter used to induce the S112A mutation in this study is not activated before adipocyte maturation, the PPARγ S112A mutation is expressed only in the later stages of differentiation. In contrast, PPARγ expression probably occurs earlier during differentiation in the S112A knock-in mice due to the endogenous promoter activity of the PPARγ gene. This may explain, at least in part, the increase in the number of small adipocytes and serum adiponectin levels and thereby, the increased insulin sensitivity, in the knock-in mice [10]. Secondly, since PPARγ knock-in mice exhibit high PPARγ activity throughout the body due to intrinsic PPARγ promoter expression, the phenotype of the knock-in mice may result from increased insulin action in the skeletal muscles and liver. In fact, liver-or muscle-tissue-specific PPARγ-KO mice have been reported to show glucose intolerance and progressive insulin resistance [16, 17]. Our S112A mice showed elevated PPARγ activity only in adipose tissue.

TZD-mediated PPARγ activation increases the small adipocyte number [8], thereby increasing the production of adiponectin, or directly upregulates adiponectin by activating adiponectin gene transcription [8]. Moreover, TZD also increases insulin sensitivity by lowering the plasma FFA [8]. In contrast, S112A mice with increased PPARγ activity showed comparable adipocyte size and serum levels of adiponectin and FFA to those in the wild-type mice. The effects of the

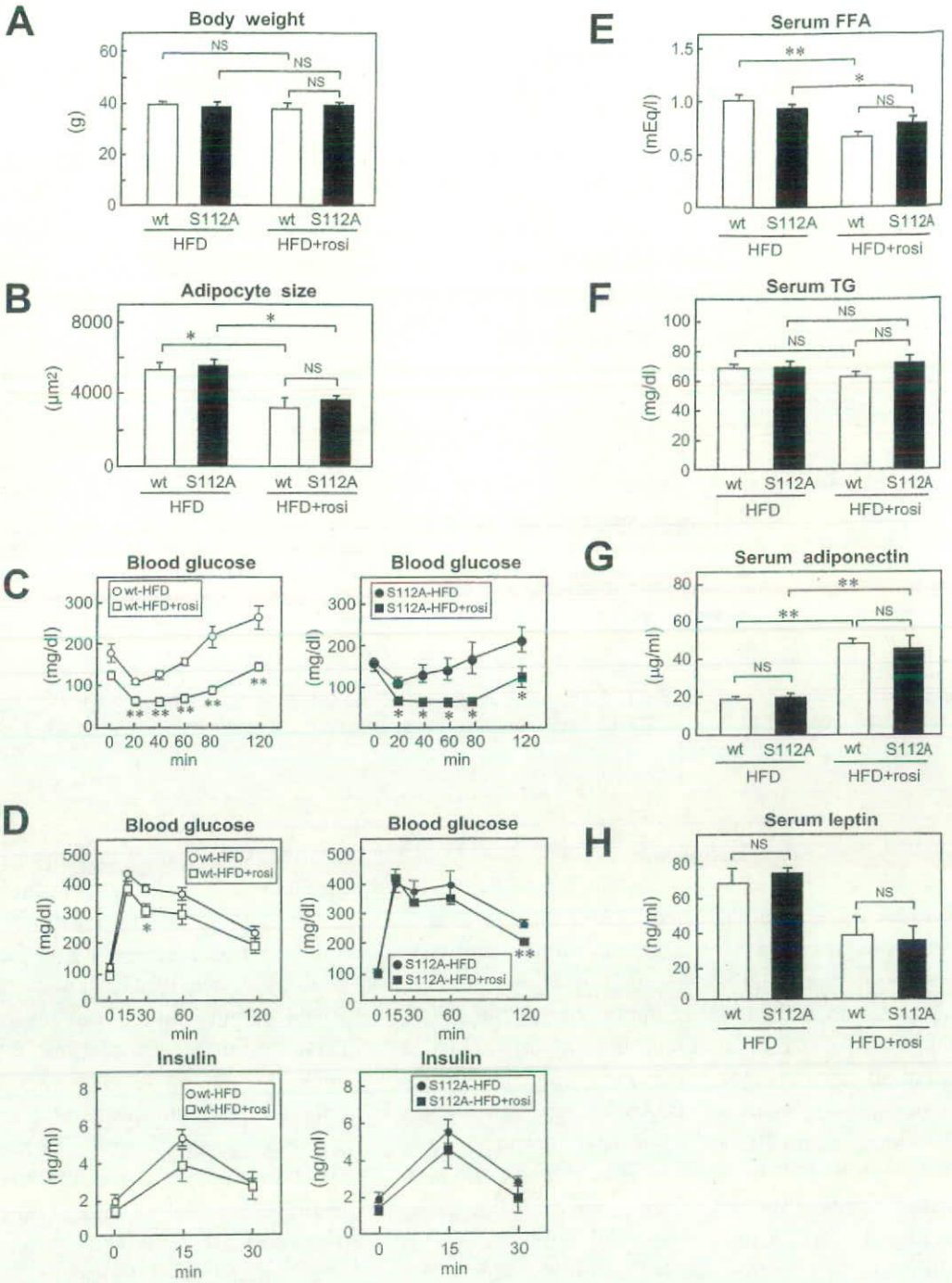

Fig. 4. Rosiglitazone increased insulin sensitivity to a similar degree in both the mouse genotypes. The mice were fed a HFD for 6 weeks and then treated or not treated with rosiglitazone for 15 weeks (A, B, E–H), or 6 weeks (C, D). body weight (A), adipocyte size (B), insulin tolerance test (C), glucose tolerance test (D), and serum FFA (E), TG (F), adiponectin (G) and leptin levels (H). Values are expressed as means ± S.E. ($n = 4$) *$P<0.05$, **$P<0.01$. NS, no significant difference.

increase in PPARγ activity in mature adipocytes associated with the S112A mutation on the insulin sensitivity and adipocyte size were distinct from those of PPARγ activation via rosiglitazone. One possibility is that the PPARγ activation induced by rosiglitazone is more marked than that observed in the S112A mice. It

might also be possible that the amount of PPARγ ligands available in adipocytes is far lower than the amount of PPARγ receptors under physiological conditions.

