The Pro12Ala substitution in the peroxisome proliferator activated receptor gamma 2 is associated with an insulin-sensitive phenotype in families with familial combined hyperlipidemia and in nondiabetic elderly subjects with dyslipidemia

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Abstract

Dyslipidemias and insulin resistance often present simultaneously, as in familial combined hyperlipidemia (FCHL), and therefore may have a common genetic background. In our previous study the Pro12Ala substitution of peroxisome proliferator receptor γ 2 (PPARγ2) associated with insulin sensitivity, low body mass index (BMI) and high-density lipoprotein (HDL) cholesterol levels. In this study, we investigated the role of this substitution in dyslipidemias. Therefore, 228 nondiabetic members of FCHL families and 866 nondiabetic elderly subjects with (n=217) and without dyslipidemia (n=649) were genotyped. The allele frequencies of the Pro12Ala substitution did not differ between elderly subjects with or without dyslipidemia or 27 probands with FCHL. However, this substitution was associated with low fasting insulin levels both in FCHL family members (P=0.036 adjusted for gender and age) and elderly subjects with dyslipidemia (P=0.050) but not in elderly subjects without dyslipidemia (P=0.080). In addition, the Ala12 allele of PPARγ2 was associated with low BMI (P=0.034) and low total triglycerides (P=0.027), and increased HDL-cholesterol (P<0.001) in elderly subjects with dyslipidemia (n=299) but not among any other study groups. We conclude that the Ala12 isoform of PPARγ2 ameliorates the insulin resistance and unfavorable lipid and lipoprotein profiles in FCHL and hyperlipidemic elderly subjects. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Familial combined hyperlipidemia; Genetics; Insulin resistance; PPARγ

1. Introduction

Hypertriglyceridemia alone [1,2] or in combination with hypercholesterolemia [3] have been associated with insulin resistance. In familial combined hyperlipidemia (FCHL) both variable dyslipidemias [4,5] and insulin resistance [6–9] are often present. In FCHL patients a defect in adipose tissue metabolism has been proposed to lead, via increased free fatty acid (FFA) levels both to increased hepatic lipoprotein synthesis and insulin resistance [9–11]. Therefore genes that regulate fat cell metabolism are likely to be important for the understanding of insulin resistance in FCHL.

Peroxisome proliferator activated receptor γ (PPARγ) is a transcription factor belonging to the nuclear hormone receptor family that plays an important role in adipocyte differentiation and gene expression [12,13]. Therefore, the PPARγ gene is a potential candidate gene both for dyslipidemias and insulin resistance. The PPARγ gene is transcribed into three different mRNAs [14,15], which give rise to two distinct proteins, i.e. PPARγ1 and PPARγ2 [16]. PPARγ2 is distinct from PPARγ1 since it contains 28 additional amino acids compared to PPARγ1, conferring a distinct biological activity. PPARγ2 has been shown to be associated with low BMI and increased HDL-cholesterol levels in familial combined hyperlipidemia (FCHL) patients [17].
Twenty-seven families with FCHL including 228 family members (27 probands, 129 of their first-degree relatives, 44 of their second-degree relatives and 28 spouses) formed the study population. Twenty-five of the probands of FCHL families were male survivors of myocardial infarction at young age (< 55 years, n = 18) or their first-degree relatives (n = 7, all females), who were first studied in 1978–1980 [31] and restudied in 1992–1994. Two probands fulfilling similar lipid criteria were selected from the Coronary Angiography Register of the Kuopio University Hospital. The criteria for FCHL were: total cholesterol ≥ 7.7 mmol/l and/or total triglycerides ≥ 2.2 mmol/l in women and ≥ 2.4 mmol/l in men at least in one of the visits. These lipid criteria were based on lipid levels of 250 control subjects from a random population sample who participated in the same study as control families [31]. The cut-off points were 80th percentile for total cholesterol, and 90th percentile for total triglycerides. The 80th percentile for total cholesterol was used because of high cholesterol level among Finns living in eastern Finland. In order to meet the criteria for FCHL, all probands who were included had to have at least two affected first-degree relatives with different types of dyslipidemias. None of the study subjects had tendon xanthomas or defects of the LDL receptor which explain about 90% of all cases of familial hypercholesterolemia in this area [32].

