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Inhibitory Effect of a Proline-to-Alanine Substitution at Codon 12 of Peroxisome Proliferator-Activated Receptor-γ 2 on Thiazolidinedione-Induced Adipogenesis

Jiro Masugi, Yoshikazu Tamori,[1] Hiroyuki Mori, Takashi Koike, and Masato Kasuga
Second Department of Internal Medicine, Kobe University School of Medicine, Kobe, Japan

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Peroxisome proliferator-activated receptor-γ (PPARγ) is a member of the nuclear hormone receptor superfamily of transcription factors and appears to be a key regulator of adipogenesis. Members of the thiazolidinedione class of insulin-sensitizing agents act as high-affinity ligands for PPARγ, indicating that PPARγ is also important in systemic insulin action. To determine whether Pro[12] → Ala (P12A) mutation in PPARγ gene contributes to the development of obesity or insulin sensitivity, we examined the effects of the P12A mutation on the function of PPARγ by expression of the mutant protein in COS or 3T3-L1 cells. The abilities of the P12A mutant of PPARγ to mediate both transcriptional activation of a luciferase reporter gene construct containing the peroxisome proliferator response element and adipogenesis induced by a thiazolidinedione drug were reduced compared with those of the wild-type protein. These results suggest that the P12A substitution in PPARγ gene may be associated with abnormalities of adipose tissue formation and insulin sensitivity.

Peroxisome proliferator-activated receptor-γ (PPARγ) belongs to the nuclear hormone receptor superfamily and a central regulator of adipose cell differentiation (1). Like other members of the PPAR family, PPARγ forms a heterodimer with the retinoid X receptor (RXR), and this dimer binds to enhancers of adipocyte-specific genes that contain a peroxisome proliferator response element (PPRE) (2, 3). Effective modulation of the transcription of such adipocyte-specific genes by the PPARγ-RXR heterodimer requires the presence of a PPAR activator and an RXR ligand (1, 3).

Thiazolidinediones (TZDs), a class of insulin-sensitizing compounds effective in the treatment of Type 2 (non-insulin-dependent) diabetes mellitus (4, 5), bind to PPARγ with high affinity (6), and no other receptor for the TZDs has been identified. The rank order of potency for the antidiabetic activities of these compounds closely matches the rank order of their affinities for PPARγ (7). Furthermore, each TZD drug activates PPARγ in the same concentration range as that in which it exhibits antidiabetic activity (7). Agonists of the RXR also increase insulin sensitivity in diabetic and obese mice (8). These observations thus suggest that the insulin-sensitizing effects of TZDs are mediated through activation of PPARγ.

A Pro[115] → Gln mutation in PPARγ2 has been implicated as a rare cause of morbid obesity (9). Overexpression of this mutant protein in murine fibroblasts resulted in an increase in the rates of both differentiation into adipocytes and accumulation of triglycerides, compared with those apparent in cells expressing wild-type PPARγ2 (9). Although several studies have also detected a Pro[12] → Ala (P12A) polymorphism of PPARγ2 (10–18), the effects of this mutation remain unclear. For example, Deeb *et al.* (14) showed that the P12A substitution in PPARγ2 is associated with a reduced body mass index (BMI) and improved insulin sensitivity among Finns. In contrast, Beamer *et al.* (12) showed that the P12A variant is associated with an increased BMI in two Caucasian populations.

Since the results of the subjects with P12A polymorphism in PPARγ gene are controversial, we examined the ability of the mutant protein to activate transcription and to induce adipogenesis to assess the significance of P12A mutation in PPARγ gene.

MATERIALS AND METHODS

Materials. 3T3-L1 cells and COS cells were obtained from American Type Culture Collection; human embryonic kidney 293 cells and

Abbreviations used: PPARγ, peroxisome proliferator-activated receptor-γ; RXR, retinoid X receptor; PPRE, peroxisome proliferator response element; TZD, thiazolidinedione; BMI, body mass index; PCR, polymerase chain reaction; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium.

[1] To whom correspondence should be addressed at Second Department of Internal Medicine, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Fax: +81-78-382-2080. E-mail: tamori@med.kobe-u.ac.jp.

