Subcutaneous or Visceral Adipose Tissue Expression of the PPARγ Gene Is Not Altered in the Fatty (fa/fa) Zucker Rat

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We cloned 537 basepairs (bp) of rat partial peroxisome proliferator-activated receptor γ2 (PPARγ2) cDNA and examined the effect of fasting or obesity on the expression of two isoforms of rat PPARγ, γ1 and γ2, in either subcutaneous or mesenteric adipose tissue specimens using an RNase A protection assay. In Wistar rats, expression of both isoforms was dramatically reduced after 48 hours of fasting in the two fat tissue specimens. In comparing genetically obese (fa/fa) Zucker rats and lean control rats, no significant difference was observed in expression of the two isoforms in either type of adipose tissue. From these findings, we conclude that the adipose tissue level of rat PPARγ depends on nutritional deprivation but is not closely associated with either obesity or insulin resistance in obese Zucker rats.

A NEW SUBCLASS of the peroxisome proliferator-activated receptor (PPAR) family, PPARγ, is preferentially expressed in fat cells and plays an essential role in adipogenesis.[1] PPARγ exists as two spliced variants from a single gene, namely PPARγ1 and PPARγ2, which differs from γ1 only by 30 additional amino acids at the N-terminal extremity.[2-10] Forced expression of either PPARγ1 or PPARγ2 in fibroblast and myoblast cell lines results in efficient adipocyte differentiation in a PPARγ activation–dependent manner.[1,11] Moreover, the antidiabetic thiazolidinedione, which increases insulin sensitivity of target tissues in non–insulin-dependent animal models[12] and humans,[13] was shown to be a high-affinity ligand for PPARγ1 and PPARγ2.[14] PPARγ is negatively regulated by tumor necrosis factor alpha, which has been recognized as a leading candidate for inhibition of insulin action.[15] Based on these findings, PPARγ has been implicated as one of the crucial factors responsible for the mechanisms of obesity and insulin sensitivity.

Although the degree of adipose tissue expression of PPARγ has been examined in murine models of obesity and in obese humans, conflicting results were obtained between these species. Namely, no change in the expression of PPARγ in perigonadal fat in two of three murine models of obesity was demonstrated,[16] whereas significantly higher expression of PPARγ2 in subcutaneous fat in obese humans was reported.[17] However, nutritional deprivation by either fasting or a low-calorie diet causes a relatively dramatic decrease in γ2 expression in both species.[16,17] In the rat, even the cDNA structure of PPARγ1 or PPARγ2 remains unclear, and thus, regulation of the PPARγ gene in this species is still poorly understood.

In the present study, we cloned rat partial PPARγ2 cDNA and established a RNase A protection assay that permits simultaneous quantitation of both γ1 and γ2 isoforms. Using this method, we examined the regulation of the rat PPARγ gene by nutritional changes in Wistar rats and the regulation by obesity in Zucker fatty rats as an established animal model of obesity and insulin resistance.[18-20] In particular, regional differences in the expression of both isoforms were examined between subcutaneous and mesenteric fat, since marked differences in the metabolism and physiology of these two fat tissues have been observed and thus implicated in the pathogenesis of various metabolic complications in Zucker fatty rats[20,21] and in human subjects.[22]

MATERIALS AND METHODS

Animals and Experimental Protocols

Male Wistar rats, male genetically obese (fa/fa) Zucker rats, and lean (Fa/?) control rats were bred under specific pathogen-free conditions at the Kyushu University Animal Center (Fukuoka, Japan). They were maintained on a 12-hour light/dark photoperiod and fed a commercial pelleted chow ad libitum. The following experiments were approved by the animal center and performed according to the ethical guidelines for animal experiments at our institution. The effect of fasting was examined in Wistar rats at 18 weeks of age. One group (n = 3) was normally fed a laboratory chow diet, and the other group (n = 3) was completely fasted for 48 hours. Both groups were allowed to drink tap water ad libitum. After 48 hours, the rats were killed and abdominal subcutaneous and mesenteric (visceral) fat tissue was removed and immediately frozen at −80°C. Next, basal expression of PPARγ in adipose tissue was compared between male obese Zucker rats (n = 6) and control lean rats (n = 6). Both obese and lean rats were killed at 18 weeks of age, and abdominal subcutaneous and mesenteric (visceral) fat tissue specimens were removed and immediately frozen at −80°C. Blood concentrations of glucose and insulin at 18 weeks were measured as previously described,[23] and the respective values and body weight are shown in Table 1.