Elderly subjects were a random sample of inhabitants in Kuopio aged 65–74 years during the baseline study in 1986–1988 [33]. From the original sample of 1910 inhabitants 1069 nondiabetic subjects participated in the baseline study and 896 in the follow-up study in 1990–1991 [34]. Only subjects who participated in the follow-up study could be used here because DNA samples were taken during that visit. We divided elderly subjects into two subgroups according to the same lipid criteria used for FCHL. Table 1 shows the clinical characteristics of study subjects.

All study subjects had normal glucose tolerance according to the World Health Organization criteria [35]. normal liver, kidney and thyroid function tests and none of them had a history of excessive alcohol intake.

2. Methods

2.1. Study subjects

All subjects participating in this study were Finnish. The Finnish population is genetically relatively homogenous, originating mainly from southern (European) and eastern (Asian) immigration 2000 years ago [29,30].
2.3. Analytical methods

Plasma glucose levels in the fasting state and after an oral glucose load were measured by the glucose oxidase method (2300 Stat Plus, Yellow Springs Instrument Co., Yellow Springs, OH). For the determination of plasma insulin, blood was collected in EDTA-containing tubes and after centrifugation the plasma was stored at −70°C until the analysis. Plasma insulin concentration was determined by a commercial immunoassay (Phadeseph Insulin RIA 100; Pharmacia Diagnostics AB, Uppsala, Sweden). Lipoprotein fractionation was performed by the use of ultracentrifugation and selective precipitation [36] as previously described [1]. Cholesterol and triglyceride levels from whole serum and from lipoprotein fractions were assayed by automated enzymatic methods (Boehringer-Mannheim, Mannheim, Germany). Apolipoprotein B (apoB) was determined by a commercial immunoturbidometric method (Kone Instruments, Espoo, Finland).

2.4. Determination of the Pro12Ala and Pro115Gln substitutions of the PPARγ2 gene

DNA was prepared from peripheral blood leukocytes by proteinaseK-phenol-choloroform extraction method. For amplification of exon B of the PPARγ2 gene we used primers forward 5’-GACAAA-ATATCAGTGTGAATTACAGC-3’ and reverse 5’-CCCAATAGCCGTATCTGGAGG-3’ [26], and for amplification of the region spanning codon 113 we used primers forward 5’-TGAATTCAAGTGAGG-3’ and reverse 5’-CAGAAGCTTATCTCCACA-GAC-3’ [27]. PCR was done in 6 μl volume containing 50 ng of genomic DNA, 3 pmol of each primer, 10 mmol/l Tris-HCL (pH 8.8), 50 mmol/l KCl, 1.5 mmol/l of MgCl2, 0.1% Triton X-100, 100 μmol/l dNTP, 0.25 units of DNA polymerase (Dynazyme DNA polymerase, Finnzymes, Espoo, Finland) and 0.55 μCi of [32P]dCTP. PCR conditions were denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 66°C for 30 s and extension at 72°C for 30 s with final extension at 72°C for 4 min. Variants were detected with the single strand conformation polymorphism (SSCP) analysis. PCR products were first diluted 4–10-fold with 0.1% SDS and 10 mmol/l EDTA and then diluted (1:1) with loading mix (95% formamide, 20 mmol/l EDTA, 0.05% bromphenol blue, 0.05% xylene cyanole). After denaturation at 98°C for 3 min, samples were immediately cooled on ice, and 2 μl of each sample was loaded onto 6% nondenaturing polyacrylamide gel (acrylamide/N,N-methylene-bis-acrylamide ratio 49:1) containing 10% glycerol. Samples were run at two different gel temperatures (29°C and 37–39°C). The gel was autoradiographed overnight at −70°C with intensifying screens. Amplified segments from subjects with a variant pattern in SSCP analysis were purified by electrophoresis on a 1% low-melting point agarose gel and directly sequenced with the use of Sequenase (Amersham Life Sciences, Cleveland, OH) as previously described [37].

2.5. Statistical analysis

All basic statistical analysis were performed with the SPSS/WIN programs (version 7.5, SPSS, Chicago, IL).

