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ARTICLE

The common PPAR-γ2 Pro12Ala variant is associated with greater insulin sensitivity

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Several genetic variants of peroxisome proliferator-activated receptor-γ2 (PPAR-γ2) have been identified, among which Pro12Ala, a missense mutation in exon 2, is highly prevalent in Caucasian populations. Up to now, conflicting results with regard to the association between this mutation and complex traits, such as obesity, insulin sensitivity and Type 2 diabetes, have been reported. We investigated the influence of the Pro12Ala polymorphism of PPAR-γ2 on insulin sensitivity in a large Italian population sample, $n = 1215$, in whom extensive clinical and biochemical analyses were performed. To estimate the insulin sensitivity status, the homeostasis model assessment of insulin resistance (HOMA-IR) was calculated; in the obese/overweight subjects an oral glucose tolerance test (OGTT) was also performed and the Matsuda insulin sensitivity index (ISI) calculated. The insulin secretion index (homeostasis model assessment of percent β-cell function, HOMA-β%) was utilized to evaluate β-cell function. The effect of the Pro12Ala polymorphism on quantitative variables was tested using multiple linear regression analysis. X12Ala (either Pro12Ala or Ala12Ala) genotype was associated with significantly lower fasting insulin levels compared to Pro/Pro ($P = 0.01$ after correction for multiple comparisons) in all subjects. Consistent with this finding, significantly lower HOMA-IR was observed in X12Ala carriers ($P = 0.013$ after correction for multiple comparisons) in all cohort. Moreover, no significant interaction effect was observed between body mass index and X12Ala polymorphism and between gender and X12Ala polymorphism in modulating insulin sensitivity. Our observations substantially extend previous findings and demonstrated that X12Ala variant is significantly associated with greater insulin sensitivity.

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Introduction

Insulin resistance is a key factor in the development of Type 2 diabetes (T2DM) and multiple mechanisms contribute to its pathogenesis. Among these, the role of adipose tissue and obesity are of great significance. The metabolism and development of adipocytes is under complex regulation and recently the importance of the

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transcription factor peroxisome proliferator-activated receptor-γ2 (PPAR-γ2) in these processes has been recognized.

PPAR-γ are members of the nuclear hormone receptor family of transcription factors, known to control the expression of genes involved in the regulation of several metabolic processes.[1,2] Alternative use of promoters and differential splicing of the PPAR-γ gene results in three mRNA isoforms: PPAR-γ1, -γ2 and -γ3.[3] The PPAR-γ2 is specific for adipose tissue; this characteristic makes it a likely candidate gene to be involved in the regulation of adipogenesis and lipid storage, insulin and glucose metabolism. Furthermore, this molecule controls the expression of secretory proteins such as leptin, adiponectin[4] and tumor necrosis factor α,[5] which may act as modulators of insulin sensitivity in the skeletal muscles.[1,6,7] These findings strongly suggest that PPAR-γ2 may play a pivotal role in the whole body insulin sensitivity by influencing both adipocyte metabolism and insulin-stimulated process in nonadipose tissues.

Several genetic variants of PPAR-γ2 have been identified, among which Pro12Ala, a missense mutation in exon 2,[8] is highly prevalent in Caucasian populations.[2]

Up to now, conflicting results with regard to the association between this mutation and complex traits, such as obesity, insulin sensitivity and T2DM, have been reported. Some of these proposed that the X12Ala (either Pro12Ala or Ala12Ala) genotype may be related to improved insulin sensitivity[9,10] and protection from T2DM,[10,11] but this conclusion is still controversial.

The disparate findings may be partly attributed to insufficient power in some studies as well as to the heterogeneity of the population studied, in terms of clinical phenotypes. These factors highlight the need for much larger studies.

In view of these considerations, we evaluated the association of PPAR-γ2 Pro12Ala polymorphism with measures of insulin sensitivity in a large population of individuals and assessed the relation of this genetic variant with the clinical and metabolic abnormalities of the insulin resistance state.