cDNA Cloning of Rat PPARγ2

Total RNA of adipose tissue from the Wistar or Zucker rats was extracted using Isogen (Waco Junyaku, Osaka, Japan). The total RNA from Wistar rats was used as a template for cDNA synthesis. First, 413 basepairs (bp) of partial rat PPARγ1 cDNA were obtained by reverse transcription–polymerase chain reaction (RT-PCR)[24] using sense/antisense primers (5′ATTCTGGCCCACCAACTTCGG3′/5′TATCATAAATAAGCTTCAATCGGATGGTTC3′) based on the mouse PPARγ1 cDNA sequence.[5] Rat PPARγ1 cDNA contained the common N-terminal region of both γ1 and γ2. Next, to obtain rat cDNA containing the γ2-specific region, 5′-RACE (rapid amplification of cDNA ends) was performed using the Marathone cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA). Namely, rat PPARγ2 cDNA was obtained by amplification from the adapter-ligated double-stranded cDNA using a sense (adapter) primer and an antisense primer (cDNA-specific primer, 5′TATCATAAATAAGCTTCAATCGGATGGTTC3′). Finally, the 5′-RACE product was subcloned into pBluescript SK (−)

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Table 1. Body Weight and Blood Concentrations of Glucose and Insulin in Lean and Fatty (fa/fa) Zucker Rats

Parameter	Lean (n = 6)	Fatty (n = 6)
Body weight (g)	380.1 ± 13.7	510.4 ± 9.3†
Glucose (mmol/L)	5.86 ± 0.80	7.14 ± 0.46
Insulin (nmol/L)	0.24 ± 0.05	5.77 ± 1.81*

NOTE. Values are the mean ± SE.
*$P < .05$ *v* lean.
†$P < .01$ *v* lean.

(Stratagene, La Jolla, CA), and four clones were sequenced by the dideoxy chain-termination[25] method to determine the correct sequence.

RNase A Protection Assay

To determine the expression level of rat PPARγ1 and PPARγ2 mRNA in adipose tissue, a RNase A protection assay was performed.[7] Based on the sequence of rat PPARγ2 cDNA clarified by the above-mentioned method, 444 bp of the PCR product using sense/antisense primers P1/P2 (Fig 1) were subcloned into pBluescript SK (−). The two primers were designed to amplify a region that includes 90 bp of the γ2-specific sequence and 354 bp common to both γ1 and γ2. The plasmid was linearized with *Hin*dIII and then used as a template for sense [^{32}P]-labeled riboprobe synthesis. The use of the antisense probe in a RNase A protection assay permits quantitation of PPARγ mRNA transcripts as protected bands of either 444 nucleotides (nt) (PPARγ2) or 354 nt (PPARγ1). A [^{32}P]-labeled RNA probe (480 nt) complementary to the 444-bp DNA template plus 36 bp of pBluescript was prepared using T7 RNA polymerase in the presence of [α^{32}P]-UTP (Amersham, Bucks, UK) using an in vitro transcription system (Clontech). A RNA probe complementary to 185 bp of rat β-actin plus 119 bp of pBluescript was also prepared by similar procedures. Procedures for hybridization of the cRNA probe with the total RNA (5 μg) from adipose tissue, treatment of the samples with RNase A1 and RNase T1, and electrophoresis on a denaturing polyacrylamide gel (5%) were all performed exactly as previously described.[7] The results were visualized by autoradiography, and the signal intensity of protected RNA bands was quantified by densitometric scanning (FUSIX BAS2000; Fuji Photo Film, Tokyo, Japan). PPARγ1 and PPARγ2 expression were normalized to that of β-actin. A statistical analysis was performed using the *t*-independent Student's *t* test.

