NOTE

Impact of Increased PPARγ Activity in Adipocytes in vivo on Adiposity, Insulin Sensitivity and the Effects of Rosiglitazone Treatment

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Abstract. Peroxisome proliferator-activated receptor (PPARγ), a transcription factor belonging to the nuclear receptor superfamily, is essential for adipogenesis. PPARγ is recognized as a major target for the insulin-sensitizing effects of the thiazolidinediones. Previous studies have demonstrated that heterozygous PPARγ-deficient mice are protected from high-fat diet (HFD)-induced adipocyte hypertrophy, obesity and insulin resistance, which suggests that PPARγ may have a pivotal role in adipocyte hypertrophy, obesity and insulin resistance. In this study, we generated transgenic mice with the gain-of-function PPARγ Ser112Ala mutation (S112A mice) using the aP2 promoter, to elucidate the impact of increased PPARγ activity in mature adipocytes. Despite a 2–3-fold increase in the adipocyte PPARγ2 gene expression and PPARγ activity, the S112A mice showed comparable adiposity and insulin sensitivity to wild-type mice under both normal and HFD conditions. Although the expression levels of the PPARγ target genes involved in lipid metabolism, such as aP2 and stearoyl-CoA desaturase 1, were upregulated in the white adipose tissue of the S112A mice, the serum levels of free fatty acid, triglyceride, adiponectin and leptin, as well as the oxygen consumption, were comparable between the wild-type and S112A mice under the HFD condition. Moreover, treatment with rosiglitazone ameliorated insulin resistance and glucose intolerance to a similar degree in the two genotypes under the HFD condition. 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.

Key words: Peroxisome proliferator-activated receptor γ, Rosiglitazone

ligands, are widely used in the treatment of type 2 diabetes [1]. TZD-induced PPAR\(\gamma\) activation increases the plasma levels of the insulin-sensitizing adipokine, adiponectin [8], and simultaneously decreases the plasma levels of free fatty acid (FFA) and insulin-resistance-causing adipokines such as TNF\(\alpha\) and resistin [8]. Thus, PPAR\(\gamma\) activation by TZD ameliorates insulin resistance, whereas heterozygous PPAR\(\gamma\)-deficient mice are protected from HFD-induced adipocyte hypertrophy, obesity and insulin resistance [3, 4].

PPAR\(\gamma\) activity is regulated by mitogen-activated protein (MAP) kinase phosphorylation of the serine 112 residue, which reduces its transcriptional activity [9]. The PPAR\(\gamma\) Ser112Ala (S112A) mutation has been shown to be non-phosphorylable and indeed more active than wild-type PPAR\(\gamma\) [9]. S112A knock-in mice showed improved insulin sensitivity without body weight gain [10]. A gain-of-function PPAR\(\gamma\) mutation in humans had no impact on the insulin resistance, but induced obesity [11]. These phenotypes can probably be explained by the induction of PPAR\(\gamma\) expression in the early stage of differentiation of preadipocytes [12]. In contrast, overexpression of wild-type PPAR\(\gamma\) in mature 3T3L1 adipocytes increased both the cell size and intracellular TG content [13]. Although expression of PPAR\(\gamma\)2 is maintained at a high level even in mature adipocytes [14], the physiological role of increased PPAR\(\gamma\) activity in mature adipocytes in vivo remains unknown.

To understand the role of increased PPAR\(\gamma\) activity in mature adipocytes in vivo, we produced transgenic mice with the PPAR\(\gamma\)2 S112A mutation to activate PPAR\(\gamma\) under the aP2 promoter. Moreover, we examined the effect of increased PPAR\(\gamma\) activity in mature adipocytes on TZD-induced amelioration of insulin sensitivity.

Materials and Methods

Generation of transgenic PPAR\(\gamma\)2-S112A mutant mice

The mouse PPAR\(\gamma\)2 cDNA (1.8 kb) with the S112A mutation, generated by PCR mutagenesis, was subcloned into the KpnI-SacI site of pBluescript SK(−) (Stratagene, La Jolla, CA, USA). PPAR\(\gamma\)2-S112A cDNA was ligated into the unique EcoRI site between the rabbit \(\beta\)-globin intron and the polyadenylation signal. The resultant fragment was ligated into the Smal site of a plasmid containing the aP2 promoter in pBluescript SK(−). The DNA fragment was excised from its plasmid by digestion with ClaI-NotI, then purified and microinjected into the pronuclei of fertilized eggs obtained from C57Bl/6N mice (Nippon CREA Co. Ltd., Tokyo, Japan). Transgenic founder mice were identified by Southern blot analysis of tail DNAs. F1 offspring were then crossed with C57Bl/6N mice to establish a transgenic lineage. Male mice with access to food ad libitum and reared under a light-dark cycle of 12 h were used for the experiments at 8–10 weeks of age. All experimental procedures conformed to the guidelines of the Animal Care Committee of the University of Tokyo.

Genotyping by PCR

Genotype was determined by PCR analysis of genomic DNA obtained from tail snips. To detect the S112A transgene, the sense primer corresponded to sequences in the rabbit \(\beta\)-globin intron (5’-TTATTGGTAGAAACAACCTACATCCT-3’), and the antisense primer corresponded to sequences in the coding region of PPAR\(\gamma\) (5’-ATATTTGTAATCAGCAACCATTGGGG-3’). To detect the wild-type allele, the sense primer corresponding to sequences in the coding region of PPAR\(\gamma\) (5’-AACCCTGGAATCAGCTCTGTGGACC-3’) and the same antisense primer as that indicated above for detection of the S112A transgene, were used. The three primers and a genomic DNA template were mixed in a tube. The thermal reaction cycles consisted of an initial step at 94°C for 5 min, followed by 35 cycles of 94°C for 30 seconds, 55°C for 1 min and 72°C for 1 min, with a final step at 72°C for 5 min. The wild-type allele yielded a 393 base-pair (bp) product, and the transgene, a 529 bp product.

