The role of PPARγ as a thrifty gene both in mice and humans

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The biological role of peroxisome proliferator-activated receptor γ (PPARγ) was investigated by gene targeting and case–control study of the Pro12Ala PPARγ2 polymorphism. Homozygous PPARγ-deficient embryos died at 10·5–11·5 days post conception (dpc) due to placental dysfunction. Heterozygous PPARγ-deficient mice were protected from the development of insulin resistance due to adipocyte hypertrophy under a high-fat diet, whose phenotypes were abrogated by PPARγ agonist treatment. Heterozygous PPARγ-deficient mice showed overexpression and hypersecretion of leptin despite the smaller size of adipocytes and decreased fat mass, which may explain these phenotypes at least in part. This study reveals a hitherto unpredicted role for PPARγ in high-fat diet-induced obesity due to adipocyte hypertrophy and insulin resistance, which requires both alleles of PPARγ. A Pro12Ala polymorphism has been detected in the human PPARγ2 gene. Since this amino acid substitution may cause a reduction in the transcriptional activity of PPARγ, this polymorphism may be associated with decreased insulin resistance and decreased risk of type 2 diabetes. To investigate this hypothesis, we performed a case–control study of the Pro12Ala PPARγ2 polymorphism. In an obese group, subjects with Ala12 were more insulin sensitive than those without. The frequency of Ala12 was significantly lower in the diabetic group, suggesting that this polymorphism protects against type 2 diabetes. These results revealed that in both mice and humans, PPARγ is a thrifty gene mediating type 2 diabetes.

Type 2 diabetes: Obesity: Thiazolidinediones

Hypertrophic obesity due to adipocyte hypertrophy is closely linked to major health issues such as diabetes, hyperlipidemia, hypertension, and cardiovascular diseases (Spiegelman & Flier, 1998). The prevalence of hypertrophic obesity, which often develops in adulthood, is increasing sharply under a high-fat diet and sedentary lifestyle in Western countries and Japan. Insulin resistance, which is usually associated with hypertrophic obesity, is believed to be the major underlying mechanism of these diseases. Although an excess of hormones and metabolic nutrients produced by hypertrophic adipocytes such as tumor necrosis factor α (TNFα) and free fatty acids have been proposed to cause insulin resistance in peripheral tissues such as skeletal muscles and liver (Hotamisligil *et al.* 1993; Boden, 1997), the precise mechanism of insulin resistance remains unclear (Spiegelman & Flier, 1998).

Thiazolidinedione (TZD), a new class of anti-diabetic drugs which increase insulin sensitivity, can directly bind and activate PPARγ and can stimulate adipocyte differentiation (Spiegelman 1998; Spiegelman & Flier, 1998; Lehmann *et al.* 1995). Although TZD can ameliorate insulin resistance in both human and animal models of obese type 2 diabetes, the link between its potency to promote adipocyte differentiation and amelioration of

Abbreviations: BMI, body mass index; HOMA, homeostasis model assessment; PPARγ, peroxisome proliferator-activated receptor γ; TZD, thiazolidinedione.

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insulin resistance has been largely elusive (Spiegelman & Flier, 1998; Saltiel & OleFsky, 1996). We have previously shown that treatment of Zucker fa/fa rats with TZD caused an increase in the number of small adipocytes, and a concomitant decrease in the number of large adipocytes which had produced an excess amount of TNFα and free fatty acids, thereby ameliorating insulin resistance (Okuno *et al.* 1998). These results led us to propose that stimulation of PPARγ with potent synthetic agonists such as TZD may result in adipocyte differentiation and a consequent increase in the number of small adipocytes, thereby rendering the body more sensitive to insulin (Okuno *et al.* 1998). Nevertheless, the physiological roles of PPARγ in mature adipocytes and regulation of insulin sensitivity *in vivo* remain largely unclear.

To investigate the role of PPARγ *in vivo*, we have generated PPARγ-deficient mice by gene targeting (Kubota *et al.* 1999). Homozygous PPARγ-deficient mice were embryonic lethal due to placental dysfunction. Quite unexpectedly, heterozygous PPARγ-deficiency in mice prevented the development of adipocyte hypertrophy and insulin resistance under a high-fat diet. This effect was blocked by use of PPARγ agonist, indicating that decreased activity of PPARγ in PPARγ (+/−) mice indeed confered protection from adipocyte hypertrophy and insulin resistance under a high-fat diet. Heterozygous PPARγ-deficient mice showed overexpression and hypersecretion of leptin despite the smaller size of adipocytes and decreased fat mass, which may explain these phenotypes at least in part. This study provides the evidence that both PPARγ alleles are required for high-fat diet-induced obesity due to adipocyte hypertrophy and insulin resistance, a hitherto unpredicted role of PPARγ in mature adipocytes (Kubota *et al.* 1999).