Fig 1. Partial nt sequence and deduced amino acid sequence of rat PPARγ2 cDNA. Bold letters indicate the first 30–amino acid sequence specific to PPARγ2. The sequence starting from the ATG codon at amino acid position 31 designated by the arrow is common to both PPARγ1 and PPARγ2. Sense and antisense PCR primers, P1 and P2, used to make a DNA template for cRNA synthesis are thickly underlined. Italic letters indicate differences in amino acids in human PPARγ2 (7-10). The number at right indicates the amino acid number.

RESULTS

Sequence analysis of the 5′-RACE product revealed 537 bp of partial rat PPARγ2 cDNA, including a 45-bp 5′-untranslated sequence and γ2-specific 90-bp sequence encoding an additional 30 amino acids N-terminal to the first ATG codon of rat PPARγ1. The amino acid sequence of rat PPARγ2 was identical to the corresponding region of mouse PPARγ2 except for the fact that ^{8}Pro and ^{54}Asp replace ^{8}Ser and ^{54}Glu in the mouse, respectively. However, the rat sequence was different from the corresponding region of human PPARγ2, especially in the γ2-specific region (Fig 1).

Clarification of the γ2-specific sequence in the rat has enabled us to determine adipose tissue expression of the γ1 and γ2 isoforms relative to β-actin by a RNase A protection assay in this species. We first studied the effect of fasting on the expression of both isoforms in Wistar rat adipose tissue. Under normal feeding conditions, expression of γ1 was 1.6 times higher than expression of γ2 in both mesenteric and subcutaneous fat. Expression of both PPARγ1 and PPARγ2 isoforms in mesenteric fat was 1.8 times higher than in subcutaneous fat. Fasting for 48 hours caused a 35% and 38% reduction in the expression of γ1 and γ2 isoforms in subcutaneous adipose tissue, respectively. Similarly, the treatment also caused a 43% and 46% reduction in the expression of PPARγ1 and PPARγ2 isoforms in mesenteric adipose tissue, respectively. Thus, the γ1/γ2 ratio did not significantly change with fasting treatment in either type of adipose tissue (Figs 2 and 3).

We next examined the expression of γ1 and γ2 isoforms in adipose tissue of obese or lean Zucker rats. The respective size of the protected band for PPARγ1 and PPARγ2 was the same in Zucker rats as in Wistar rats, thus indicating that the corresponding sequence of PPARγ2 cDNA in Zucker rats is the same as in Wistar rats. In Zucker obese and control lean rats, mRNA levels of both isoforms were significantly lower in mesenteric fat than in subcutaneous fat. However, in comparing obese and lean rats, there was no significant difference in expression of the two isoforms in either type of adipose tissue. The γ1/γ2 ratio in either subcutaneous or mesenteric adipose tissue was not significantly different between lean and fatty rats (Figs 4 and 5).

DISCUSSION

We revealed a partial PPARγ2 cDNA sequence including the γ2-specific 90-bp nt sequence that encodes an additional 30 amino acids N-terminal to the first ATG codon of rat PPARγ1, as in mouse or human PPARγ2.[6-10] The significance of the presence of two isoforms, γ1 and γ2, in tissue is still not well understood, since no functional difference between these two isoforms has been demonstrated.[1,14] However, it was recently reported that a ligand-independent activation function of isoform γ2 is fivefold to sixfold greater than that of γ1[26] and that a relatively predominant change in expression of the γ2 isoform rather than γ1 in adipose or nonadipose tissue occurs under certain conditions,[16,17] thus suggesting some functional and regulational differences between these two isoforms. In this