Table 1
Clinical characteristics of the study groups

<table>
<thead>
<tr>
<th></th>
<th>Elderly nondiabetic subjects</th>
<th>FCHL family members (n = 228)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without dyslipidemia (n = 649)</td>
<td>With dyslipidemia (n = 217)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>264/385</td>
<td>63/154b</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>72.9 ± 2.9</td>
<td>73.0 ± 2.8</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.7 ± 4.2</td>
<td>27.7 ± 4.1c</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.94 ± 0.08</td>
<td>0.94 ± 0.08</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>153 ± 22</td>
<td>160 ± 25</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>81 ± 10</td>
<td>84 ± 10</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.7 ± 0.7</td>
<td>6.0 ± 1.1c</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>69.0 ± 39.0</td>
<td>95.4 ± 61.2a</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>6.14 ± 0.88</td>
<td>7.52 ± 1.29d</td>
</tr>
<tr>
<td>Total triglycerides (mmol/l)</td>
<td>1.33 ± 0.40</td>
<td>2.50 ± 1.42d</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.39 ± 0.33</td>
<td>1.30 ± 0.42a</td>
</tr>
<tr>
<td>Apolipoprotein B (g/l)</td>
<td>1.03 ± 0.20</td>
<td>1.42 ± 0.22d</td>
</tr>
</tbody>
</table>

* All values for continuous variables are mean ± SD. HDL indicates high-density lipoprotein.
** P < 0.05.
*** P < 0.01.
**** P < 0.001 when compared to elderly people without dyslipidemia.
The frequencies between the study groups were compared with the chi-square test. The effect of the variants on continuous variables was tested by ANCOVA in unrelated elderly subjects and by family-based association analysis with the program ASSOC in FCHL families [38]. The ASSOC program uses linear regression analysis allowing the quantitative trait to have familial correlation among individuals. The likelihood for the pedigree is computed with a linear regression model in which the quantitative trait is the dependent variable and the genetic variation, discrete and/or continuous covariates are independent variables. Residual variation is modeled assuming an additive polygenic pattern of correlation among relatives. Using this model the likelihood for each pedigree was maximized twice, with and without the genetic variant in the model. The difference in natural logarithms of these two maximized likelihoods follows chi-square distribution from which the corresponding $P$-value is taken with two degree of freedom (three groups of genotypes) or with one degree of freedom (subjects with or without a definite genotype). Gender was included as a discrete covariate and age as a continuous covariate in all analyses. Insulin and triglyceride values were logarithmically transformed before analysis in order to achieve normal distribution. All data are presented as mean ± SD. $P$-value $<0.05$ was considered statistically significant.

### 3. Results

Nondiabetic elderly subjects with dyslipidemia had higher BMI ($P = 0.002$, adjusted for age and gender) and higher levels of glucose ($P = 0.005$), insulin ($P < 0.001$), total cholesterol ($P < 0.001$), total triglycerides ($P < 0.001$) and apoB ($P < 0.001$) and lower levels of HDL-cholesterol ($P < 0.001$) than subjects without dyslipidemia. In addition to being more dyslipidemic (higher total cholesterol, total triglycerides and apoB, $P < 0.001$), FCHL family members were younger ($P < 0.001$) and had higher gender- and age-adjusted insulin levels ($P = 0.026$) compared to elderly subjects without dyslipidemia (Table 1).

Allele frequencies of the Pro12Ala polymorphism of the PPARγ2 gene did not differ between elderly subjects with (0.17) or without (0.14) dyslipidemia or probands with FCHL (0.15, $P = \text{ns}$). Genotype frequencies followed the Hardy–Weinberg equilibrium in all study groups. The Pro113Gln substitution was not found in any of the FCHL family members.

In FCHL family members, fasting insulin levels were associated with the Pro12Ala substitution of the PPARγ2 gene ($72.6 \pm 72.0$ in subjects with the Pro12Pro genotype vs. $83.4 \pm 59.4$ in subjects with the Pro12Ala genotype vs. $46.8 \pm 16.8$ pmol/l in subjects with the Ala12Ala genotype, $P = 0.036$ ANCOVA over
Table 2
Body mass index, waist-to-hip ratio and fasting glucose, insulin and lipid and lipoprotein levels according to the Pro12Ala substitution of the PPARγ2 gene in the study groups