S112A mice treated with rosiglitazone showed similar insulin sensitivity to the wild-type mice treated with rosiglitazone. The possibility that rosiglitazone did not activate the S112A allele cannot be excluded, however, rosiglitazone binds PPARγ and activates both PPARγ1 and PPARγ2 [18]. In fact, it has been reported that PPARγ2 S112A is activated as much as or more than wild-type PPARγ by rosiglitazone [19, 20]. We also believe that PPARγ S112A is activated by rosiglitazone, although why rosiglitazone-treated S112A mice exhibited similar insulin sensitivity to wild-type mice still remains to be clarified.

In conclusion, whereas the 50% decrease in PPARγ activity showed protection from HFD-induced obesity and insulin resistance, in the present study, the 2–3-fold increase in PPARγ2 expression and PPARγ activity failed to show obesity and insulin resistance even under the HFD condition.

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ORIGINAL ARTICLE

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Crucial role of insulin receptor substrate-2 in compensatory β-cell hyperplasia in response to high fat diet–induced insulin resistance

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In type 2 diabetes, there is a defect in the regulation of functional β-cell mass to overcome high-fat (HF) diet–induced insulin resistance. Many signals and pathways have been implicated in β-cell function, proliferation and apoptosis. The co-ordinated regulation of functional β-cell mass by insulin signalling and glucose metabolism under HF diet–induced insulin-resistant conditions is discussed in this article. Insulin receptor substrate (IRS)-2 is one of the two major substrates for the insulin signalling. Interestingly, IRS-2 is involved in the regulation of β-cell proliferation, as has been demonstrated using knockout mice models. On the other hand, in an animal model for human type 2 diabetes with impaired insulin secretion because of insufficiency of glucose metabolism, decreased β-cell proliferation was observed in mice with β-cell–specific glucokinase haploinsufficiency ($Gck^{+/-}$) fed a HF diet without upregulation of IRS-2 in β-cells, which was reversed by overexpression of IRS-2 in β-cells. As to the mechanism underlying the upregulation of IRS-2 in β-cells, glucose metabolism plays an important role independently of insulin, and phosphorylation of cAMP response element-binding protein triggered by calcium-dependent signalling is the critical pathway. Downstream from insulin signalling via IRS-2 in β-cells, a reduction in FoxO1 nuclear exclusion contributes to the insufficient proliferative response of β-cells to insulin resistance. These findings suggest that IRS-2 is critical for β-cell hyperplasia in response to HF diet–induced insulin resistance.

Keywords: β-cell hyperplasia, cAMP response element-binding protein, forkhead protein FoxO1, glucokinase, glucose, high-fat diet, insulin receptor substrate-2
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Introduction

The prevalence of type 2 diabetes has increased markedly in both Western and Asian countries, and this increase can be explained by drastic changes, such as a high-fat (HF) diet and a sedentary lifestyle. Excess white adipose tissue is now linked to obesity-related health problems in the current environment of excessive nutrition. Some of the adipokines, a group of hormones secreted by adipose tissue, have been shown to directly or indirectly affect insulin sensitivity through modulation of insulin signalling and the molecules involved in glucose and lipid metabolism [1]. Hypertrophic adipocytes secrete an excess of certain hormones and nutrients, which have been reported to cause insulin resistance and produce less adiponectin, which has been reported to increase

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insulin sensitivity [2]. Under normal circumstances, this insulin resistance would be compensated for by increased insulin secretion, but in type 2 diabetes, there is a defect in the regulation of functional β-cell mass to overcome HF diet–induced insulin resistance. In fact, decreased β-cell mass as well as impaired insulin secretion was reported in both Western and Asian patients with type 2 diabetes [3–5]. Although many signals and pathways have been implicated in β-cell function, proliferation and apoptosis, the co-ordinated regulation of functional β-cell mass by insulin signalling and glucose metabolism under HF diet–induced insulin-resistant conditions has not been fully elucidated [6–10].

This article focuses on the role of insulin signalling for the regulation of β-cell mass in knockout mouse models [10]. We also discuss the regulation of β-cell mass by glucose metabolism using mice with haploinsufficiency for β-cell–specific glucokinase ($Gck^{+/-}$) on a HF diet and the striking role of insulin receptor substrate (IRS)-2 for the regulation of β-cell mass in this model [11]. In addition, we examine the molecular mechanisms of upstream and downstream IRS-2 signalling with discussion of the recent emerging concepts that cAMP response element-binding protein (CREB) and FoxO1 mediate β-cell–mass changes [12,13]. Finally, we frame the challenges involved in designing therapeutic approaches that manipulate IRS-2 signalling in β-cells for the treatment of type 2 diabetes [14,15] (figure 1).

Regulation of β-Cell Mass by Insulin Signalling in Knockout Mouse Models

IRSs are evolutionarily conserved adaptor proteins, which are required for various biological processes, such as nutrient metabolism, cell-cycle control, apoptosis and differentiation. IRS-1 and IRS-2 are the two major substrates for insulin receptor tyrosine kinase and insulin-like growth factor (IGF)-1 receptor kinase [16–19]. The physiological roles of these proteins *in vivo* have been evaluated using gene-targeting strategies [10,20]. Total IRS-1 knockout ($IRS\text{-}1^{-/-}$) mice are growth retarded and insulin resistant [21,22], but because of compensatory hyperinsulinaemia associated with β-cell hyperplasia in response to insulin resistance, they do not develop diabetes. Total IRS-2 knockout ($IRS\text{-}2^{-/-}$) mice, on the other hand, develop type 2 diabetes associated with hepatic insulin resistance, a lack of compensatory β-cell hyperplasia and leptin resistance [23–26] (figure 2).