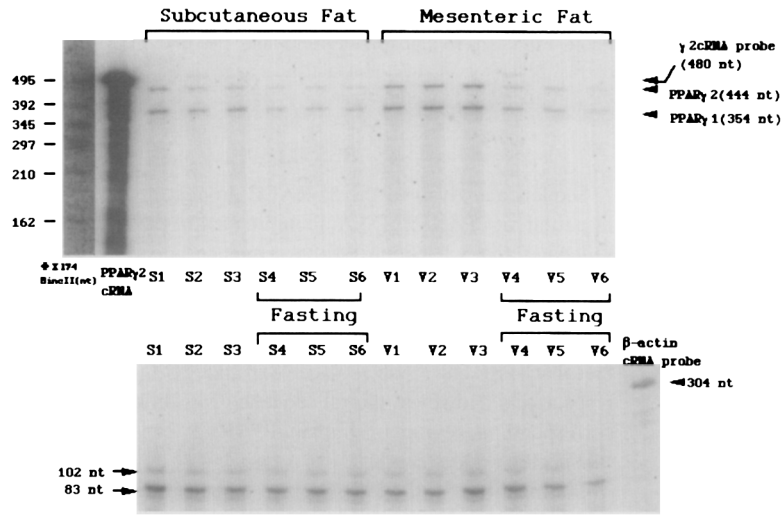

Fig 2. RNase protection assay demonstrating the expression of rat PPARγ1, PPARγ2, and β-actin in subcutaneous and mesenteric adipose tissue specimens from Wistar rats treated with or without 48 hours of fasting. S1-S6 and V1-V6 indicate subcutaneous and visceral (mesenteric) adipose tissue from rats 1-6, respectively. The 444 and 354 nt fragments indicate protected bands of rat PPARγ2 and PPARγ1, respectively. The protected bands for rat β-actin consisted of 2 fragments, namely 102 and 83 nt fragments, because of 1 base nt mismatch, and only the 83 nt fragment was used as an expression control against rat PPARγ. The [^{32}P]-end-labeled ΦX174-*Hin*cII fragment was used as a size marker.

respect, simultaneous quantitation of γ1 and γ2 expression is most informative for elucidating the physiological significance of PPARγ in tissue.

Despite the initial finding of exclusive expression of the γ2 isoform in mouse cultured fat cells,[5] the predominant expression of PPARγ1 over γ2 was observed in all rat adipose tissue specimens examined in the present study, as in human and mouse adipose tissue.[7,16,17] However, the ratio of γ1/γ2 (1.6 to 3) found in the rats was relatively smaller than in humans (2 to 10)[9,17] and mice (2 to 5).[16] The different degrees of promoter usage for γ1 and γ2 may lead to the different ratios among species. In Wistar rats, fasting for 48 hours caused a dramatic reduction in expression of the γ1 and γ2 isoforms in both mesenteric and subcutaneous adipose tissue. Our findings basically support the recent report showing a reduced expression of both isoforms in the fasted mouse perigonadal pad,[16] except that the downregulation was more specifically observed in γ2 in the mouse,[16] whereas no such specificity was evident in our experiment. This may simply be due to the species difference or the difference in the experimental protocol, since the effect of feeding and fasting in the previous study was sequentially evaluated using an identical mouse,[16] thus resulting in a more dramatic change in the γ2 isoform. Nevertheless, the reduced adipose tissue expression of PPARγ in rodents and humans by nutritional deprivation may be partly caused by the decrease of insulin secretion, considering that insulin upregulates expression of the PPARγ gene in cultured fat cells.[17]

There are two types of obesity: subcutaneous fat and intraabdominal visceral fat. In humans, visceral fat deposition has been shown to be more frequently accompanied by the complications of hyperinsulinemia, hyperlipidemia, and cardiovascular disease than the subcutaneous type.[22] A spontaneous

