Association of the *PRO12ALA* Polymorphism of the *PPAR-γ2* Gene With Oxidized Low-Density Lipoprotein and Cardiolipin Autoantibodies in Nondiabetic and Type 2 Diabetic Subjects

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Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a key component in adipocyte differentiation and fat-specific gene expression and may modulate macrophage functions, like proinflammatory activities, and stimulate oxidized low-density lipoprotein (ox-LDL) uptake. We hypothesized that the *Pro12Ala* polymorphism of the *PPAR-γ2* gene may affect the immune response to ox-LDL. Therefore, we investigated the association of the *Pro12Ala* polymorphism of the *PPAR-γ2* gene with ox-LDL autoantibodies, as well anticardiolipin antibodies, in a 10-year prospective study. The *Pro12Ala* polymorphism was genotyped in 119 nondiabetic subjects (age, 45 to 64 years; body mass index [BMI], 19 to 46 kg/m^2) and 70 type 2 diabetic patients (age, 45 to 65 years; BMI, 19 to 46 kg/m^2) by the polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) method. Ox-LDL autoantibodies and anticardiolipin antibodies were determined at baseline and after 10 years of follow-up. At baseline, the *Pro12Ala* polymorphism was not associated with ox-LDL autoantibodies in nondiabetic subjects, whereas type 2 diabetic patients having the *Pro12Ala* or the *Ala12Ala* genotypes tended to have higher levels of ox-LDL autoantibodies than did type 2 diabetic patients with the *Pro12Pro* genotype. At the 10-year follow-up, diabetic subjects having the *Ala12* allele had higher ox-LDL autoantibody levels than did diabetic subjects with the *Pro12Pro* genotype (*P* = .043 after adjustment for age, gender, BMI, and hemoglobin A_{1c} [HbA_{1c}] at 5 years). In nondiabetic subjects and regarding anticardiolipin antibodies, no such relationship was observed. We conclude that the *Pro12Ala* polymorphism of the *PPAR-γ2* gene was associated with increased ox-LDL autoantibodies in type 2 diabetic subjects. Genotype may therefore modulate the oxidative modification of LDL in hyperglycemic milieu.

PEROXISOME PROLIFERATOR-activated receptor-γ (PPAR-γ) is a nuclear receptor that regulates adipocyte differentiation, fat-specific gene expression, and insulin action.[1,2] Alternative use of promoters and differential splicing of the *PPAR-γ* gene result in 4 mRNA isoforms: PPAR-γ1, PPAR-γ2, PPAR-γ3, and PPAR-γ4.[3-5] PPAR-γ1 mRNA is expressed at low levels in many tissues, and PPAR-γ3 mRNA expression is confined to adipose tissue, colon epithelium, and macrophages, whereas PPAR-γ2 mRNA is most abundantly and relatively specifically expressed in the adipose tissue.[3-7] A missense mutation that results in a substitution of proline for alanine in codon 12 has been found in the *PPAR-γ2* gene-specific exon B.[8,9] This polymorphism has been reported to modulate body weight and insulin sensitivity. Increased body mass index (BMI) in those with the *Ala12* allele as compared to those with the *Pro12Pro* genotype has been reported,[10-13] but no difference[14-17] and even lower BMI[18] has been reported in other studies.

Functional studies[18] suggested that the Pro12Ala mutant isoform of PPAR-γ2 had reduced ability to activate transcription in COS cells and to induce adipogenesis in 3T3-L1 cells. Likewise, some studies have reported increased insulin sensitivity in subjects with the *Ala12* allele compared to that in subjects with the *Pro12Pro* genotype,[19-22] but increased insulin resistance has also been observed.[23]

The role of PPAR-γ with respect to atherosclerosis is a complex issue and evidently a double-edged sword: some of its functions are proatherogenic, whereas others are antiatherogenic. PPAR-γ may in theory affect foam cell formation, modulate the inflammatory response, and influence plaque stability and affect the concentrations of fibrinogen and C-reactive peptide.[24] PPAR-γ is expressed in activated monocyte and tissue macrophages and even in foam cells of atherosclerotic lesions,[24,25] although the physiological role of these transcription factors in macrophages has remained unresolved. PPAR-γ activation by agonists like thiazolidinediones or 15-deoxy$\Delta^{12,14}$ prostaglandin J_2 induces activation of the class B scavenger receptor CD36.[25] As a consequence, PPAR-γ–activated THP-1 cells take up increased amounts of oxidized low-density lipoproteins (ox-LDL).[26] Further, it is known that both thiazolidinediones and 15-deoxy$\Delta^{12,14}$ prostaglandin J_2 have anti-inflammatory effects that are independent of PPAR-γ. Recently Chawla et al[27] showed that CD36 is a target gene for PPAR-γ and that thiazolidinediones induce CD36 expression in wild-type but not in PPAR-γ–deficient macrophages. However, PPAR-γ expression was not required for its ligands to exert anti-inflammatory effect in macrophages.