Considering that insulin resistance caused by the absence of IRS-2 in peripheral tissues may affect leptin sensitivity and β-cell proliferation, specific disruption of the IRS-2 gene in β-cells and the hypothalamus is needed

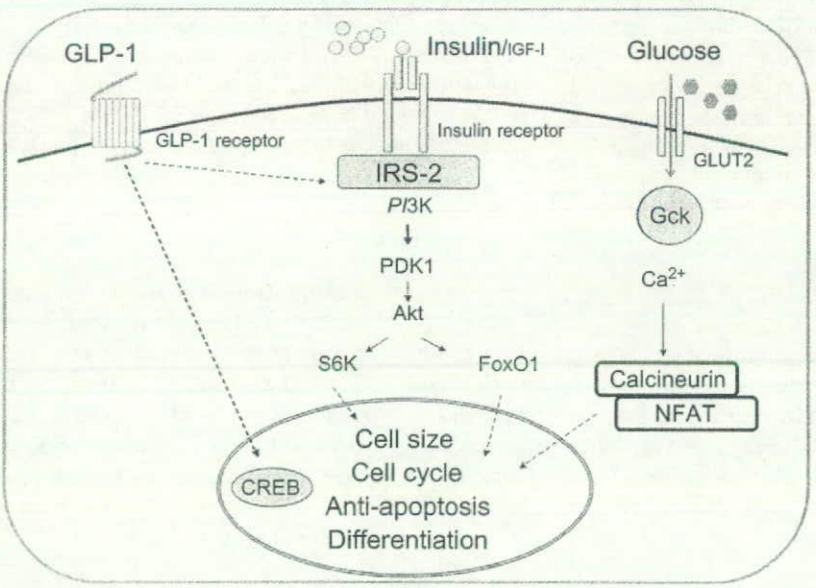

Fig. 1 Schema showing adaptive β-cell growth with crucial signals and pathways. Many signals and pathways have been implicated in β-cell function, proliferation and apoptosis. The coordinated regulation of functional β-cell mass by insulin signalling and glucose metabolism under high-fat diet–induced insulin resistance is discussed in the body of this article. CREB, cAMP response element-binding protein; GLP, glucagon-like peptide; IGF, insulin growth factor; IRS, insulin receptor substrate; PI, phosphoinositide.

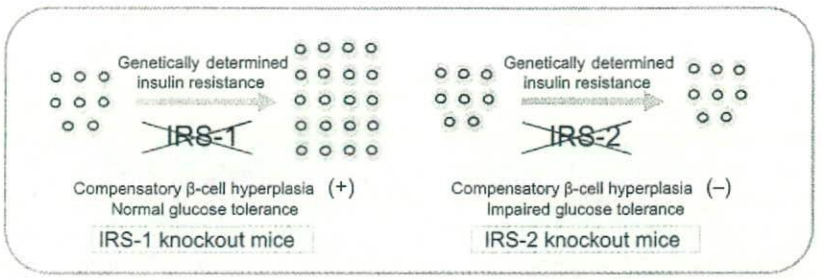

Fig. 2 Schema showing the difference between insulin receptor substrate (IRS)-1 and IRS-2 for compensatory β-cell hyperplasia in response to genetically-determined insulin resistance. Total IRS-1 knockout mice are insulin resistant, but because of compensatory hyperinsulinaemia associated with β-cell hyperplasia, they do not develop diabetes. In contrast, total IRS-2 knockout mice develop type 2 diabetes associated with insulin resistance and a lack of compensatory β-cell hyperplasia.

to determine the roles of IRS-2 at these sites more precisely. Recently, we generated β-cell–specific IRS-2 knockout and hypothalamus-specific IRS-2 knockdown (βHT-IRS-2 KO) mice by crossing IRS-2 floxed (IRS-2 flox/flox) mice and transgenic mice expressing Cre recombinase under the control of the rat insulin II promoter (RIP-Cre mice) [27]. The expression of IRS-2 mRNA in βHT-IRS-2 KO mice was reduced by approximately 90% in islets and markedly reduced in the arcuate nucleus of the hypothalamus, whereas the expression of IRS-2 in the liver, muscle and adipose tissue of βHT-IRS-2 KO was indistinguishable from that in control mice. The βHT-IRS-2 KO mice were obese and leptin resistant. Furthermore, despite normal insulin sensitivity during caloric restriction, these mice also displayed glucose intolerance and impaired glucose-stimulated insulin secretion. Both the β-cell mass and β-cell proliferation rate were significantly reduced in adult βHT-IRS-2 KO mice, but not in the young animals. Using the same gene-targeting strategy, another group independently revealed that IRS-2 signalling promoted regeneration of adult β-cells and central control of nutrient homeostasis, which prevented obesity and diabetes in mice [28].

As Cre recombinase expression in the hypothalamus seen in our RIP-Cre mice resulted in the development of obesity and leptin resistance, which potentially influence β-cell function, pancreas-specific IRS-2 knockout mice (P-IRS-2 KO) were generated in which Cre recombinase expression was driven by the promoter of the pancreatic and duodenal homeobox factor 1 (Pdx-1) gene [29]. Morphometric analysis in adult P-IRS-2 KO mice revealed a reduced total β- and α-cell masses but preservation of pancreatic mass. Moreover, reduced Ki67 staining was detected in β-cells in P-IRS-2 KO mice, demonstrating a reduced proliferation rate. Together, these findings suggest that the expression of IRS-2 in β-cells plays crucial roles in the regulation of β-cell mass.

IRS-2 function was clearly shown to be sufficient for regulation of proliferation and apoptosis in β-cells *in vitro*. High expression of IRS-2 by adenoviral infection, but not of IRS-1, induced proliferation and protected human β-cells from hyperglycaemia-induced apoptosis [30]. In contrast, decreasing endogenous IRS-2 in a β-cell line, using adenoviral-mediated expression of IRS-2 antisense, caused marked apoptosis, which was further enhanced in the presence of FFA. This was associated with decreased phosphorylated Akt and increased caspase-9 activation [31].