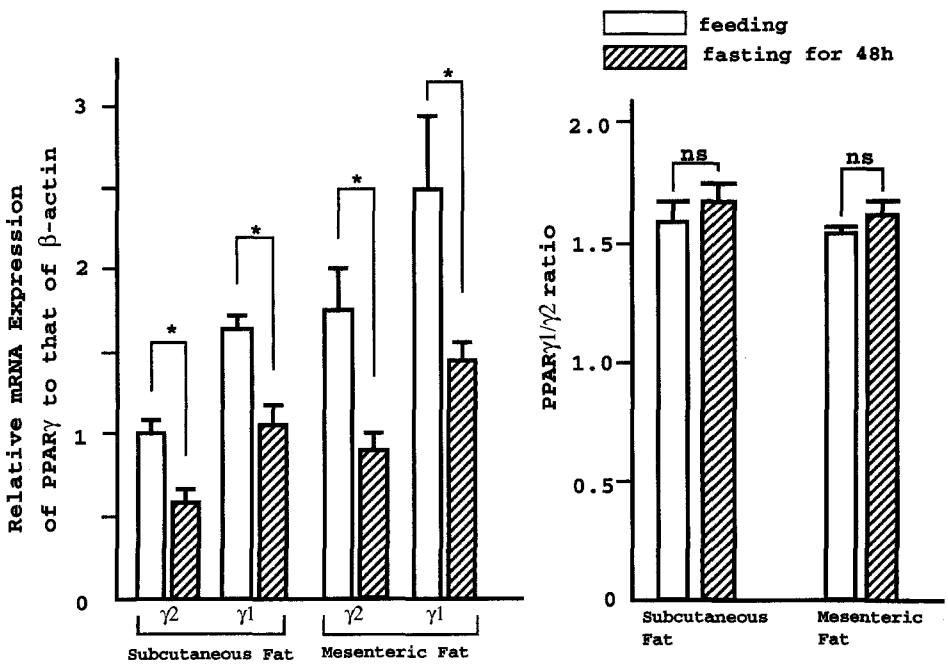

Fig 3. Statistical analysis of the effect of fasting on expression of rat PPARγ mRNA relative to β-actin mRNA and the γ1/γ2 ratio in adipose tissue of Wistar rats based on results in Fig 2. Values are the mean ± SE. NS, nonsignificant. *$P < .05$ *v* feeding.

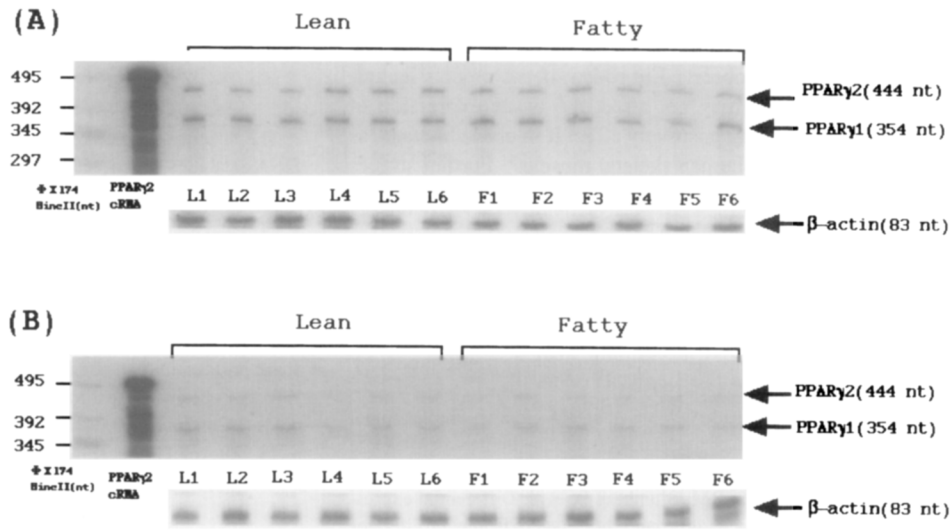

Fig 4. RNase protection assay demonstrating expression of rat PPARγ1, PPARγ2, and β-actin in subcutaneous (A) and mesenteric (B) adipose tissue from lean and fatty Zucker rats. L1-L6 and F1-F6 indicate lean and fatty rats, respectively. Only the 83 nt fragment of β-actin was used as an expression control (see Fig 2).