Subjects and methods

Subjects

We studied 1215 unrelated subjects who reside in the Lazio region (central Italy). Of these, normal-weight subjects (body mass index (BMI) <25; $n=400$) were randomly selected from a population of free-living individuals screened for coronary artery disease risk factors. Overweight/obese subjects (BMI >25, $n=815$) were consecutively recruited from the metabolic Day Hospital of the Department of Clinical Sciences of University 'La Sapienza' in Rome. Exclusion criteria were as follows: previous diagnosis of diabetes according to ADA criteria,[12] chronic liver diseases and any treatment known to interfere with insulin sensitivity and metabolic syndrome-related parameters (hypolipemic drugs, insulin sensitizers, corticosteroid hormone, acetylsalicylic acid, β-blockers, oral contraceptives, thiazide diuretics, ACE inhibitors). All subjects gave their written informed consent to participate in the study after being informed of its nature. The study protocol was approved by the Ethical Committee of the University 'La Sapienza' in Rome.

In all subjects BMI, blood pressure, waist circumference (measured midway between the lowest rib margin and the iliac crest), hip circumference (measured over the great trocanthers) were measured as well as fasting glucose, insulin plasma levels and lipid profile (total, and HDL cholesterol and triglycerides). Furthermore, a 75-g oral glucose tolerance test (OGTT) was performed in all overweight/obese patients with glucose and insulin measurements at 30, 60, 90 and 120 min.

Methods

Cholesterol and triglyceride concentrations were determined in the plasma by Technicon RA-1000 Autoanalyzer; HDL was measured after precipitation of ApoB-containing lipoproteins with phototungstic acid/$MgCl_2$. LDL–cholesterol was determined by the Friedewald formula.[13] Glucose levels were calculated by the glucose oxidase method (Autoanalyzer, Beckman Coulter, USA). Plasma insulin was measured on frozen samples by radioimmunoassay (Adaltis insulin kit, Bologna, Italy), according to the manufacturing, with a limit of detection of $<2.0\,\mu$U/ml.[14]

Insulin resistance status was estimated according to homeostasis model assessment of insulin resistance (HOMA-IR) following the formula previously described:[15] fasting insulin (μU/ml) $\times$ fasting glucose (mmol/l)/22.5. Insulin sensitivity index (ISI) was calculated according to the formula $10\,000/\sqrt{(\text{glucose}_0 \times \text{insulin}_0 \times \text{glucose}_{\text{mean}} \times \text{insulin}_{\text{mean}})}$.[16]

To evaluate β-cell function, the insulin secretion index (homeostasis model assessment of percent β-cell function, HOMA-β %) was computed as: [20 $\times$ fasting insulin (mU/l)]/[(fasting glucose (mmol/l)-3.5].[15]

In 100 subjects, insulin-stimulated glucose disposal measurements were performed by the euglycemic hyperinsulinemic clamp performed as previously described.[17] The values obtained by the indexes approach correlated well with those obtained with the glucose clamp technique (data not shown).

Genotype analysis

The 200 bp of sequence surrounding PPAR-γ2 Pro12Ala was provided to Applied Biosystems to develop Taqman Allelic Discrimination (AD) Assays® using their assay by design platform (Foster City, CA, USA). Genotyping

of the Pro12Ala AD was performed using primers forward 5′-TTATGGGTGAAACTCTGGGAGATT-3′ and reverse 5′-TGCAGACAGTGTATCAGTGAAGGA-3′ and the Taqman MGB probes: Fam-TTCTGGGTCAATAGG; Vic-CTTTCTGC GTCAATAG. A measure of 4 μl of a 10ng/μl stock of DNA was dispensed into 384-well PCR plates using a Biomek FX robot (Fullerton, CA, USA) to which 6 μl of a mix containing primers, probes and reagent mix (ABI, Foster City, CA, USA) were added according to the manufacturers' instructions. These were sealed with optical seals and incubated at 95°C 10 min followed by 40 cycles of 95°C 15 s and 60°C 1 min before analysis on a 7900HT plate reader (ABI, Foster City, CA, USA). Individual genotypes were determined using SDSv2.1 software (ABI, Foster City, CA, USA)

Statistical analysis

Statistical analysis was performed using SPSS statistical software, version 12 (SPSS, Illinois, USA). Genotypic and allelic distributions were compared using the χ^2 test. The effect of the Pro12Ala polymorphism on the quantitative variables was investigated using multiple linear regression. We adjusted the crude effect of Pro12Ala polymorphism taking account of BMI, gender and age. The Pro12Ala polymorphism was introduced as a dichotomous variable in the analysis. In addition, the effects of the interaction between genotype and BMI and between genotype and gender were included in the model. Data for insulin, triglycerides, HOMA-IR, HOMA-β and ISI were transformed using $\log_{10}$ to normalize their distributions.