To more precisely determine the roles of IRS-2 in β-cells, we attempted to establish a pancreatic β-cell line lacking IRS-2 expression. Considering the difficulty of directly establishing a β-cell line from IRS-2 knockout mice, we first established several SV40-transformed IRS-2 flox/flox β-cell lines and selected one line in which functional glucose-stimulated insulin secretion was preserved [32]. In the IRS-2 flox/flox β-cell line, the expression of IRS-2 was efficiently disrupted by infection with an adenovirus expressing Cre recombinase. When the expression level of the IRS-2 protein virtually disappeared (1 week after infection with the adenovirus), cell proliferation was arrested, whereas no morphological changes were seen after infection of the wild-type β-cell line with the same adenovirus. These findings clearly indicate that IRS-2 is crucially involved in the regulation of physiological β-cell proliferation (I. Takamoto, University of Tokyo, N. Kubota, University of Tokyo, J. Miyazaki, Osaka University and T. Kadowaki, University of Tokyo, unpublished results). The cell cycle–related and apoptosis-related molecules involved in β-cell survival via downstream IRS-2 signalling should be examined in the future.

On the other hand, even in the absence of insulin resistance, β-cells deficient in IRS-1 exhibit a compensatory increase in IRS-2, which is associated with islet

growth and is characterized by both proliferative and anti-apoptotic effects in experiments using a transplantation approach [33]. Furthermore, isolated islets from IRS-1 knockout mice and SV40-transformed IRS-1–deficient β-cell lines exhibit marked insulin secretory defects in response to glucose. This defect can be partially restored by transfecting the cells with IRS-1 [34]. IRS-1 appears to have different biological functions from IRS-2 because IRS-1 has no apparent influence on β-cell growth or survival. Rather, IRS-1 possibly plays a role in regulating intracellular calcium ion handling in β-cells at the level of the rough endoplasmic reticulum [35]. Interestingly, analysis of gene expression patterns in P-IRS-2 KO islets revealed that IRS-1 mRNA levels were unaltered, demonstrating that there is no compensatory upregulation of this gene [29], which contrasts with the upregulation of IRS-2 reported in IRS-1 deficient islets [33].

In summary, IRS-1 and IRS-2 appear to have distinct roles in β-cell function and mass for reasons that are still unclear. The underlying molecular mechanisms also await full clarification in future studies.

Regulation of β-Cell Mass by Glucose

While it has long been known that glucose and its metabolism can stimulate β-cell proliferation *in vitro* and *in vivo* [6,36,37], the precise mechanism of this process remains largely unknown. To unravel the molecular mechanism by a gene-targeting strategy, we used mice with haploinsufficiency for β-cell–specific glucokinase ($Gck^{+/-}$) as an animal model of human type 2 diabetes with impaired insulin secretion [11,38]. As glucokinase (Gck) catalyses the conversion of glucose into glucose 6-phosphate, which is a critical process in glucose sensing for insulin secretion in pancreatic β-cells, $Gck^{+/-}$ mice display glucose intolerance associated with a reduction in insulin secretion in response to glucose [38]. Therefore, $Gck^{+/-}$ mice fed a HF diet are an appropriate animal model for human type 2 diabetes [11]. In addition, mutations of the Gck gene in humans have been identified in maturity onset diabetes of the young (MODY2) patients [39].

On a HF diet, the wild-type mice maintained normal glucose tolerance as a result of compensatory hyperinsulinaemia, whereas the $Gck^{+/-}$ mice developed severe diabetes because of the lack of compensatory hyperinsulinaemia, despite similar degrees of obesity and insulin resistance. A histological analysis showed that the HF diet caused islet hyperplasia in the wild-type mice but, surprisingly, not in the $Gck^{+/-}$ mice. In addition, the estimation of β-cell proliferation based on the rate of 5-bromo-2′-deoxyuridine incorporation revealed that the failure of compensatory β-cell hyperplasia in the $Gck^{+/-}$ mice fed with the HF diet was associated with lack of a compensatory increase in β-cell proliferation, in contrast to the situation in wild-type mice fed with the HF diet [11].

A DNA microarray analysis as a means of systematically examining the gene expression profiles of the islets again pointed out the striking role of IRS-2 in the control of β-cell mass in response to HF diet–induced as well as genetically determined insulin resistance [25]. Of all genes that were examined, the expression of IRS-2 was most downregulated in the islets of $Gck^{+/-}$ mice fed with the HF diet, compared with the islets of wild-type mice fed the HF diet [11] (figure 3). In addition, the expression of PDK-1, IGF-1 receptor the prolactin receptor and cyclin D2 were also markedly lower. Moreover, by western blot analysis, upregulation of IRS-2 expression in the islets occurred in the wild-type mice fed the HF diet, but not in the $Gck^{+/-}$ mice on the same HF diet [11]. Taking into consideration the crucial roles of IRS-2 in the knockout mouse models, a reduction in IRS-2 seemed to explain the impaired β-cell hyperplasia in $Gck^{+/-}$ mice fed the HF diet (figure 4).

To directly test our hypothesis that a reduction in IRS-2 explains the impaired β-cell hyperplasia in $Gck^{+/-}$ mice fed the HF diet, $Gck^{+/-}$ mice crossed with β-cell–specific IRS-2 transgenic (βIRS-2Tg) mice were analysed [11]. When the expression of IRS-2 protein in βIRS-2TgGck$^{+/-}$ mice was comparable to that in the wild-type mice, β-cell proliferation was rescued. This resulted in a significantly larger β-cell area in βIRS-2TgGck$^{+/-}$ mice than in $Gck^{+/-}$ mice and partially prevented diabetes development on the HF diet [11]. Consistent with the experiments involving β-cell–specific IRS-2 transgenic mice that were independently reported by others, the upregulation of IRS-2 in β-cells promoted β-cell growth and survival, preventing the onset of diabetes caused by IRS-2 disruption or HF diet–induced obesity [40].

