Biochemical and Biophysical Research Communications **271,** 212–216 (2000)
doi:10.1006/bbrc.2000.2605, available online at http://www.idealibrary.com on IDEAL®

The Pro12Ala Polymorphism in PPAR γ2 May Confer Resistance to Type 2 Diabetes

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Received March 24, 2000

Peroxisome proliferator-activated receptor γ (PPARγ) has been implicated in adipocyte differentiation. Recently it was reported that heterozygous deficiency of PPARγ led to the protection from high-fat diet-induced insulin resistance in an animal model. 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 Japanese diabetic and non-diabetic subjects. The frequency of Ala12 was significantly lower in the diabetic group. In an overweight or obese group, subjects with Ala12 were more insulin sensitive than those without. These results suggest that the PPARγ is a thrifty gene and that the Pro12Ala PPARγ2 polymorphism protects against type 2 diabetes in the Japanese.

Key Words: **peroxisome proliferator-activated receptor γ; Pro12Ala polymorphism; insulin resistance; type 2 diabetes; thrifty gene.**

Peroxisome proliferator-activated receptor γ (PPARγ) plays a major role in adipocyte differentiation (1) and is a molecular target of the insulin sensitizers, thiazolidinediones (2). However, the physiological role of PPARγ remains unclear. Recently we have generated mice lacking PPARγ. Homozygous PPARγ-deficient mice were embryonic lethal due to placental dysfunction (3). Unexpectedly, heterozygous PPARγ-deficient mice were partially protected from high-fat diet-induced obesity and insulin resistance, suggesting that PPARγ may mediate high-fat diet-induced obesity and insulin resistance. Thus, it appears that the amount of PPARγ plays a critical role in adipocyte hypertrophy and development of insulin resistance under a high-fat diet. PPARγ is therefore viewed as a "thrifty gene" (3, 4). High-fat diet is a major cause of the explosive increase in obesity and diabetes in western countries and Japan. It is likely that two alleles of wild-type PPARγ may interact with environmental factors such as high-fat diet leading to an increase in the incidence of type 2 diabetes. A Pro12Ala substitution has been detected in the PPARγ2 gene (5). This amino acid is located in the PPARγ2 domain that enhances ligand-independent activation (6). The non-conservative substitution of proline to alanine may cause a conformational change in the protein, which may affect its activity. It has been reported that a mutant PPARγ2 with Ala12 had reduced transactivation activity (7). Based on the fact that this amino-acid is highly conserved and that codon 12 in the mouse PPARγ2 gene is also proline (5), we hypothesized that subjects with this polymorphism may be partially protected from type 2 diabetes. To test this hypothesis, we performed a case-control study of the PPARγ 2 Pro12Ala polymorphism in unrelated non-diabetic and diabetic subjects.

MATERIALS AND METHODS

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 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 Department of Metabolic

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0006-291X/00 $35.00

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] **

Note. 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.

Diseases, Tokyo University. All the subjects enrolled in this study were of full Japanese ethnicity. A total of 541 non-diabetic and 415 diabetic subjects were enrolled in the present study. The study was performed under informed consent from all subjects and was approved by the Ethics Committee of Tokyo University.

Biological measurements. Plasma insulin was measured by radio-immunoassay (Phadeseph Insulin RIA kit, Pharmacia K.K., Tokyo, Japan). Serum leptin was measured by radioimmunoassay (Human Lepin RIA Kit, Linco Research, Inc., St. Charles, MO). Insulin resistance and β cell function were assessed by homeostasis model assessment (HOMA) (homeostasis model assessment of insulin resistance (HOMA-IR) = fasting insulin (mU/l) × glucose (mmol/l)/22.5, homeostasis model assessment of β cell function (HOMA-β) = 20 × fasting insulin (mU/l)/(fasting glucose (mmol/l) − 3.5)), as described elsewhere (8).