In humans, a Pro12Ala substitution has been detected in the PPARγ2 gene (Yen *et al.* 1997). This amino acid is located in the PPARγ2 domain that enhances ligand-independent activation. The non-conservative substitution of proline to alanine may cause a conformational change in the protein, which may affect its activity. Indeed, a mutant PPARγ2 with Ala12 had reduced transactivation activity (Deeb *et al.* 1998). Based on the fact that this amino acid is highly conserved and that codon 12 in the mouse PPARγ2 gene is also proline (Yen *et al.* 1997), we hypothesized that subjects with this polymorphism may be partially protected from type 2 diabetes. To test this hypothesis we performed an association study regarding this polymorphism. Frequency of subjects bearing Ala allele was significantly higher in non-diabetic subjects associated with the decreased risk of type 2 diabetes. In obese subjects, Ala allele bearers were more insulin sensitive than non-bearers.

These findings provide profound insights into the role of PPARγ mediating insulin resistance and type 2 diabetes.

High-fat diet-induced adipocyte hypertrophy requires both PPARγ alleles

Heterozygous PPARγ-deficient mice showed normal weight gain and insulin sensitivity under a standard diet. To determine the role of PPARγ in high-fat diet-induced obesity and insulin resistance, we studied body weight gain and white adipose tissue mass of wild-type and heterozygous PPARγ-deficient mice under either a high-carbohydrate diet or a high-fat diet. Body weight gain under a high-carbohydrate diet for 15 weeks was not distinguishable between wild-type and heterozygous PPARγ-deficient mice. Under a high-fat diet, wild-type mice gained significantly more body weight than that under a high-carbohydrate diet. In contrast, heterozygous PPARγ-deficient mice had little weight gain under a high-fat diet. Although the total white adipose tissue mass of epididymal, retroperitoneal, and perirenal fat pads was significantly larger under a high-fat diet in wild-type mice, an increase in white adipose tissue mass under a high-fat diet was inhibited by more than 70 % in heterozygous PPARγ-deficient mice (Kubota *et al.* 1999).

Histological analyses of epididymal fat pads after direct fixation and quantification of adipocyte size revealed that adipocytes from wild-type mice and heterozygous PPARγ-deficient mice under a high-fat diet were significantly larger than those from both types of mice under a high-carbohydrate diet. However, the size of adipocytes from heterozygous PPARγ-deficient mice was significantly smaller than that of adipocytes from wild-type mice under a high-fat diet (Kubota *et al.* 1999). The number of adipocytes from both types of mice under either a high-fat or a high-carbohydrate diet was similar.

Heterozygous deficiency of PPARγ protects against high-fat diet-induced insulin resistance

We next investigated whether heterozygous PPARγ-deficient mice were associated with insulin resistance or insulin sensitivity. Before a high-fat diet, the glucose lowering effect of insulin was similar between wild-type and heterozygous PPARγ-deficient mice. Surprisingly, after a 15-week high-fat diet, the glucose lowering effect of insulin was markedly larger in heterozygous PPARγ-deficient mice than in wild-type mice. Thus, heterozygous PPARγ-deficient mice showed insulin sensitivity rather than insulin resistance as compared to wild-type mice under a high-fat diet (Kubota *et al.* 1999).

Mechanism for protection from high-fat diet-induced adipocyte hypertrophy and insulin resistance-role of leptin

To determine the mechanism for the protection against increased fat mass due to adipocyte hypertrophy and development of insulin resistance under a high-fat diet in heterozygous PPARγ-deficient mice, we first studied food intake. Food intake after a high-fat diet was significantly lower in heterozygous PPARγ-deficient mice than in wild-type mice. We next studied energy expenditure. Rectal temperature of heterozygous PPARγ-deficient mice was significantly higher than that of wild-type mice under a high-fat diet, indicating that energy expenditure was increased. The total brown adipose tissue mass was approximately 40 % smaller in heterozygous PPARγ-deficient mice than in wild-type mice under a high-fat diet. Importantly, the adipocytes in brown adipose tissue in heterozygous PPARγ-deficient mice were approximately