Moreover, our experiments using other animal models with hyperphagia and insulin resistance, such as ob/ob mice, db/db mice and KKAy mice, have shown that the expression level of IRS-2 in islets consistently correlated with the β-cell mass (I. Takamoto and M. Ohsugi, University of Tokyo, unpublished results). Thus, IRS-2 is critical for the regulation of β-cell mass in response to both HF diet–induced and genetically determined insulin resistance.

Mechanism for the Upregulation of IRS-2 in β-Cells

What is the impact of HF diet on IRS-2 in β-cells? To address this simple question, we initially investigated the mechanisms of upregulation of IRS-2 in β-cells under

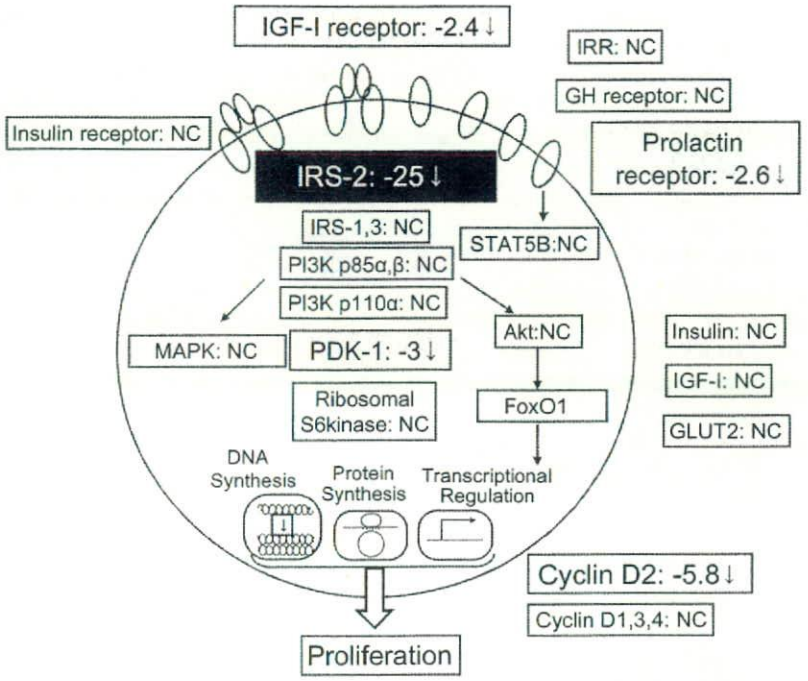

Fig. 3 Gene expression profiles of the islets based on a DNA microarray analysis. The numbers before arrows in the boxes refer to the fold-change of $Gck^{+/-}$ mice fed the high fat (HF) diet for 20 weeks compared with wild-type mice fed the HF diet for 20 weeks. Described based on the data from Terauchi *et al.* [11] with permission. IGF, insulin growth factor; NC, not changed.

HF diet–induced insulin resistance conditions. Two mechanisms are theoretically possible. One is a direct effect of specific nutrients of the HF diet, but there is little evidence to support this hypothesis. The other possibility is an indirect effect of the elevation of plasma insulin and/or glucose secondary to insulin resistance. Because of the complexity of the mechanisms by which glucose and its metabolites can exert their effects on β-cell proliferation and because glucose also stimulates insulin secretion, which in turn can feed back on β-cell proliferation, it is a challenge to separate the proper effects of glucose from those of insulin in experiments using β-cells.

It is well known that the hepatic IRS-2 mRNA level is very high during fasting and markedly reduced by refeeding, whereas hepatic IRS-1 levels do not show a significant change [41]. At least in the liver, the IRS-2 promoter was reported to be activated by forkhead protein FoxO1 through an insulin response element and nuclear sterol regulatory element–binding protein (SREBP)-1c interfered with the binding of FoxO1. During refeeding, the

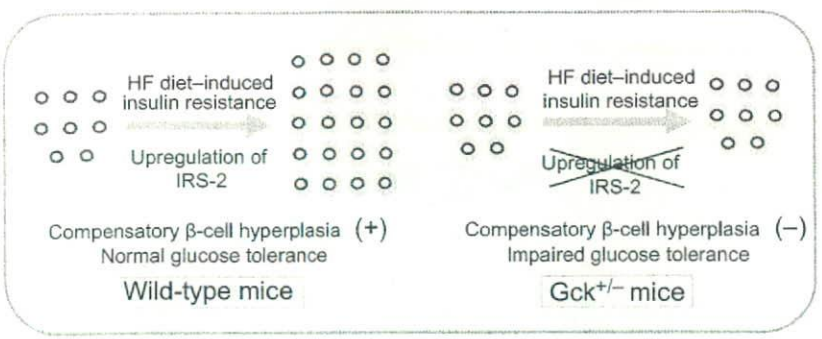

Fig. 4 Schema showing how the upregulation of insulin receptor substrate (IRS-2) is required for compensatory β-cell hyperplasia in response to high fat (HF) diet–induced insulin resistance. Lacking the upregulation of IRS-2 in β-cells is critically responsible for the impaired β-cell hyperplasia in $Gck^{+/-}$ mice fed with the HF diet.

increase in insulin levels inactivated hepatic FoxO1 by phosphorylation and nuclear exclusion, but the elevation of glucose and insulin markedly induced hepatic SREBP-1c expression. Thus low FoxO1 and high SREBP-1c levels in liver nuclei were prominent in the fully fed state, resulting in a reduction of IRS-2 expression [41].