Results

Observed genotype frequencies of the polymorphism were in agreement with the Hardy–Weinberg expectations. The frequency of Ala allele was 9.15% in the whole cohort (Pro/Pro: 83%; Pro/Ala: 15.7%; Ala/Ala: 1.3%), similar to that reported in other Caucasian populations (Table 1). Impaired fasting glucose occurred in 60 (4.9%) of all subjects; according to the results of OGTT, impaired glucose tolerance occurred in 134 (16.5%) of overweight and obese subjects. The prevalence of women in menopausal status was 24% in our cohort.

The Pro12Ala variant was not associated with BMI at the $P=0.05$ level. No significant differences in age, gender, blood pressure, waist/hip ratio were noted between the X12Ala carriers and wild-type individuals (Table 1). We observed significantly lower fasting glucose levels in X12Ala carriers compared to Pro/Pro ($P=0.02$) in all subjects. X12Ala genotypes were associated with significantly lower fasting insulin levels compared to Pro/Pro ($P=0.0008$) in all cohort. Consistent with this finding, a significantly lower HOMA-IR was observed in X12Ala carriers ($P=0.001$) compared to wild-type individuals in all subjects. Although not significant, the insulin secretion index HOMA-β tended to be reduced in Ala carriers. The lipid profile did not show any significant differences in the groups analyzed (Table 2) in all individuals.

Among the overweight/obese subjects, Ala carriers had a significantly higher ISI index compared to wild-type individuals ($P=0.01$). However, once we have corrected for multiple comparisons, using Bonferroni test, the fasting glucose ($P=0.26$) and ISI OGTT ($P=0.13$) significances

Table 1 Clinical features of subjects according to Pro12Ala PPAR-γ2 genotype

	Pro/Pro	*Pro/Ala or Ala/Ala*	*P-value*[a]
n (men/women)	1008 (303/705)	207 (63/144)	
Age (years)	42.6±13.6	42.9±13.3	0.75
BMI (kg/m^2)	32.8±9	32.4±10	0.34
Systolic BP (mmHg)	128±19.84	129±20.10	0.43
Diastolic BP (mmHg)	81.3±11.53	81.5±11	0.78
Waist/hip	0.84±0.08	0.83±0.09	0.12

[a]Data are given as means and SD. All comparisons are adjusted for age, BMI, gender, PPAR-γ2 × BMI interaction and PPAR-γ2 × gender interaction.

Table 2 Biochemical parameters of subjects according to Pro12Ala PPAR-γ2 genotype

	Pro/Pro (n = 1008)	*Pro/Ala or Ala/Ala (n = 207)*	*P-value*[a]
Total cholesterol (mmol/l)	5.31±1.11	5.29±1.13	0.91
HDL cholesterol (mmol/l)	1.12±0.37	1.14±0.39	0.25
Triglycerides (mmol/l)	1.3±0.82	1.25±0.84	0.21
Fasting glucose (mmol/l)	4.75±0.59	4.68±0.57	0.02
Fasting insulin (pmol/l)	129±75	114±70	0.0008
HOMA-IR (arbitrary units)	3.66±2.3	3.23±2	0.001
HOMA-β% (arbitrary units)	275±159	264±154	0.18
ISI during OGTT (arbitrary units)[a]	4.77±2.58	5.21±2.87	0.01

[a]Data are given as means and SD. All comparisons are adjusted for age, BMI, gender, PPAR-γ2 × BMI interaction and PPAR-γ2 × gender interaction.

were no longer statistically significant at the 5% level. The significance levels of fasting insulin and HOMA-IR remained significant after correction for multiple comparisons ($P = 0.01$ and 0.013, respectively). No significant interaction effect was observed between BMI and X12Ala polymorphism (HOMA-IR *P*-value interaction 0.51, fasting insulin *P*-value interaction 0.55 and fasting glucose *P*-value interaction 0.66) and between gender and X12Ala polymorphism in modulating insulin sensitivity (HOMA-IR *P*-value interaction 0.71, fasting insulin *P*-value interaction 0.68 and fasting glucose *P*-value interaction 0.75).