Genetic analysis. Genotyping was carried out on genomic DNA extracted from peripheral blood leukocytes using a QIAamp DNA Blood Mini kit (QIAGEN K.K., Tokyo, Japan). The Pro12Ala polymorphism was detected by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The sequences of the primers were 5′-TCTGGGAGATTCTCCTATTGGC-3′ (forward primer) and 5′-CTGGAAGACAACTACAAGAG-3′ (reverse primer). The forward primer contained one nucleotide mismatch (underlined), which made it possible to use the restriction enzyme Hha I (New England Biolabs, Beverly, MA) for the detection of the Pro12Ala polymorphism. The conditions for PCR were: PCR in a 50 μl reaction mixture containing 50–500 ng of genomic DNA, 0.2 μM of the primers, 2.5 mM of KCl, and 1.25 U of Ampli Taq Gold (PE Biosystems Japan, Chiba, Japan). The reaction mixtures were incubated at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 30 s. PCR products were digested with Hha I at 37°C overnight, and resolved by 3% ethidium bromide-stained agarose gel electrophoresis. The Pro12 allele gives one 154-bp fragment whereas the Ala12 allele gives 132- and 22-bp fragments.

Statistical analysis. Clinical variables are expressed as mean ± standard error of the mean (SEM). The proportions of genotypes or alleles were compared by χ^2 analysis. Odds ratio and 95% CIs nonadjusted or adjusted for age, gender, and body mass index (BMI) were calculated by logistic regression analysis. Differences in the clinical characteristics between subjects with and without the polymorphism were evaluated by two-tailed t-test. All the statistic analyses were performed using SAS for WINDOWS ver 6.12 software (SAS Institute Inc., Cary, NC).

RESULTS

Association between Pro12Ala polymorphism in PPARγ and type 2 diabetes. The allelic frequency of Ala12 in the total 956 subjects was 0.032, similar to a previous report in the Japanese population (9). The genotypic distribution of the Pro12Ala polymorphism was in Hardy-Weinberg equilibrium (Pro/Pro, 93.7%; Pro/Ala, 6.2%; Ala/Ala, 0.1%). The frequency of subjects bearing the Ala12 allele was lower in the diabetic group (3.6%) than in the non-diabetic group (8.3%) ($P = 0.003$) (Table 1). The allelic frequency of Ala12 was significantly lower in type 2 diabetic group (0.018) than in non-diabetic group (0.043) ($P = 0.003$) (Table 1). Subjects with the Ala12 allele had a decreased risk for type 2 diabetes (odds ratio (OR) = 0.413, 95% CI; 0.220–0.735). After adjustment for age, gender, and BMI, this reduced risk was still observed (OR = 0.324, 95% CI; 0.152–0.658).

Effect of Pro12Ala polymorphism in PPARγ2 on clinical variables. We also tested whether there were differences in clinical parameters between subjects with and without the Pro12Ala polymorphism. To assess the insulin resistance and β cell function of subjects, we used the homeostasis model assessment (HOMA-IR and HOMA-β, respectively) (8). Values obtained by HOMA has been shown to correlate well with those from the glucose clamp technique (10). Neither HOMA-IR nor HOMA-β was different between subjects with and without the Ala12 allele (Table 2). Any other clinical variables were not different between subjects with and without the Ala12 allele both in non-diabetic and diabetic groups (Table 2).

As type 2 diabetes itself may have effects on insulin and glucose levels, we performed a further analysis to investigate any effect of the Pro12Ala polymorphism on insulin resistance in non-diabetic subjects. 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 BMI (lean, BMI < 22 kg/m^2: normal, 22 kg/m$^2 \leq$ BMI $<$ 25 kg/m^2: overweight or obese, 25 kg/m$^2 \leq$ BMI). Overweight or obese subjects were defined as described in the previous study (11). 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 overweight or obese group, both fasting plasma insulin (57.4 ± 5.23 vs 70.6 ± 2.53 pmol/l, $P = 0.03$) and HOMA-IR (1.89 ± 0.18 vs 2.35 ± 0.09, $P = 0.03$) were lower in subjects with the Ala12 allele than those without (Fig. 1). Since the average BMI of subjects with the Ala12 allele (27.5 ± 0.46 kg/m^2) was comparable to that of those without (27.7 ±