Oxidative modification of LDL renders it more atherogenic compared to native LDL (nat-LDL) and is more rapidly taken up by macrophages; it also induces immunogenic responses.[28] Ox-LDL is found in atherosclerotic lesions[29] and autoantibodies against ox-LDL have been detected in human and animal plasma and atherosclerotic lesions.[30] Antiphospholipid antibodies contain antibodies against cardiolipin, phosphatidylserine, and phosphatidylethanolamine and have been linked with inflammatory and autoimmune disease, like lupus erythematosus.[31] The predictive role with respect to the development of

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Submitted May 20, 2002; accepted September 3, 2002.

Supported by the grants from Academy of Finland and the EVO fund (Grant No. 5106) of the Kuopio University Hospital.

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0026-0495/03/5202-0022$35.00/0

doi:10.1053/meta.2003.50039

cardiovascular events of ox-LDL autoantibodies and of anticardiolipin antibodies has been observed.[28,32]

Based on the above data, we hypothesized that the *Pro12Ala* polymorphism of the *PPAR-γ2* gene may affect the immune response, in particular to ox-LDL. Therefore, we investigated the association of the *Pro12Ala* polymorphism of the *PPAR-γ2* gene with ox-LDL antibodies and anticardiolipin antibodies in a Finnish type 2 diabetic and noniabetic subjects in a 10-year follow-up study.[33-36]

MATERIALS AND METHODS

Subjects and Study Design

The formation and representativeness of the baseline, 5-, and 10-year study populations have been described earlier in detail.[33-36] Briefly, the original study population consisted of 133 (70 men and 63 women) middle-aged newly diagnosed patients with type 2 diabetes, and 144 (62 men and 82 women) nondiabetic subjects selected from the population registers of the study area. Both groups were recruited during the years 1979 to 1981, and the groups were collected from a defined area of 180,000 inhabitants in the county of Kuopio in eastern Finland. The diagnosis of diabetes was made in the clinical setting, and it was confirmed by an oral glucose tolerance test (OGTT) according to the World Health Organization 1980 diagnostic criteria.[37] The groups were re-examined after 5 and 10 years during 1985 to 1986 and 1991 to 1992, respectively. During the 10-year follow-up, 36 (20 men and 16 women) type 2 diabetic patients and 8 (3 men and 5 women) nondiabetic subjects died. In addition, DNA samples could not be obtained from all subjects of the original study and, therefore, the screening for the *Pro12Ala* polymorphism of the *PPAR-γ2* gene was performed in 70 (38 men and 32 women) type 2 diabetic patients and 119 (55 men and 64 women) nondiabetic subjects who participated both in the baseline and 10-year follow-up examinations and who also had laboratory examinations. The study was approved by the Ethics Committee of the University of Kuopio and the Kuopio University Hospital. Informed consent was given by all subjects.

Biochemical and Anthropometric Measurements

For the present study, data from the baseline and 10-year examinations were used. The methods for performing the OGTT and the determinations of fasting blood glucose and plasma insulin have been presented earlier in detail.[33-36] Standing height without shoes was measured to the nearest 0.5 cm. Body weight was measured with an electric weighing equipment (model 708; Seca, Hamburg, Germany) with the subjects barefoot and in light clothing. BMI was calculated as weight (kg)/height $(m)^2$.

Screening for the Pro12Ala Polymorphism of the PPAR-γ2 Gene

The *Pro12Ala* polymorphism of the *PPAR-γ2* gene was detected by the polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) method as previously reported.[10]