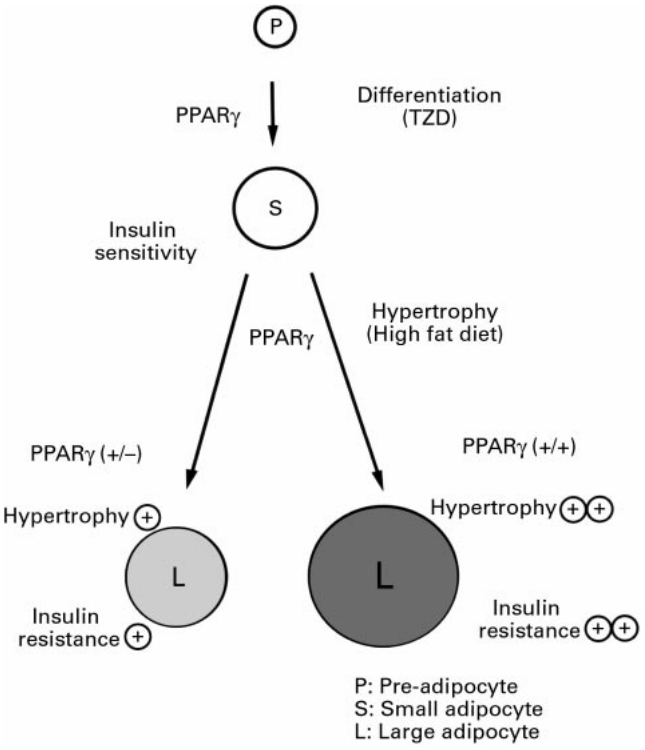

Fig. 1. PPARγ mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance.

36 % smaller than those from wild-type mice, which may be consistent with the higher energy expenditure in heterozygous PPARγ-deficient mice under a high-fat diet (Kubota *et al.* 1999).

Leptin may be a candidate molecule to account for the reduced food intake and increased energy expenditure in heterozygous PPARγ-deficient mice (Friedman & Halaas, 1998). We thus studied the expression of leptin mRNA in white adipose tissue. Expression levels of leptin were similar between wild-type and heterozygous PPARγ-deficient mice under a high-carbohydrate diet. Under a high-fat diet, expression of leptin was increased only modestly in wild-type mice. Surprisingly, expression levels of leptin were more markedly increased in heterozygous PPARγ-deficient mice despite the fact that adipocytes under a high-fat diet were significantly smaller than in wild-type mice. Moreover, serum leptin levels were approximately 1·8-fold higher in heterozygous PPARγ-deficient mice under a high-fat diet than those in wild-type mice despite the fact that mass of white adipose tissue under a high-fat diet was significantly less than in wild-type mice. We next addressed whether this increase in leptin expression levels reflected an increased ability of heterozygous PPARγ-deficient adipocytes to express and secrete leptin. Thus, we measured leptin mRNA levels by ribonuclease protection assay of EF cells during *in vitro* adipocyte differentiation experiments from wild-type, heterozygous and homozygous PPARγ-deficient embryos. Interestingly, while leptin mRNA in homozygous mutants was completely abrogated, it was increased in heterozygous mutants (data not shown) despite the decreased triglyceride contents. Moreover, secretion of leptin into the medium was significantly higher with EF cells from heterozygous PPARγ-deficient embryos than those from wild-type embryos. We next examined the leptin sensitivity in wild-type and heterozygous PPARγ-deficient mice under a high-fat diet. Leptin administered as a daily intraperitoneal injection of 5 mg/g body weight per day caused a similar degree of reduction in food intake and body weight in the two mouse genotypes. These results indicated that the leptin sensitivity was similar between the two mouse genotypes (Kubota *et al.* 1999).

Roles of PPARγ in the regulation of adipocyte size and insulin sensitivity: a novel model

Based upon the experimental results we propose the following model with respect to the roles of PPARγ in the regulation of adipocyte size and insulin sensitivity (Fig. 1). The amount of PPARγ plays a critical role in adipocyte hypertrophy and development of insulin resistance under a high-fat diet. In wild-type mice, a high-fat diet promotes adipocyte hypertrophy that converts small adipocytes (S) into large adipocytes (L), which in turn