TABLE 2

Characteristics of Non-Diabetic and Type 2 Diabetic Subjects

	Non-diabetic subjects (n = 541)			Type 2 diabetic subjects (n = 415)		
	Pro/Pro	Pro/Ala + Ala/Ala	P-value	Pro/Pro	Pro/Ala + Ala/Ala	P-value
Gender (female/male)	258/238	28/17	0.19	152/248	4/11	0.37
Age (years)	69.1 ± 0.26	69.1 ± 0.80	0.97	61.8 ± 0.53	58.3 ± 2.62	0.20
BMI (kg/m^2)	23.7 ± 0.14	24.4 ± 0.49	0.15	23.5 ± 0.20	22.9 ± 0.92	0.10
Systolic blood pressure (mm Hg)	131.4 ± 0.80	135.0 ± 2.42	0.54	131.5 ± 0.96	126.6 ± 4.47	0.44
Diastolic blood pressure (mm Hg)	77.5 ± 0.43	79.4 ± 9.1	0.54	75.4 ± 0.62	71.7 ± 2.66	0.37
Fasting glucose (mmol/l)	5.16 ± 0.02	5.12 ± 0.09	0.78	7.96 ± 0.13	7.15 ± 0.43	0.35
HbA1c (%)	5.15 ± 0.01	5.10 ± 0.03	0.13	7.94 ± 0.10	8.33 ± 0.45	0.56
Fasting insulin (pmol/l)	53.0 ± 1.24	51.8 ± 3.22	0.72	48.9 ± 1.42	34.8 ± 3.64	0.19
Total cholesterol (mmol/l)	5.39 ± 0.24	5.67 ± 0.14	0.33	5.25 ± 0.05	4.99 ± 0.11	0.41
Triglyceride (mmol/l)	1.31 ± 0.04	1.46 ± 0.13	0.62	1.55 ± 0.06	1.23 ± 0.13	0.40
High density lipoprotein (mmol/l)	1.57 ± 0.02	1.72 ± 0.08	0.26	1.30 ± 0.02	1.29 ± 0.11	0.90
HOMA-IR[a]	1.72 ± 0.04	1.67 ± 0.11	0.64	2.43 ± 0.08	1.64 ± 0.21	0.22
HOMA-β[b]	94.1 ± 2.43	99.8 ± 8.2	0.50	38.7 ± 1.34	27.3 ± 4.25	0.26

Note. Values are means ± SEM. [a, b]indicates homeostasis model assessment of insulin resistance and pancreatic β cell function, respectively as described under Materials and Methods.

0.15 kg/m^2, P = 0.73), these results suggest that subjects bearing the Ala12 allele were more insulin sensitive than those without.

Serum leptin levels were also measured in non-diabetic subjects. The plasma leptin levels tended to be higher in subjects with the Ala12 allele (8.02 ± 1.04 ng/ml) than in those without (6.27 ± 0.20 ng/ml) (P = 0.08).

DISCUSSION

The aim of this study was to investigate the impact of the proline to alanine substitution in the human PPARγ2 gene on glucose metabolism and susceptibility for type 2 diabetes. First, this study indicated that the proline to alanine substitution in codon 12 in PPARγ2 is associated with a decreased risk of type 2 diabetes. Second the present data showed that insulin sensitivity was higher in subjects with the Ala12 allele than those without in the overweight or obese group. This is consistent with a previous report (7) in which the average BMI of subjects was comparable to that of our overweight or obese group. This suggests that having the Ala12 allele may protect against insulin resistance which would normally arise in obese subjects. It is possible that genetic or environmental factors causing obesity may interact with the PPARγ gene, leading to the differences in insulin sensitivity between subjects