Autoantibodies Against Ox-LDL

Autoantibodies were determined for 91 diabetic patients and 82 control subjects from the samples obtained in 1979 through 1981. At the 10-year examination these antibodies were measured for all diabetic patients participating in the follow-up study (n = 92) and for 80 of the control subjects for whom the baseline serum samples were also available. All serum samples were stored at −70°C until analyzed. Thus both genotype and anti–ox-LDL autoantibodies were measured in 75 nondiabetic and 55 diabetic subjects. Autoantibodies against ox-LDL were measured according to a modification of a published method.[29,38] The enzyme-linked immunosorbent assay (ELISA) used for the determinations does not measure antibodies against acetylated LDL or other types of nonoxidized LDL modifications. The three determinations made for each sample were identical except for antigen coating of microtiter plates (MaxiSorb, Nunc, Roskilde, Denmark): one plate was coated with nat-LDL, a second with 24-hour copper-oxidized LDL, and a third with postcoat only (see below). Plates were coated with antigen (5 μg/mL) in phosphate-buffered saline (PBS) overnight at 4°C. To prevent oxidation of nat-LDL, PBS contained 0.27 mmol/L EDTA and 20 μmol/L butylated hydroxytoluene (BHT). Plates were washed 3 times with PBS containing 0.5% Tween 20 and twice with water. Plates were blocked with 2% bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO) and washed as above. Serum samples in PBS containing 1% BSA, 0.27 mmol/L EDTA, 20 μmol/L BHT, and 0.05% Tween 20 were placed by pipette on plates at 1:20, 1:50, and 1:100 dilutions. Plates were incubated overnight at 4°C and washed. Horseradish peroxidase–conjugated anti-human IgG (Cappel, Organon Teknika Corp, Durham, NC) diluted 1:5,000 in the previous buffer was added, and plates were incubated at 4°C for 4 hours. After washing, the plates were incubated with peroxidase substrate (*o*-phenylenediamine) for 5 minutes. Color development was stopped with 2 mol/L H_2SO_4, and absorbances were measured at 492 nm by using a Multiskan microplate reader (Eflab, Helsinki, Finland). The reaction volume was 50 μL for each step except 150 μL for postcoating. All measurements were done in duplicate. Baseline and 10-year follow-up samples were always measured simultaneously on the same plate. Postcoat values for each dilution were subtracted from the analyzed samples. Results were calculated as the ratio of ox-LDL to nat-LDL at each dilution. Coefficients of variation for autoantibodies against ox-LDL and nat-LDL, as well as for the ox-LDL/nat-LDL ratio were below 10 %.[38] Between-run precision was calculated for the internal standard sample tested in duplicate on every plate in 10 different assays including a total of 38 plates. The tests were performed on 10 different days, with 3 to 5 plates tested on every day. Coefficients of variation for ox-LDL and nat-LDL absorbance values and the ox-LDL/nat-LDL ratio were 12.0 %, 11.4 %, and 8.0 %, respectively. In the present article "anti-ox-LDL antibodies" refers to this ratio.

Anticardiolipin Antibodies

Autoantibodies against cardiolipin were also determined with an ELISA method.[39] One half of a flat-bottomed microtiter plate (Maxisorp, Nunc) was coated with absolute ethanol and another half with cardiolipin (Sigma Chemical Co) (50 μg/mL in absolute ethanol) 25 μL/well. Ethanol was evaporated to dryness under a stream of nitrogen and plates were incubated at room temperature for 24 hours to oxidize cardiolipin. The plates were blocked with PBS containing 2% BSA, 0.27 mmol/l EDTA, and 20 μmol/l BHT 150 μL/well for 2 hours. Plates were washed 3 times with PBS containing 0.05% Tween 20 (Wellwash 4 MK II, Labsystems Oy, Helsinki, Finland). Serum samples were diluted (1:50) in PBS containing 1% BSA, 0.05% Tween 20, 0.27 mmol/l EDTA, and 20 μmol/L BHT and pipetted on plates 50 μL/well. Plates were incubated for 2 hours and washed as above. Horseradish peroxidase–conjugated anti-human IgG (Cappel, Organon Teknika Corp) diluted 1:4,000 in the sample buffer was placed on the plates 50 μL/well and incubated for 2 hours. After washing, plates were incubated with peroxidase substrate (tetramethylbenzidine [TMB] as chromogen) 50 μL/well for 30 minutes in the dark. Color development was stopped with 0.5 mol/L H_2SO_4 50 μL/well. Absorbances were measured at 450 nm (Multiskan microplate reader, Labsystems Oy). All incubations were performed at room temperature. The results were calculated by subtracting the binding to ethanol-coated wells from the binding to cardiolipin-coated wells after subtracting the mean background binding to the wells. The intra-assay coefficient of variation was 11.1 % and interassay, 10.0 %.