In vivo glucose homeostasis

Glucose tolerance test: The mice were fasted for more than 16 h before the study. They were then given 1.0 mg/gram (body weight) of glucose orally. Blood samples were collected at different time-points and the blood level of glucose was measured immediately with an automatic blood glucose meter (Glutest Pro: Sanwa Chemical, Nagoya, Japan). Whole blood specimens were collected and centrifuged, and the serum samples obtained after separation were stored at −20°C. Insulin levels were determined with an insulin
radioimmunoassay (RIA) kit (BIOTRAK: Amersham Biosciences, Buckinghamshire, UK) using rat insulin as the standard.

Insulin tolerance test: Mice were given free access to chow before the study. They were administered an intraperitoneal injection of 0.75 mU/gram (body weight) of human insulin (Humulin R: Lilly, Indianapolis, IN, USA). Blood samples were taken at different time-points from the tail vein and the blood levels of glucose were measured with an automatic blood glucose meter.

RNA preparation, Northern blot analysis and real-time quantitative PCR

Total RNA was prepared from epididymal WAT with TRIzol (invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Northern blot analysis was performed using the standard protocol. A 20 μg sample of total RNA was electrophoresed through denaturing formaldehyde-agarose (1%) gel and then transferred to a Hybond N+ nylon membrane (Amersham Biosciences). Real-time quantitative PCR was performed on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) using TaqMan PCR Master Mix Reagent Kit (Applied Biosystems), with cyclophilin as the internal control. The primers and probes for the other genes were purchased from Applied Biosystems.

Generation of probe and RNase protection assay (RPA)

A partial murine PPARγ cDNA probe was generated by reverse transcriptase-PCR using total RNA and primers designed to amplify a region including 90 bp of the PPARγ2 transcript and a 185 bp region common to both PPARγ1 and PPARγ2, as previously described [15]. The PPARγ cDNA PCR product was subcloned into the PstI-EcoRI site of the pBluescript SK(−) (Stratagene). The antisense cRNA was transcribed with [α-32P]UTP (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA) using the T7 RNA polymerase (MAXIscript: Ambion, Inc., Austin, TX, USA). A 10 μg sample of total RNA was subjected to RPA using an RPAIII kit (Ambion, Inc.) and hybridized overnight with the cRNA probe in Hybridization III Buffer (Ambion, Inc.) at 42°C. After digestion with RNaseA/RNaseT1 diluted 1 : 100, the protected fragments were separated on 8 M urea/5% polyacrylamide gels and analyzed quantitatively using an imaging plate and BAS2000 (Fuji Film, Tokyo, Japan).

Diets and rosiglitazone treatment

The normal diet (MF) was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The diet was composed of 5.3% (wt/wt) fat, 23.6% protein, 54.4% carbohydrate, 2.9% dietary fiber, and 6.1% minerals. The HFD, containing 32% safflower oil, 33.1% casein, 17.6% sucrose and 5.6% cellulose, was prepared as described previously [3]. Rosiglitazone (BRL49653), procured from GlaxoSmithKline (Brentford Middlesex, UK), was given at a 0.01% (wt/wt) mixture with the HFD.

Histochemistry

Epididymal adipose tissue was removed from each animal, fixed in 10% formaldehyde/PBS, and stored at 4°C until use. The tissue specimens were routinely processed for paraffin embedding, and 5-μm sections were cut and mounted on silanized slides. The adipose tissue was stained with hematoxylin and eosin, and the total adipocyte area was analyzed with Win ROOF software (Mitani Co., Ltd., Chiba, Japan). The white adipocyte area was measured in 400 or more cells per mouse in each group as described previously [3].

Blood sample assays

The serum levels of FFA and TG were determined by a nonesterified fatty acid-C test and triglyceride L-type test (Wako Pure Chemical Industries, Japan), respectively. The serum levels of adiponectin and leptin were assayed with a mouse adiponectin immunoassay kit (Otsuka Pharmaceutical, Tokushima, Japan) and Quantikine M kit (R&D Systems, Minneapolis, MN), respectively.

Energy expenditure

Oxygen consumption was measured every 3 minutes for 24 h in fasting mice using an O2/CO2 metabolism
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 PPAR\( \gamma \) activity in the S112A mouse adipocytes**

Transgenic mice with the PPAR\( \gamma \)2 S112A mutation expressed under the control of the aP2 promoter were established to investigate the physiological role of increased PPAR\( \gamma \) 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 transgens carrying 2 copies expressed PPAR\( \gamma \)2 in the adipose tissue. While a 3-fold higher PPAR\( \gamma \)2 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 PPAR\( \gamma \)1 (Fig. 1C). The mRNA levels of aP2, stearoyl-CoA desaturase (SCD)1 and C/EBP\( \alpha \), downstream target genes of PPAR\( \gamma \), were significantly upregulated in the S112A mice (Fig. 1D), confirming that enhanced PPAR\( \gamma \) 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 PPAR\( \gamma \)2 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 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-
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.
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

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**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.
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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