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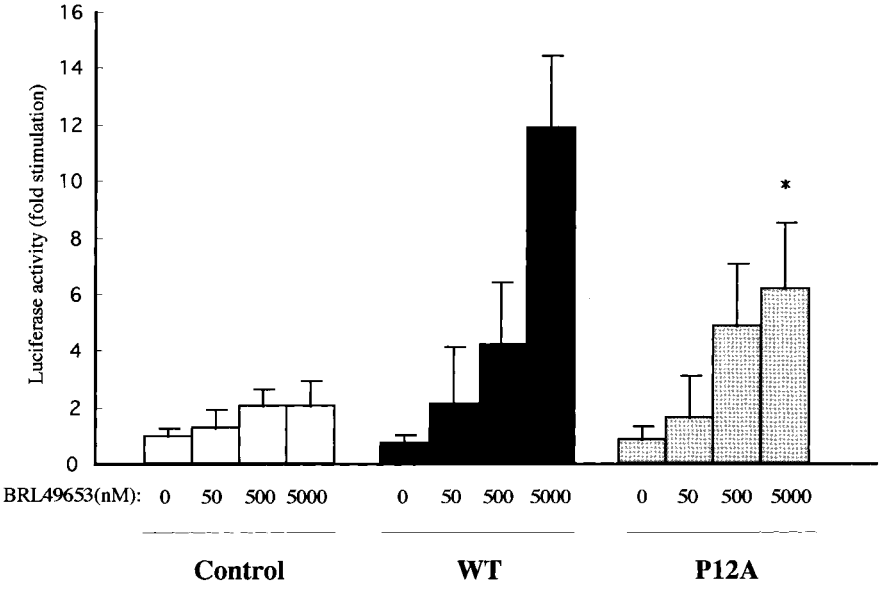

FIG. 1. Transcriptional activities of PPARγ2-WT and PPARγ2-P12A. COS cells were transfected with the reporter plasmid PPRE3-tk-luc, pSV-β-galactosidase, pSG5-RXR, and either pcDL-SRα alone (control) or pcDL-SRα containing PPARγ2-WT or PPARγ2-P12A cDNA. After incubation of cells in the presence of the indicated concentrations of BRL49653 for 24 h, cell lysates were prepared and assayed for luciferase and β-galactosidase activities. Luciferase activity was normalized by β-galactosidase activity and expressed as fold stimulation by BRL49653. Data are means ± SD ($n = 8$); *$p < 0.01$ vs. corresponding value for cells overexpressing PPARγ2-WT (t test).

restriction endonucleases were from Takara Shuzo (Shiga, Japan); mouse monoclonal antibody 12CA5 to the hemagglutinin (HA) epitope tag was from Boehringer Mannheim; charcoal-treated calf serum was from Sigma; and Dulbecco's modified Eagle's medium (DMEM) and Lipofectin were from Gibco. The TZD drug BRL49653 was kindly provided by SmithKline Beecham (Harlow, UK). DNA-TPC and the pAdex1CAwt vector were kindly provided by Izumu Saito (University of Tokyo, Japan). A reporter plasmid containing three copies of the acyl-coenzyme A oxidase gene PPRE upstream of the thymidine kinase promoter and luciferase gene (PPRE3-tk-luc) and the expression vector pSG5-mouse RXR were kindly provided by Kazuhiko Umesono (University of Kyoto, Japan) and Keiko Ozato (National Institutes of Health, Bethesda, MD), respectively.

Construction of plasmids. Mouse PPARγ2 cDNA was obtained by reverse transcription and PCR with mRNA from 3T3-L1 adipocytes. Appropriate restriction enzyme sites and a sequence encoding a COOH-terminal HA epitope tag were introduced into the wild-type cDNA by PCR with the upstream primer 5′-ATTCTAGAGTTATG-GGTGAAACTCTGGGA-3′ and the downstream primer 5′-TAG-GTACCTCATGCGTAGTCGGGAACATCGTACGGATACAAGTCCT-TGTAGATCTCCTG-3′. The PCR product was cloned into the Xba I-Kpn I site of pBluescriptII-KS vector (Stratagene) to yield pBS-PPARγ2-WT-HA. A corresponding plasmid encoding the P12A mutant was obtained by PCR-mediated mutagenesis with pBS-PPARγ2-WT-HA. The sequences of both wild-type and P12A clones were confirmed experimentally.