However, this is not the case in the islets. In striking contrast to the decrease observed in the liver, the mRNA level of IRS-2 in pancreatic islets was markedly increased by refeeding (I. Takamoto and M. Ohsugi, unpublished results). Recent publications support the concept that glucose itself, within physiologically relevant levels, can regulate IRS-2 expression levels in a dose-dependent manner [43]. Experiments in rat primary islet β-cells suggested that glucose metabolism was necessary for increased IRS-2 expression and that inhibition of glucose-induced rise in β-cell cytosolic calcium prevented the increase in IRS-2 expression, indicating that this process was calcium dependent. In addition, glucose stimulation of IRS-2 expression can be separated from effects of insulin by using somatostatin that inhibits insulin secretion. Moreover, we have also observed that glucose metabolism in β-cells can upregulate IRS-2 expression levels independently of insulin both *in vitro* and *in vivo*, and the Ser 133 phosphorylation of CREB triggered by calcium-dependent signalling may be the critical pathway (M. Ohsugi, University of Tokyo, unpublished results) (figure 5).

Glucagon-like peptide-1 (GLP-1) receptors are abundantly expressed in β-cells and neurons [44]. The GLP-1 receptor, which has seven transmembrane-spanning regions, is coupled to Gs, which increases cAMP levels through the activation of adenylyl cyclase. GLP-1 is involved in the protein kinase A (PKA) pathway via the elevation of cAMP, which enhances glucose-stimulated insulin secretion in β-cells. In addition, GLP-1 reportedly increases pancreatic β-cell survival, leading to an increase in β-cell mass [14]. Exendin-4 (Ex-4) is a long-acting GLP-1 receptor agonist that is now used in the treatment of human diabetes. In fact, Ex-4 increased cAMP levels in a murine β-cell line (MIN6 cells) and in human islets, subsequently promoting IRS-2 expression – in which CREB phosphorylation was crucially involved [45]. Furthermore, in human islets, the anti-apoptotic action of Ex-4 exposed to cytokines was partially lost when CREB function was impaired by adenoviral infection with dominant negative mutant forms of CREB [45]. To date, cAMP and the subsequent phosphorylation of CREB are thought to be regulators of IRS-2 expression in β-cells [12]. While the activation of PKA is a common consequence of the activation of GLP-1 activation of the tyrosine phosphorylation of IRS-2, downstream IRS-2 activates the phosphoinositide (PI) 3–kinase cascade and appears to be important for β-cell survival and growth.

In accordance with the critical role of CREB in the regulation of IRS-2 expression in β-cells, Ser 133 phosphorylation of CREB was also impaired in $Gck^{+/-}$ mice fed a HF diet compared with the wild-type mice fed a HF diet, despite the cAMP content of the islets in both groups being unchanged [11]. We hypothesized that impaired calcium signalling in β-cells caused by the glucokinase

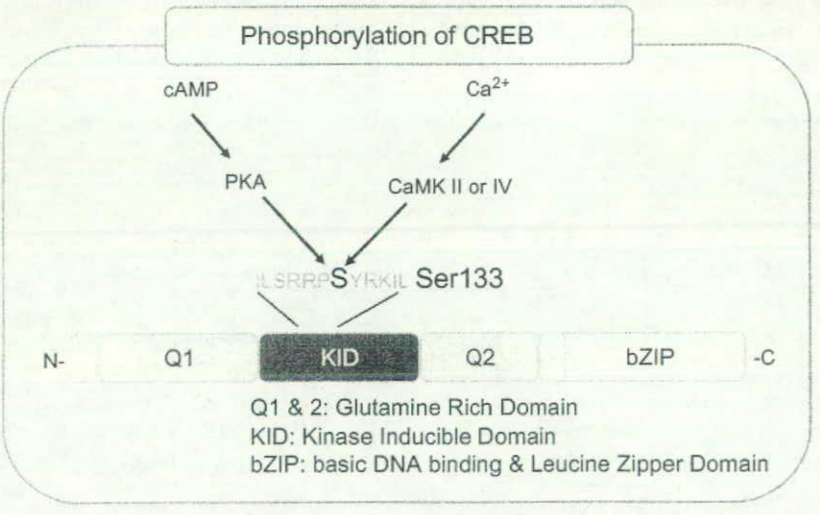

Fig. 5 Schema showing phosphorylation of cAMP response element-binding protein (CREB) triggered by cAMP–protein kinase A (PKA) pathway and calcium-dependent signalling in β-cells. Phosphorylation of CREB is thought to be a key regulator of insulin receptor substrate-2 expression in β-cells.

haploinsufficiency failed to promote the full Ser 133 phosphorylation of CREB in response to HF diet–induced insulin resistance.

Downstream from Insulin Signalling Via IRS-2 in β-Cells

As mentioned above, FoxO1 has been reported to be an activator of IRS-2 in the liver, but there is no clear evidence that the promoter of IRS-2 is activated by FoxO1 in β-cells. Rather, β-cell FoxO1 is recognized as a prominent transcriptional effector downstream from insulin signalling via IRS-2. FoxO1 activity is inhibited by PI 3–kinase/Akt signalling by phosphorylation-dependent nuclear exclusion. The most brilliant genetic result is that FoxO1 haploinsufficiency reverses β-cell mass in IRS-$2^{-/-}$ mice [13]. This appears to be mediated, at least in part, by increased Pdx-1 expression. The transcription factor Pdx-1 plays important roles in β-cell differentiation, proliferation and function [47]. Pdx-1 transcription is regulated by another forkhead transcription factor, FoxA2 (also known as HNF3β) [48]. As FoxO1 and FoxA2 share common DNA-binding sites in the Pdx-1 promoter, FoxO1 competes with FoxA2 for binding to the Pdx-1 promoter, resulting in the inhibition of Pdx-1 transcription [13]. Conversely, a decrease in Akt phosphorylation results in enhanced nuclear FoxO1, which is inhibitory toward β-cell mass expansion [49]. Thus, FoxO1 seems to play an important role as a prominent transcriptional effector of downstream from insulin signalling in β-cells.