<table>
<thead>
<tr>
<th></th>
<th>Elderly subjects without dyslipidemia</th>
<th>Elderly subjects with dyslipidemia</th>
<th>FCHL family members</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pro/Pro (n = 457)</td>
<td>Pro/Ala (n = 176)</td>
<td>Ala/Ala (n = 16)</td>
</tr>
<tr>
<td></td>
<td>Pro/Pro (n = 157)</td>
<td>Pro/Ala (n = 57)</td>
<td>Ala/Ala (n = 3)</td>
</tr>
<tr>
<td></td>
<td>Pro/Pro (n = 164)</td>
<td>Pro/Ala (n = 58)</td>
<td>Ala/Ala (n = 6)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>181/276</td>
<td>78/98</td>
<td>5/11</td>
</tr>
<tr>
<td>Age (years)</td>
<td>73.0 ± 2.9</td>
<td>72.8 ± 2.8</td>
<td>73.1 ± 3.3</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.7 ± 3.9</td>
<td>27.3 ± 4.4</td>
<td>26.1 ± 4.5</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.94 ± 0.08</td>
<td>0.94 ± 0.08</td>
<td>0.93 ± 0.08</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>153 ± 22</td>
<td>153 ± 22</td>
<td>160 ± 18</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>81 ± 10</td>
<td>82 ± 9</td>
<td>83 ± 11</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.7 ± 1.1</td>
<td>5.6 ± 0.8</td>
<td>5.5 ± 0.5</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>68.4 ± 33.6</td>
<td>65.4 ± 42.7</td>
<td>55.2 ± 22.2</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>6.20 ± 0.99</td>
<td>6.09 ± 0.88</td>
<td>6.30 ± 0.83</td>
</tr>
<tr>
<td>Total triglycerides (mmol/l)</td>
<td>1.37 ± 0.50</td>
<td>1.33 ± 0.45</td>
<td>1.18 ± 0.32</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.40 ± 0.35</td>
<td>1.38 ± 0.30</td>
<td>1.50 ± 0.30</td>
</tr>
<tr>
<td>Apolipoprotein B (g/l)</td>
<td>1.04 ± 0.18</td>
<td>1.05 ± 0.18</td>
<td>1.05 ± 0.15</td>
</tr>
</tbody>
</table>

All values are mean ± SD. HDL indicates high-density lipoprotein.

* P = 0.034,
* P = 0.050,
* P = 0.036,
* P = 0.027,
* P < 0.001 over the three genotypes after adjustment for gender, age.
the three genotypes) (Fig. 1, Table 2). Subjects with the Ala12Ala genotype had lower levels of fasting insulin than subjects with the Pro12Pro and Pro12Ala genotypes ($P = 0.010$), fasting insulin ($P = 0.034$), HDL-cholesterol ($P = 0.002$) and total triglycerides ($P = 0.007$) were associated with the Pro12Ala substitution of the PPARγ gene. No associations between the Pro12Ala substitution and these parameters could be shown in the subgroup of elderly subjects without dyslipidemia ($n = 649$, Table 2). However, in dyslipidemic elderly subjects ($n = 217$) BMI ($27.0 \pm 4.4$ vs. $28.2 \pm 4.0$ vs. $23.3 \pm 2.8$ kg/m$^2$, $P = 0.034$), fasting insulin levels ($85.2 \pm 54.6$ vs. $102.0 \pm 65.4$ vs. $54.6 \pm 10.8$ pmol/l, $P = 0.050$), total triglyceride levels ($2.09 \pm 1.34$ vs. $2.26 \pm 1.28$ vs. $1.07 \pm 0.35$ mmol/l, $P = 0.027$) and HDL-cholesterol levels ($1.33 \pm 0.39$ vs. $1.23 \pm 0.33$ vs. $2.24 \pm 0.41$ mmol/l, $P < 0.001$) differed among the three genotypes (Fig. 1, Table 2). No differences were seen between subjects with Pro12Pro and Pro12Ala genotypes. However, the subjects with the Ala12Ala genotype had higher HDL-cholesterol ($P < 0.001$) levels than subjects with other genotypes. Still, BMI ($P = 0.097$), fasting insulin ($P = 0.200$) or total triglycerides levels ($P = 0.088$) did not differ significantly between dyslipidemic elderly subjects with or without the Ala12Ala genotype. We did not find any association between the Pro12Ala substitution and waist-to-hip ratio, blood pressure, fasting glucose, total cholesterol or apoB levels in elderly subjects (Table 2).

4. Discussion

Insulin resistance and dyslipidemias often occur simultaneously [2,39] as in FCHL [6–9], indicating that insulin resistance and dyslipidemias may have a common genetic background. In our study the Pro12Ala substitution of the PPARγ2 gene was associated with low fasting insulin levels in FCHL families and in dyslipidemic elderly subjects. Furthermore, the Pro12Ala substitution was also associated with low BMI, low total triglycerides and high HDL cholesterol levels in dyslipidemic elderly subjects, whereas no statistically significant associations were observed in elderly subjects without dyslipidemia. This implies that the Pro12Ala substitution by itself does not have a major causative role in FCHL or dyslipidemias in elderly subjects, but it may modify fasting insulin and lipid levels in subjects with a genetic or environmental predisposition to dyslipidemias.