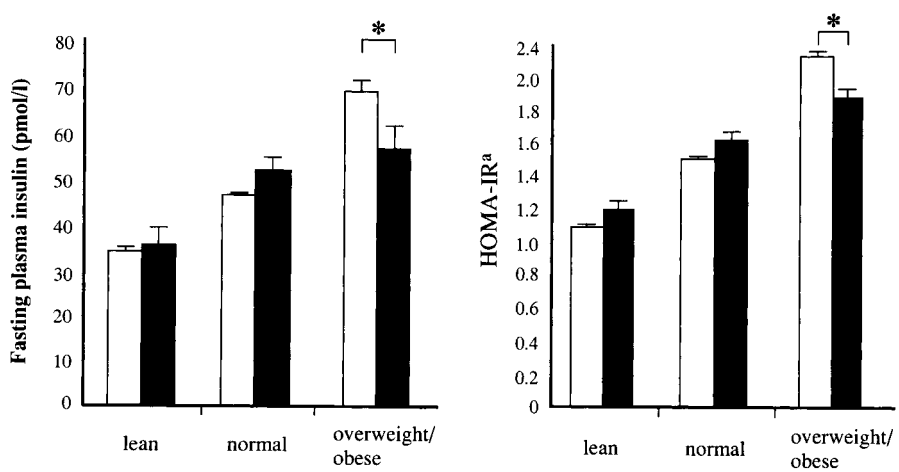

FIG. 1. Influence of the PPARγ Pro12Ala polymorphism on fasting plasma insulin (left panel) and insulin resistance index (right panel), in non-diabetic subjects with (solid bars) or without (open bars) the Ala12 allele. Subjects were subdivided into three groups according to BMI (lean, BMI < 22 kg/m^2: normal, 22 kg/m^2 ≤ BMI < 25 kg/m^2: overweight and obese, 25 kg/m^2 ≤ BMI. Means ± SEM are shown. [a]indicates homeostasis model assessment of insulin resistance as described under Material and Methods. *P < 0.05.

with and without this substitution in overweight and obese subjects.

In contrast to our results, no significant association was detected between the Pro12Ala polymorphism and type 2 diabetes in German (12) and Italian populations (13). The reasons for this discrepancy are not clear. It has been pointed out that the case-control methodology is not necessarily robust because of population stratification, especially in heterogeneous ethnic groups such as Caucasians. Japanese are an ethnically fairly homogenous group. Thus it is likely that the influence of population stratification in this study was smaller than that in previous studies. In addition, since nondiabetic subjects in our study were over 60 years of age and had no family history of diabetes at the time of registration, they can be considered to be "true" controls in that they are not likely to have genetic susceptibility to diabetes. The control group in the previous studies might have included a substantial number of subjects who possess some genetic susceptibility to type 2 diabetes but do not yet have overt diabetes.

We failed to detect any difference in BMI between subjects with Ala12 and those without. It was reported that the Pro12Ala polymorphism was associated with higher BMI in a Caucasian population (14, 15). In those studies, however, though the association was seemingly strong in an extremely obese cohort, it was much less clear in the less obese cohort, the average BMI of which was still much higher than our subjects. Increased insulin sensitivity might predispose subjects with this variant to obesity under some environmental and/or genetic backgrounds in Caucasians which may be different from ours.

How then does this proline to alanine substitution protect against insulin resistance and type 2 diabetes? In heterozygous PPARγ-deficient mice, food intake was decreased and body temperature was higher, indicating increased energy expenditure (3). The expression of leptin in white adipocytes and serum leptin were higher in heterozygous PPARγ-deficient mice than in wild-type mice. As sensitivity to exogenously administered leptin was comparable between both types of mice, we concluded that protection from high-fat diet-induced insulin resistance resulted at least partially from augmented effect of leptin. Treatment with PPARγ agonists led to a reduction of leptin expression levels in adipocytes (16). C/EBPα, which is important for adipocyte differentiation, promotes expression of leptin through binding to the leptin gene promoter. It has been postulated that PPARγ inhibits this effect of C/EBPα (17). Thus we have proposed that the higher leptin level in heterozygous PPARγ-deficient mice may be caused by a partial release from inhibition of leptin gene expression due to a relative reduction in the activities of PPARγ (3). We postulated that differences in leptin levels may account for the present results and therefore examined the serum levels of leptin in subjects with or without the Ala12 allele. The leptin levels were higher in subjects with the Ala12 allele than those without. Thus it is possible that higher levels of leptin might be associated with a relatively increased insulin sensitivity in subjects bearing the Ala12 allele.

In conclusion, the present findings suggest that the Ala12 allele of the PPARγ2 gene may protect subjects with this polymorphism from type 2 diabetes. PPARγ may therefore be an important thrifty gene.

ACKNOWLEDGMENTS

This work was supported by Health Sciences Research Grants (Research on Human Genome and Gene Therapy) from the Ministry of Health and Welfare (to T. Kadowaki). We thank Yuko Okada for technical assistance. This work was also supported by grant-in-aid from Ministry of Education, Science, Sports and Culture (to T. Kadowaki).

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