genetic obesity model, the Zucker fatty (fa/fa) rat, in contrast to the Zucker lean rat, is characterized by hyperphagia, hyperinsulinemia, hypertriglyceridemia, and excessive deposition of lipid throughout the body, similar to human obesity.[20,21] It has been recently reported that a missense mutation from glutamine at codon 269 to proline in the leptin receptor might play a role in the pathogenesis of obesity.[27] Even so, the mechanism for excessive fat deposition and metabolic abnormalities including insulin resistance in the Zucker fatty rat has yet to be elucidated. In Zucker fatty rats, it has also been reported that there is a larger volume of mesenteric fat cells and the cell number also increases faster than subcutaneous fat cells.[20] It is therefore of interest to examine whether the altered expression of the PPARγ gene may be associated with obesity and the regional metabolic differences in adipose tissue of the Zucker fatty rat. However, when comparing obese and lean rats, no significant difference in the expression of both isoforms was observed in either mesenteric or subcutaneous adipose tissue. These results suggest that the adipogenicity or regional metabolic difference in fat tissue of the Zucker rat may not be simply determined by altered expression of the PPARγ gene. Enhanced expression of lipogenetic enzymes such as acyl-coenzyme A synthetase and lipoprotein lipase may be essential for the adipogenicity in obese Zucker rats.[28] Although it still remains unclear as to what determines the different metabolic characteristics between subcutaneous and visceral fat, some genes are reported to be predominantly expressed in the visceral fat of humans.[29] Plasminogen activator inhibitor-1 is one such gene, and its enhanced expression in visceral fat has been suggested to contribute to vascular disease.[30]

No change in PPARγ gene expression in adipose tissue also has been demonstrated in the *ob/ob* or GTG obese mouse.[16] In contrast, increased expression of PPARγ2 in subcutaneous fat in obese humans has been reported.[17] This may simply reflect a species difference in the regulation of the PPARγ gene in adipose tissue or a difference in the contribution of genetic or

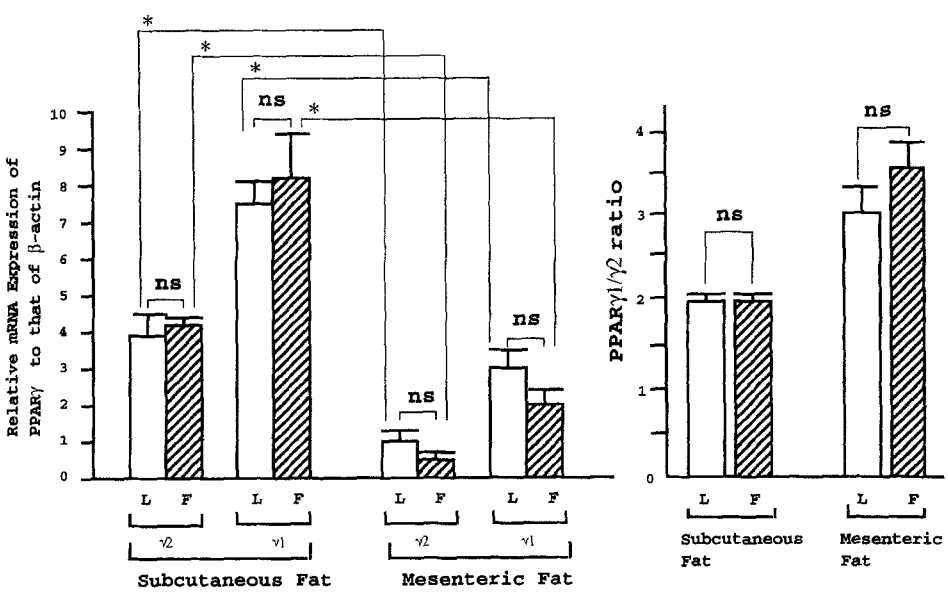

Fig 5. Statistical analysis of the expression of rat PPARγ mRNA relative to β-actin mRNA and the γ1/γ2 ratio in Zucker lean (L) and fatty (F) rats based on results in Fig 4. Values are the mean ± SE. NS, nonsignificant. *$P < .05$.

environmental factors between rodent obese models and human obese subjects. One possibility to explain the difference is that the hyperinsulinemia observed in rodent obese models including Zucker fatty rats may not affect the tissue expression of PPARγ, whereas it may positively affect it in obese humans, as strongly suggested in human skeletal muscle.[31] Further studies are needed to elucidate the significance of PPARγ in the pathogenesis of obesity and insulin resistance.

NOTE

The nt sequence data reported herein are registered in the GenBank DNA databases with accession no. AB000831.

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