Preparation of recombinant adenoviruses. Recombinant adenoviruses were prepared as described previously (19). In brief, the cDNAs encoding HA-tagged wild-type and P12A PPARγ2 proteins were subcloned separately into the pAdex1CAwt adenovirus vector (20), which contains the CAG promoter (21). The resulting constructs were transfected together with DNA-TPC into 293 cells with the use of CellPhect (Pharmacia). A single clone of each recombinant adenovirus (Adex-PPARγ2-WT and Adex-PPARγ2-P12A) was isolated through serial dilution with a plaque assay. Viral titer was determined by plaque assay.

Assay of transcriptional activation by PPARγ2. COS cells, cultured in 24-well plates and with DMEM supplemented with 10% fetal bovine serum, were transfected with the use of Lipofectin with the reporter plasmid PPRE3-tk-luc (50 ng), with the expression vector pcDL-SRα (22) containing PPARγ2-WT or PPARγ2-P12A cDNA (or pcDL-SRα alone) (50 ng), with the expression vector pSG5-mouse RXR (50 ng), and with the internal control plasmid pSV-β-galactosidase (50 ng). After 24 h, the medium was replaced by DMEM supplemented with 10% charcoal-treated calf serum in the absence or presence of the indicated concentrations of BRL49653, and cells were incubated for an additional 24 h. Cells were then lysed and assayed for luciferase and β-galactosidase activities. Luciferase activity was normalized by β-galactosidase activity and expressed as fold stimulation.

RESULTS

Transcriptional Activation by Wild-Type and Mutant PPARγ2

The transcriptional activities of the wild-type (PPARγ2-WT) and mutant (PPARγ2-P12A) PPARγ2 proteins were compared by inducing their transient expression in COS cells, with the use of the expression vector pcDL-SRα, and assaying their abilities to activate transcription of a luciferase reporter gene under the control of the PPRE. Immunoblot analysis with antibodies to the HA epitope tag confirmed that PPARγ2-WT and PPARγ2-P12A were expressed in similar amounts in the transfected cells (data not shown). The TZD BRL49653 (5 μM) increased luciferase activity about twofold in control cells transfected with the empty pcDL-SRα vector, an effect likely mediated by endogenous PPARγ (Fig. 1). In contrast, this

TZD drug markedly increased luciferase activity in a dose-dependent manner in cells overexpressing PPARγ2-WT, with an ~12-fold increase apparent at a BRL49653 concentration of 5 μM. Although BRL49653 also induced a dose-dependent increase in luciferase activity in cells overexpressing PPARγ2-P12A, the approximately sixfold increase apparent at a drug dose of 5 μM was significantly smaller than the effect of the same concentration of this drug in cells overexpressing PPARγ2-WT. The TZD-induced transcriptional activity of PPARγ2-P12A was thus less than that of PPARγ2-WT in COS cells.

Effects of PPARγ2-WT and PPARγ2-P12A on Adipogenesis

The effects of PPARγ2-WT and PPARγ2-P12A on adipogenesis were investigated by infecting 3T3-L1 preadipocytes with corresponding adenovirus vectors (Adex-PPARγ2-WT and Adex-PPARγ2-P12A, respectively) for 8 h. Two days after infection, adipogenesis was induced by incubation of the cells with the indicated concentrations of BRL49653 for 4 days and then in the absence of drug for an additional 4 days. The extent of adipogenesis was then evaluated by determining the number of differentiated adipocytes as a percentage of the total number of cells. Immunoblot analysis with antibodies to the HA epitope tag confirmed that PPARγ2-WT and PPARγ2-P12A were expressed in similar amounts in the infected cells (data not shown). Whereas only about half of noninfected cells treated with 5 μM BRL49653 were differentiated, virtually all cells expressing PPARγ2-WT were differentiated after treatment with 500 nM BRL49653 (Fig. 2). Expression of PPARγ2-P12A also promoted BRL49653-induced adipogenesis in 3T3-L1 preadipocytes, with ~100% of cells showing a differentitaed phenotype after treatment with 5 μM drug. However, only ~60% of cells expressing PPARγ2-P12A were differentiated after treatment with 500 nM BRL49653, a fraction significantly lower than the corresponding value for cells expressing PPARγ2-WT. Thus, the ability of PPARγ2-P12A to mediate TZD-induced adipogenesis was impaired compared with that of PPARγ2-WT, consistent with the results of the transcriptional activation assay. TZD-induced adipogenesis in 3T3-L1 cells appeared to be more sensitive to heterologous PPARγ than did TZD induction of luciferase gene transcription in COS cells. Thus, whereas a significant difference was apparent between the effects of PPARγ2-WT and PPARγ2-P12A on adipogenesis at a BRL49653 concentration of 50 nM (Fig. 2B), the transcriptional activities of these proteins were significantly different only at a BRL49653 concentration of 5 μM (Fig. 1).