The impact of insulin signalling in 'insulin-producing' β-cells has been intensively investigated. As the insulin-signalling pathway in peripheral tissues plays an essential role in glucose and lipid homeostasis, β-cell–specific gene-targeting strategies are needed to estimate the direct effects of insulin-signalling deletion in β-cells. In this context, a more precise role of FoxO1 in β-cells can be estimated. Mice with β-cell–specific deletion of either the insulin receptor (β$IR^{-/-}$ mice) or the IGF-1 receptor (β$IGF1R^{-/-}$ mice) have already been generated and reported. β$IR^{-/-}$ mice have defects in glucose sensing and reduced β-cell mass [8,50], whereas β$IGF1R^{-/-}$ mice show defective glucose-stimulated insulin secretion without alteration in β-cell mass [51,52]. In addition, mice lacking both insulin and IGF-1 receptors only in β-cells (β$IR^{-/-}$: β$IGF1R^{-/-}$ mice) were born with a normal complement of islet cells, but they soon developed diabetes [9]. Even in the younger normoglycemic stage, β$IR^{-/-}$: β$IGF1R^{-/-}$ mice showed reduced β-cell mass and severely compromised β-cell function with reduced PDK-1 protein expression, undetectable phosphorylated Akt and enhanced nuclear FoxO1 localization, which would lead to accelerated β-cell death owing to absence of anti-apoptotic signalling. Furthermore, when either β$IR^{-/-}$ mice or β$IGF1R^{-/-}$ mice were fed a HF diet, only β$IR^{-/-}$ mice failed to expand their β-cell mass accompanied by an increase in nuclear FoxO1 expression and a corresponding decrease in Pdx-1 expression, although β$IGF1R^{-/-}$ mice were able to expand their β-cell mass [53].

Back to our animal model for human type 2 diabetes: on a HF diet, FoxO1 was also restricted to the nucleus in most β-cells of $Gck^{+/-}$ mice, and not in wild-type mice. In addition, Pdx-1 nuclear expression in β-cells was lower in $Gck^{+/-}$ mice fed with the HF diet than in wild-type mice fed with the HF diet. Moreover, overexpression of

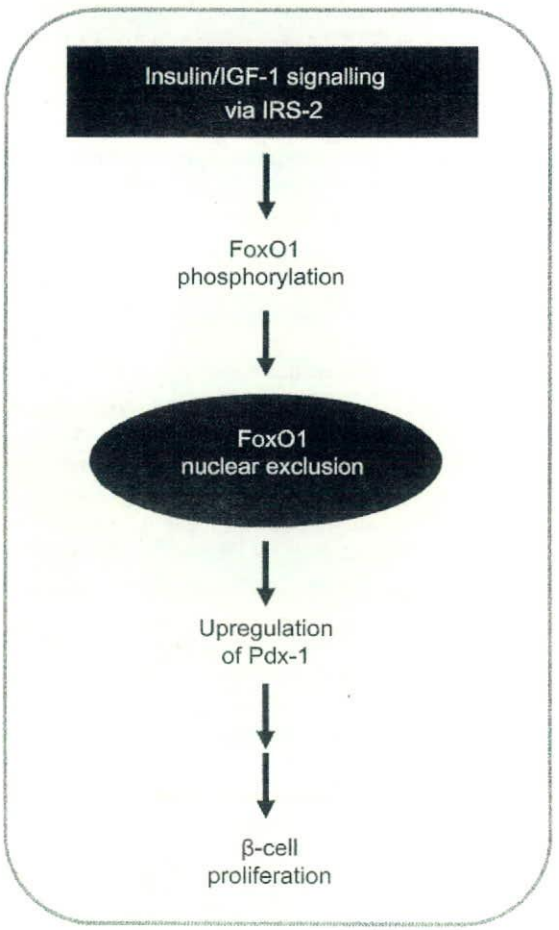

Fig. 6 Schema showing how insulin receptor substrate (IRS)-2 regulates β-cell proliferation through FoxO1 nuclear exclusion. β-cell FoxO1 is recognized as a prominent transcriptional effector downstream from insulin signalling via IRS-2. FoxO1 activity is inhibited by phosphoinositide 3–kinase/Akt signalling by phosphorylation-dependent nuclear exclusion. IGF, insulin growth factor.

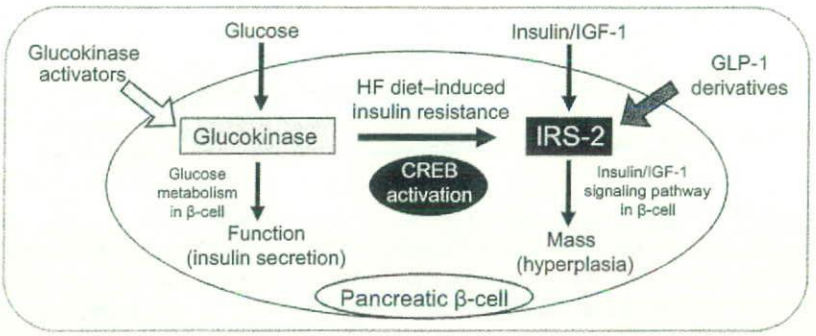

Fig. 7 Schema showing the crucial role of Insulin receptor substrate (IRS)-2 for integrating insulin signalling and glucose metabolism in β-cells. Cyclic AMP response element-binding protein (CREB) activation triggered by calcium-dependent signalling is critically important for the upregulation of IRS-2 in β-cells. Upregulation of IRS-2 via glucokinase activators in combination with GLP-1 derivatives will be an effective therapeutic strategy to improve the functional β-cell mass in type 2 diabetes. GLP, glucagon-like peptide; HF, high fat; IGF, insulin growth factor.

IRS-2 in β-cells decreased nuclear FoxO1-positive cells in the $Gck^{+/-}$ mice fed with the HF diet, indicating that the upregulation of IRS-2 in β-cells stimulated FoxO1 nuclear exclusion efficiently [11] (figure 6).

Conclusions and Prospects

An HF diet is a pivotal factor in the aetiology of type 2 diabetes patients with decreased insulin secretion. To improve the functional β-cell mass in these patients, the molecular links between decreased insulin secretion and decreased β-cell mass should be unravelled. Our findings clearly suggest that IRS-2, beyond one of the mere adaptor proteins intermediating insulin signalling, and glucokinase, beyond one of the mere enzymes in glucose metabolism, co-ordinately regulate the β-cell mass in response to HF diet–induced insulin resistance [11].