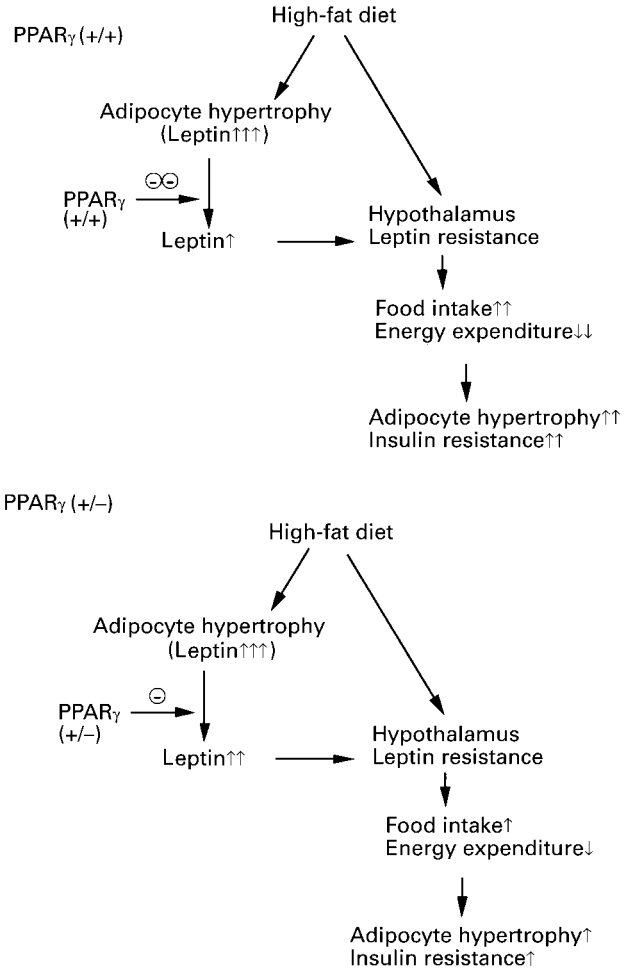

Fig. 2. Suppression of leptin expression by PPARγ plays a role in high-fat diet-induced adipocyte hypertrophy and insulin resistance.

Table 1. Genotypic and allelic distribution of the PPARγ2 Pro12Ala polymorphism in type 2 diabetic and non-diabetic subjects

Subjects	Pro/Pro	Pro/Ala + Ala/Ala	Allelic frequency of Ala12
Type 2 diabetic subjects ($N = 415$)	400 (96·4 %)	15 (3·6 %)**	0.018**
Non-diabetic subjects ($N = 541$)	496 (91·7 %)	45 (8·3 %)**	0.043**

Pro/Pro, homozygous for Pro12 allele; Pro/Ala, heterozygous for Pro12 and Ala12 alleles; Ala/Ala, homozygous for Ala12 allele. **$P < 0.005$ by χ^2 test.

induce factors such as TNFα and free fatty acids thereby causing insulin resistance. In heterozygous PPARγ deficient mice, adipocyte hypertrophy and development of insulin resistance under a high-fat diet are partially protected.

Thus, it seems likely that PPARγ plays dual roles in the regulation of insulin sensitivity. In the presence of potent synthetic ligands such as TZD, and presumably in childhood, the differentiation of adipocytes and hence the number of adipocytes are dependent upon activation of PPARγ. PPARγ stimulation results in adipocyte differentiation to generate small adipocytes and insulin sensitivity. In adults under a high-fat diet, however, adipocyte hypertrophy and hence the size of adipocytes is dependent upon the amount of PPARγ. PPARγ stimulation results in adipocyte hypertrophy to generate large adipocytes and insulin resistance.

Fig. 2 shows our model for the role of leptin in relation to adipocyte size and insulin sensitivity. In wild-type mice, high-fat diet-induced adipocyte hypertrophy and insulin resistance may be caused by impaired leptin effects due to central leptin resistance (Frederich *et al.* 1995) and relative suppression of leptin expression by two alleles of PPARγ. In heterozygous PPARγ-deficient mice, on the other hand, high-fat diet-induced adipocyte hypertrophy and insulin resistance may partially be protected by a relative preservation of leptin effects due to a partial release from suppression of leptin expression by loss of one PPARγ allele.

Pro12Ala polymorphism in PPARγ is associated with type 2 diabetes and insulin resistance

To investigate whether PPARγ plays a role in insulin resistance and onset of type 2 diabetes in humans, we compared the frequency of Ala12 bearer in the non-related type 2 diabetic and non-diabetic subjects. Non-diabetic subjects who were over the age of 60, had HbA1c values lower than 6·0 %, and no family history of type 2 diabetes were recruited from an unselected population who underwent a routine health check-up at the Hiroshima Atomic Bomb Casualty Council Health Management Center. The diabetic subjects were recruited from the outpatient clinic of the Institute for Diabetes Care and Research, Asahi Life Foundation and the Department of Metabolic Diseases, Tokyo University. This study was performed under informed consent from all subjects and was approved by the Ethics Committee of Tokyo University. The frequency of subjects bearing the Ala12 allele was significantly lower in the diabetic group (3·6 %) than in the non-diabetic group (8·3 %, $P = 0.003$) see Table 1 (Hara *et al.* 2000). Subjects with the Ala12 allele had a decreased risk for type 2 diabetes [odds ratio (OR) = 0·413, 95 %CI; 0·220–0·735] (Hara *et al.* 2000). After adjustment for age, gender, and BMI, this reduced risk was still observed (OR = 0·324, 95 %CI; 0·152–0·658) (Hara *et al.* 2000). To assess the insulin resistance and b-cell function of subjects, we used the homeostasis model assessment (HOMA-IR and HOMA-β, respectively) (Matthews *et al.* 1985). Neither