DISCUSSION

In this study, we have shown that the abilities of the mutant PPARγ2 protein to activate transcription and to induce adipogenesis in the presence of a TZD drug were impaired compared with those of the wild-type protein.

Although recent studies have examined the relation between the P12A mutation and Type 2 diabetes or obesity, the conclusions of these studies appear discordant. Thus, both a positive (12) and a negative (14) association of the mutation with BMI have been described. Furthermore, one study of Danish men showed that the Ala^{12} allele was associated with a lower BMI among lean subjects and with a higher BMI among obese subjects (17). Moreover, several studies have detected no relation of this mutation to obesity or insulin sensitivity (11, 13, 15, 18). Two studies have suggested that this mutation is associated with increased insulin sensitivity (14, 16). These contradictory results prompted us to perform a functional analysis of the P12A mutation of PPARγ2. Our observation that the P12A mutation reduced the transcriptional activity of PPARγ2 is consistent with a similar observation by Deeb *et al.* (14). One of the reasons why the conclusions concerning the relationship of the P12A mutation with obesity or insulin sensitivity are discordant may be explained by the fact that the functional impairment of the mutant is not remarkable.

Heterozygous PPARγ-deficient mice were recently shown to exhibit a reduced fat mass and to be protected from the development of insulin resistance when fed a high-fat diet (23). Moreover, despite their reduced fat mass, these mice exhibit overexpression and hypersecretion of leptin. These observations may be consistent with those of Deeb *et al.* (14) showing that the functionally impaired P12A mutant of PPARγ2 is associated with lower BMI and improved insulin sensitivity. Obese individuals (BMI, >30 kg/m^2) with the Ala^{12} allele of the PPARγ gene also exhibit a higher plasma concentration of leptin than do individuals homozygous for the wild-type allele (24). These studies also suggest that the P12A mutation impairs the function of PPARγ2, given that PPARγ has been shown to inhibit leptin gene transcription (25, 26). Further studies are required to examine the effectiveness of TZD therapy in diabetic individuals with the Ala^{12} allele of the PPARγ gene because the increased insulin sensitivity in heterozygous PPARγ-deficient mice was abrogated by treatment with a PPARγ agonist (23).

In conclusion, our data show that the abilities of the P12A mutant protein of PPARγ2 to activate transcription and to mediate adipogenesis in response to a TZD drug are impaired. Thus, it remains possible that the P12A substitution in PPARγ gene may be associated with insulin sensitivity and abnormalities of adipose tissue formation, given that PPARγ plays an important role in insulin sensitivity and development of fat mass.