Recent publications have indicated that GLP-1, an incretin hormone that promotes insulin secretion and β-cell proliferation, enhanced nuclear exclusion of FoxO1 in a PI 3-kinase–dependent manner [54]. Furthermore, glucose-dependent insulinotropic polypeptide (GIP), another incretin hormone, was reported to stimulate Ser 133 phosphorylation of CREB with an increase in the nuclear localization of transducer of regulated CREB activity 2 (TORC2), leading to activation of the anti-apoptotic Bcl-2 gene [55]. As we now recognize that the insulin-signalling cascade via IRS-2 is tightly linked to FoxO1 activity and that CREB activation with TORC2 requires calcium ion influx probably in response to glucose metabolism in β-cells [56], it seems more convincing that incretins mediate β-cell proliferation and apoptosis.

Finally, IRS-2 is critical for integrating insulin signalling and glucose metabolism in β-cells. Upregulation of IRS-2 via glucokinase activators in combination with incretin derivatives could be an effective therapeutic strategy to improve the functional β-cell mass in type 2 diabetes [14,15] (figure 7). Identification of the molecular mechanisms of β-cell survival via IRS-2 signalling is a promising approach to developing novel medications.

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Rimonabant Ameliorates Insulin Resistance via both Adiponectin-dependent and Adiponectin-independent Pathways*

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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 knock-out (*adipo*(−/−))ob/ob mice. After 21 days of 30 mg/kg rimonabant, 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 anti-obesity agents that can produce and maintain weight loss and improve the metabolic syndrome.

The newly discovered endocannabinoid system, consisting of the CB-1 (cannabinoid type-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 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 nondiabetic obese patients (15–18).

Adiponectin is an adipokine that is specifically and abundantly expressed in the adipose tissue and released into the circulation, which 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 stud-

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ies 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)[2] (26–29). We also reported that pioglitazone may induce amelioration of insulin resistance and diabetes via an adiponectin-dependent mechanism in the liver and an 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 nondiabetic 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/kg, body weight. 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/+ mice. All the mice were maintained on a C57Bl/6 background (30). All of the experiments in this study were conducted on 16–20-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% Tween 80 in saline) was administered to ob/ob and *adipo*(−/−)ob/ob mice at a 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 of 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/10 ml enriched to ~20% with [6,6-$^2H_2$]glucose (Sigma)) for 120 min. Blood was sampled via 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 was calculated as the difference between the R_d and the exogenous glucose infusion rate (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 nonreducing 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 *Escherichia coli* (21).

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

Plasma Adiponectin and Lipid Measurements—The mice were deprived of 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.

[2] The abbreviations used are: AMPK, AMP-activated protein kinase; PEPCK, phosphoenolpyruvate carboxykinase; WAT, white adipose tissue; TG, triglyceride; FFA, free fatty acid; HMW, high molecular weight.

Measurement of Adipocyte Size—Epididymal white adipose tissue and subcutaneous fat were routinely processed for paraffin embedding, and 4-μm sections were cut and mounted on silanized slides. The adipose tissue sections were stained with hematoxylin and eosin, and the total adipocyte area was manually traced and analyzed using the Win ROOF software (Mitani Co. Ltd., Chiba, Japan). The white adipocyte area was measured in 200 or more cells/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 H_2O. After an additional wash for 1 min with 60% isopropyl alcohol, 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 H_2O; the Oil Red O stock solution contained 0.5% Oil Red O in isopropyl alcohol). After one wash for 2 min with 60% isopropyl alcohol and one wash for 1 min with H_2O, 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 percentage of the area of Oil Red O staining was measured from 9–10 different sections/mouse in each experimental group. Values were expressed as percentage of area.

Analysis of O_2 Consumption—Oxygen consumption was measured every 3 min for 24 h in the fasting mice using an O_2/CO_2 metabolism measurement device (model MK-5000; Muromachikikai, Tokyo, Japan). After rimonabant treatment for 21 days, each mouse was placed in a sealed chamber (560-ml volume) with an air flow rate of 500 ml/min at room temperature. The amount of oxygen consumed was converted to ml/min 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 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 RNeasy 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 used for MCP-1 (monocyte chemoattractant protein-1), resistin, phosphoenolpyruvate carboxykinase (PEPCK), carnitine palmitoyltransferase-1A, the hepatic isoform of carnitine palmitoyltransferase-1, protein phosphatase 2C, and cyclophilin were purchased from Applied Biosystems (Foster City, CA). The relative expression levels were compared by normalization to the 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 containing 25 mM glucose and 10% fetal bovine serum at 37 °C. Confluent cultures were induced to differentiate into adipocytes by incubation in Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% fetal bovine serum, 0.25 units/ml insulin, 0.25 μM dexamethasone, and 0.5 mM isobutyl-1-methylxanthine. After 2 days, the medium was changed to Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% fetal bovine serum, and 0.025 units/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 DMSO was used as the vehicle. Prior to the start of the experiments, the differentiated adipocytes were serum-starved in Dulbecco's modified Eagle's medium 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. 1*A*), as reported previously (30). The food intake was also comparable between the untreated ob/ob and *adipo*(−/−)ob/ob mice (Fig. 1*B*). Rimonabant significantly decreased the body weight and food intake to similar degrees in the ob/ob and *adipo*(−/−)ob/ob mice (Fig. 1, *A* and *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. 1*A*). 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. 1*C*). 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. 1, *D* and *E*), and rimonabant treatment significantly reduced the average adipocyte size in the ob/ob and *adipo*(−/−)ob/ob mice to a similar degree (Fig. 1*F*). 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 knock-out mice (35). Since the involvement of adiponectin in this action of rimonabant remains unclear, we investigated the effects of rimonabant on energy expenditure.