Discussion

In the present study, we have demonstrated that the common variant of PPAR-γ2, X12Ala, present in Italian population with an allele frequency of 9.15%, is significantly associated with higher insulin sensitivity. In particular, X12Ala carriers showed significantly lower insulin levels and HOMA-IR and in subjects within the overweight/obese group a significantly higher ISI index compared to wild-type individuals (Table 2). We did not observe any significant difference between the two genotypes in the lipid profile; however, we observed a significantly lower fasting glucose level in all cohort. It is likely that improved insulin sensitivity could be responsible for the significant decrease in fasting glucose levels. To our knowledge, a significantly higher ISI index in X12Ala carriers compared to wild-type individuals has not been reported previously. The first evidence for an association between the X12Ala polymorphism in PPAR-γ2 and increased insulin sensitivity was reported by Debb *et al*.[9] The Authors found a significant increase of the Ala allele in individuals with normal glucose tolerance compared to T2DM patients and a significantly greater insulin sensitivity in nondiabetic Ala carriers. Only one[18] of the five subsequent studies[19–23] reproduced the original findings; however, a meta-analysis, including the previous five and Scandinavian parent–offspring trios, demonstrated that the Pro12Ala polymorphism significantly decreases the risk of T2DM.[11] Greater insulin sensitivity was observed in normo glucose-tolerant Swedish 70-year-old men carrying the Ala allele;[24] in subgroups of obese subjects, the increase in insulin sensitivity was also more pronounced.[25] Stumvoll and co-workers demonstrated that the Ala allele of PPAR-γ2 becomes particularly advantageous against the background of an additional, possibly disadvantageous genetic polymorphism such as the G972R variant of IRS-1.[26]

The picture, however, is still unclear as reported in a Swedish middle-aged man in whom a significantly higher body mass with predominance of abdominal distribution of adipose tissue was observed along with a marked impairment in estimated insulin sensitivity in homozygotes Ala carriers.[10] Moreover, as reported by Frederiksen *et al*,[27] in our study we did not observe an interaction between the variant and BMI on the examined variables.

The conflicting findings may be partly attributed to insufficient power in some studies, the heterogeneity of the population studied in terms of clinical phenotype (lean, obese) and type of study (population based, clinical based).

Altshuler *et al*[11] pointed out that despite the population impact of common risk alleles such as 12 Ala, their contribution will be impossible to discover by linkage analysis (requiring a genome scan of millions of sib pairs) and therefore association studies are required. Even so, studies with modest sample sizes might fail to detect true associations. Therefore, the dissection of common risk alleles involved in multifactorial diseases should entail association studies performed on large population samples as in the present study, which involves a number of subjects substantially higher compared to the majority of studies performed so far on this matter.

One key question that deserves an answer is related to the contribution of Pro12Ala polymorphism in regulating insulin secretion. Normal weight Ala carriers showed a decreased second-phase insulin secretion following elevation of serum-free fatty acids[28] and a significant lower insulin secretion was observed in T2DM patients carrying the Ala allele compared to Pro12Pro subjects.[29] In our large population study, despite a tendency for a decrease in HOMA-β%, there was no significant correlation between insulin secretion and Ala allele.

Two independent studies have observed a reduced transcriptional activity of PPAR-γ2 as a result of Pro-Ala exchange.[9,30] Our and previous observations suggest that the X12Ala polymorphism improves insulin sensitivity. These findings are in line with observations in heterozygous PPARγ-deficient transgenic mice, which have reduced transcriptional activity of PPARγ and are protected from high-fat diet obesity and insulin resistance.[31,32] However, it is still currently unclear by which mechanisms a reduced transcriptional activity of PPARγ (both in humans and in animal models) could influence insulin sensitivity. In our study, we did not observe any significant difference in lipid profile, waist/hip ratio, blood pressure and BMI between the X12Ala carriers and wild-type individuals. As it is well recognized that some adipocytokines (resistin, TNFa, adiponectin) are under the transcriptional control of PPAR-γ2, we could speculate that any of them could mediate the effect of Pro12Ala on insulin sensitivity.

In conclusion, we observed by using several measurements of insulin sensitivity, including fasting insulin, HOMA-IR and ISI, a protective effect of the X12Ala polymorphism of PPAR-γ2 on insulin resistance; future

studies investigating the functional effect of this polymorphism will help to clarify the exact role of X12Ala variant.

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