Although FCHL was originally described to have a dominant mode of inheritance [4], polygenic background is more likely [40]. Major genes for FCHL have not been yet been identified but some rare mutations in the lipoprotein lipase gene may underlie this disease [41]. In addition, the ApoAI–CIII–AIV gene complex has been implicated for the etiology of FCHL [42,43], but also negative results have been published [44,45]. Furthermore, a promising, but as yet unidentified, locus in 1q21-23 has been reported [46]. FCHL disease may in fact identify subjects with familial aggregation of several gene defects causing phenotypes of the insulin resistance syndrome [11,40]. Therefore, the search for gene defects causing FCHL may also help in resolving the genetics of the insulin resistance syndrome.

Defects in the PPARγ gene could affect transcriptional activation of several genes in adipose tissue. On the other hand, changes in adipose tissue metabolism can cause dyslipidemias [47] and insulin resistance [48] via increased FFA levels. Therefore, the association of the Ala12 variant, that has a lower receptor activity, with low BMI and fasting insulin level and high HDL-cholesterol levels in a random population sample in our previous study in Finns was not surprising. Furthermore, the Pro12Ala polymorphism may also be associated with type 2 diabetes in Japanese-Americans [26]. In the present study no differences were found in allele frequencies of this polymorphism between non-diabetic elderly subjects with or without dyslipidemia or non-diabetic FCHL probands. Therefore, it is unlikely that this substitution could have a major role in FCHL or dyslipidemias in elderly subjects.

Our finding that the association of the Pro12Ala substitution with the components of the insulin resistance syndrome (obesity, high fasting insulin, high total triglycerides and low HDL-cholesterol levels) were best seen in elderly non-diabetic subjects with dyslipidemia may be important. At least three explanations are possible. First, and most likely, the effect of the Pro12Ala variant may be relatively small, impeding detection in subjects without predisposition to dyslipidemias and other components of the insulin resistance syndrome (e.g. in elderly subjects without dyslipidemia). On the other hand, this small effect may not be seen in FCHL family members because several other more important, but still unidentified, gene defects segregate within these families. Second, there may be a direct metabolic interaction between this substitution and factors causing dyslipidemia in the general population, and maybe a less strong interaction with factors causing FCHL. Third, the codon 113 variant (Pro→Gln), described to be associated with obesity [27], was not found in FCHL family members in the present study. Therefore, no variants, which are associated with obesity, are known to be in linkage disequilibrium with the Pro12Ala substitution in the Finnish population. Finally, despite the
large number subjects in this study, the low frequency of the Pro12Ala substitution makes it difficult to draw a final conclusion about the role of this substitution in insulin resistance and dyslipidemias.

Although the effect of the Pro12Ala substitution of the PPARγ gene does not seem to be very large, these results, in combination with earlier reports [26–28], imply that variants in the PPARγ gene affect adipocyte metabolism. The Pro113Gln substitution affects the phosphorylation status of PPARγ, resulting in a constitutive activation of PPARγ and, therefore, to increased adipocyte differentiation and obesity [21–24,27]. In contrast, the Pro12Ala substitution reduces PPARγ activity and adipocyte-specific gene expression, resulting in a lower BMI and increased insulin sensitivity [26]. On the contrary, a recent study associated the Ala12 allele with high BMI in obese subjects [49]. This may indicate that the functional role of Pro12Ala substitution is different depending on the degree of energy storage. In the absence of excessive energy storage, decreased adipocyte differentiation, associated with less active PPARγ Ala 12 allele, should in fact lead to small insulin-sensitive adipocytes, and protect against the development of obesity. On the other hand, in the presence of excessive energy storage, dietary and hormonal factors will override the protective effect offered by the PPARγ Pro12Ala variant.

In summary, we found the Ala12 variant substitution of the PPARγ gene to be associated with low fasting insulin levels in nondiabetic FHCL family members and dyslipidemic elderly subjects. In addition, low BMI, low total triglyceride and high HDL-cholesterol levels were associated with this substitution in elderly subjects with dyslipidemia. Although this substitution is not a major locus for FCHL or dyslipidemias in general, it seems to have a modifying effect in both disorders, possibly by affecting insulin sensitivity.

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