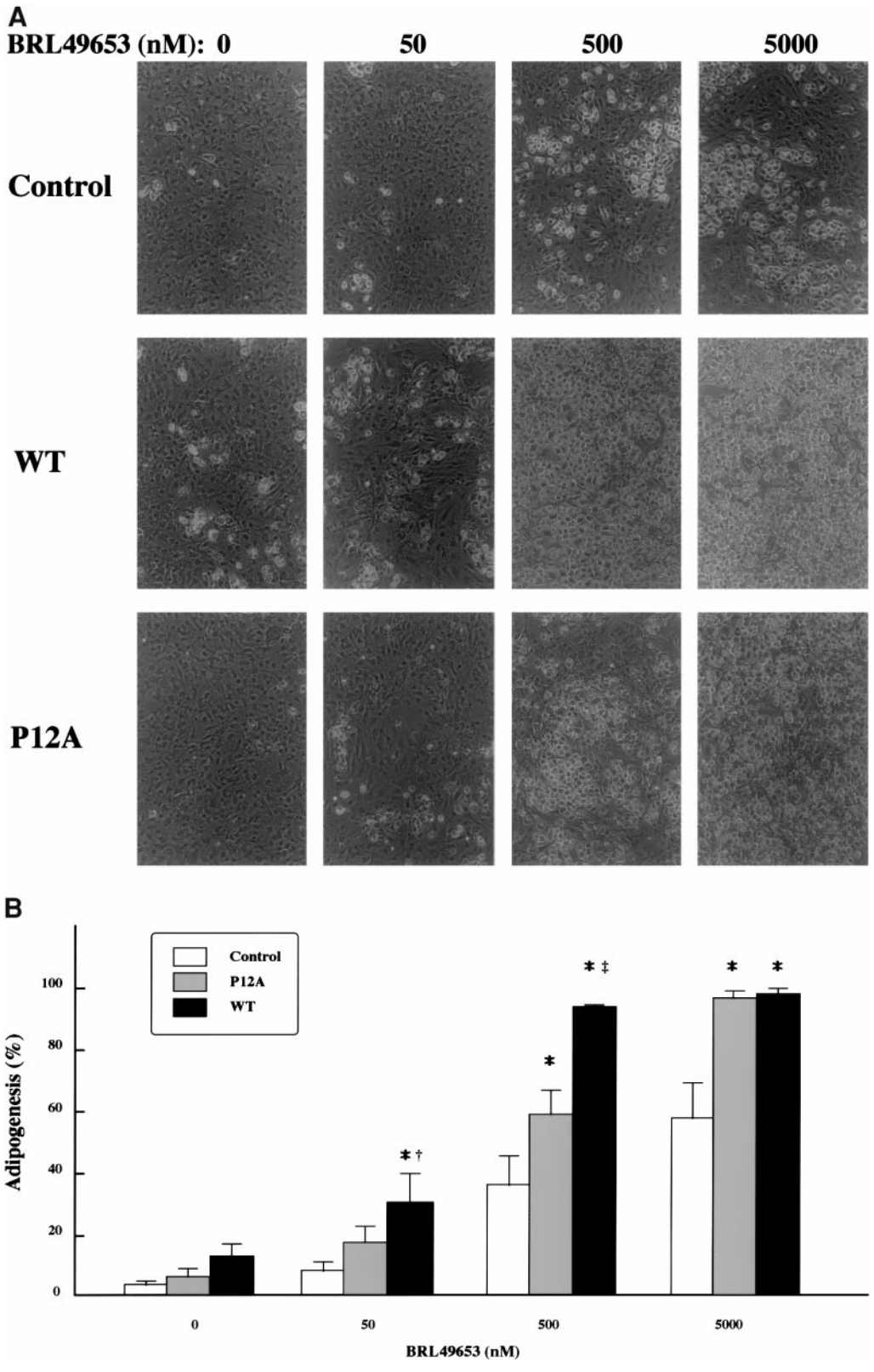

FIG. 2. Effects of PPARγ2-WT and PPARγ2-P12A on adipogenesis. 3T3-L1 preadipocytes cultured in 24-well plates with DMEM supplemented with 10% FBS were infected [or not (control)] with Adex-PPARγ2-WT or Adex-PPARγ2-P12A for 8 h at a multiplicity of infection of 10 [plaque-forming units per cell]. The cells were then incubated in fresh medium for 2 days, exposed to the indicated concentrations of BRL49653 for 4 days, and then incubated in the absence of drug for an additional 4 days; the medium was changed every 2 days. Cells were examined by light microscopy (magnification, 100×) (A), and the extent of adipogenesis was evaluated by determining the number of differentiated adipocytes as a percentage of the total number of cells (B). Data in (B) are means ± SD of four independent experiments. *$p < 0.01$ vs. corresponding value for cells incubated in the absence of BRL49653; †$p < 0.05$, ‡$p < 0.01$ vs. cells expressing PPARγ2-P12A and treated with 50 and 500 nM BRL49653, respectively (*t* test).

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