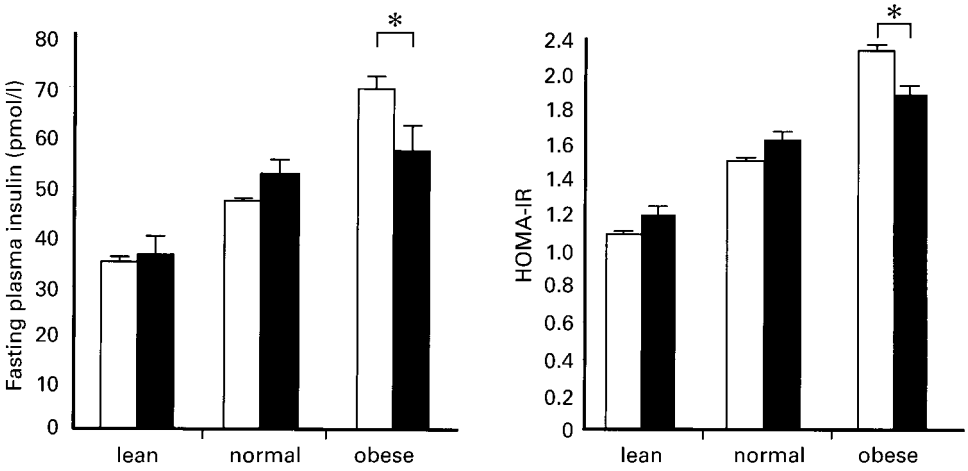

Fig. 3. Influence of the PPARγ Pro12Ala polymorphism on fasting plasma insulin (left panel) and insulin resistance index (right panel), in non-diabetic subjects with (solid bars) or without (open bars) the Ala12 allele. *$P < 0.05$.

HOMA-IR nor HOMA-b was different between subjects with or without the Ala12 allele (data not shown). Since it is well known that obesity has a confounding effect on variables relevant to insulin resistance, we subdivided non-diabetic subjects into three groups according to body mass index (BMI) (lean, BMI $<22\ kg/m^2$: normal, $22\ kg/m^2 \leq$ BMI $< 25\ kg/m^2$: obese, $25\ kg/m^2 \leq$ BMI). In lean and normal groups, neither fasting plasma insulin nor HOMA-IR were different between subjects with and without the Ala12 allele. However in the obese group, both fasting plasma insulin ($57{\cdot}4 \pm 5{\cdot}23$ *v.* $70{\cdot}6 \pm 2{\cdot}53$ pmol/l, $P = 0{\cdot}03$) and HOMA-IR ($1{\cdot}89 \pm 0{\cdot}18$ *v.* $2{\cdot}35 \pm 0{\cdot}09$, $P = 0{\cdot}03$) were lower in subjects with the Ala12 allele than those without (Fig. 3) (Hara *et al.* 2000). Since the average BMI of subjects with the Ala12 allele was comparable to that of those without, these results suggest that having the Ala12 allele may protect against insulin resistance which would normally arise in obese subjects. The plasma leptin levels tended to be higher in subjects with the Ala12 allele ($8{\cdot}02 \pm 1{\cdot}04$ ng/ml) than in those without ($6{\cdot}27 \pm 0{\cdot}20$ ng/ml) (Hara *et al.* 2000). Thus it is possible that higher levels of leptin might be associated with a relatively increased insulin sensitivity in subjects bearing the Ala12 allele. These findings suggest that the Ala12 allele of the PPARγ2 gene may protect subjects with this polymorphism from type 2 diabetes.

In conclusion, PPARγ has the pivotal role in adipocyte hypertrophy and insulin resistance induced by high-fat diet, which requires two alleles of wild type PPARγ. Partial reduction in dose or activity of PPARγ, due to genetically inherited or functional antagonist, leads to the protection against obesity and type 2 diabetes induced by a high-fat diet. Both in mice and humans, PPARγ is an important thrifty gene mediating insulin resistance and type 2 diabetes.

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