Table 1. Characteristics of the Study Population by the *Pro12Ala* Polymorphism of the *PPAR-γ2* Gene

	Nondiabetic Subjects			Type 2 Diabetic Patients		
	Pro12Pro	*Pro12Ala/Ala12Ala*	*P* Value	*Pro12Pro*	*Pro12Ala/Ala12Ala*	*P* Value
Baseline						
No. of subjects	93	26		56	14	
Sex (male/female)[a]	40/53	15/11		31/25	7/7	
Age (yr)	54.0 ± 5.4	54.0 ± 5.8	NS	55.8 ± 5.6	53.9 ± 5.1	NS
Weight (kg)	73.3 ± 13.6	75.5 ± 13.1	NS	83.7 ± 15.6	84.3 ± 17.5	NS
BMI (kg/m^2)	27.1 ± 4.6	27.1 ± 2.9	NS	30.4 ± 5.1	30.7 ± 5.9	NS
Fasting blood glucose (mmol/L)	5.5 ± 0.7	5.7 ± 0.7	NS[b]	9.9 ± 3.4	10.1 ± 2.4	NS[b]
Fasting plasma insulin (pmol/L)	89.8 ± 45.4	105.9 ± 79.3	NS[b]	134.6 ± 83.2	107.6 ± 70.0	NS[b]
Ox-LDL antibodies[a]	2.10 ± 1.13	2.07 ± 1.25	NS	2.05 ± 1.47	2.77 ± 1.35	.077[c] .091[d]
Anticardiolipin antibodies[e]	0.64 ± 0.17	0.59 ± 0.15	NS	0.57 ± 0.18	0.68 ± 0.24	.183[c] .081[d]
10-year study						
Weight (kg)	74.4 ± 14.3	79.6 ± 13.7	.023	79.4 ± 15.9	78.2 ± 16.7	NS
BMI (kg/m^2)	27.5 ± 4.9	28.5 ± 3.3	.042	28.8 ± 5.0	28.4 ± 5.1	NS
Fasting blood glucose (mmol/L)	6.0 ± 1.0	6.3 ± 1.7	NS[a]	11.9 ± 3.4	12.9 ± 2.7	NS[a]
Fasting plasma insulin (pmol/L)	70.1 ± 40.0	78.4 ± 49.6	NS[a]	91.3 ± 44.8	87.8 ± 38.2	NS[a]
Ox-LDL antibodies[f]	2.25 ± 1.77	1.85 ± 1.88	NS	1.95 ± 1.27	2.83 ± 1.04	.041[c] .043[g]
Anticardiolipin antibodies[h]	0.69 ± 0.21	0.67 ± 0.21	NS	0.61 ± 0.20	0.66 ± 0.26	NS

NOTE. Data are given as means ± SD.

[a]Number of nondiabetic subjects: *Pro12Pro* = 56 and *Pro12Ala/Ala12Ala* = 19; number of type 2 diabetic patients: *Pro12Pro* = 45 and *Pro12Ala/Ala12Ala* = 9.

[b]*P* value after adjustment for BMI.

[c]*P* value indicates the significance of the differences between the genotypes.

[d]*P* value after adjustment for age, gender, fasting glucose, and BMI.

[e]Number of nondiabetic subjects: *Pro12Pro* = 66 and *Pro12Ala/Ala12Ala* = 19; number of type 2 diabetic patients: *Pro12Pro* = 44 and *Pro12Ala/Ala12Ala* = 13.

[f]Number of nondiabetic subjects: *Pro12Pro* = 55 and *Pro12Ala/Ala12Ala* = 18; number of type 2 diabetic patients: *Pro12Pro* = 45 and *Pro12Ala/Ala12Ala* = 10.

[g]*P* value after adjustment for age, gender, BMI, and $HbA1_{1c}$ (at 5-year examination).

[h]Number of nondiabetic subjects: *Pro12Pro* = 91 and *Pro12Ala/Ala12Ala* = 26; number of type 2 diabetic patients: *Pro12Pro* = 49 and *Pro12Ala/Ala12Ala* = 11.

Statistical Analysis

The significance of differences in genotype and allele frequencies between nondiabetic subjects and type 2 diabetic patients was analyzed using 2-sided chi-square and Fisher's exact tests in the StatXact-4 program version 4.0.1 (Cytel Software Corp, Cambridge, MA). Otherwise the data were analyzed using the SPSS/WIN program version 9.0 (SPSS Inc, Chicago, IL). Normal distribution of continuous variables was verified with the Kolmogorov-Smirnov test with Lilliefors correction. Fasting blood glucose, fasting plasma insulin, ox-LDL antibodies, and anticardiolipin antibodies were logarithmically transformed to obtain a normal distribution. In statistical analysis, subjects who were heterozygous and homozygous for the *Ala12* allele were combined and compared with subjects who were homozygous for the *Pro12* allele. The association of the *Pro12Ala* polymorphism with continuous variables was tested by the univariate analysis of variance (GLM). A *P* value less than .05 (2-tailed) was considered statistically significant. Data are presented as mean ± standard deviation, unless otherwise indicated.

RESULTS

The genotype distribution of the *Pro12Ala* polymorphism of the *PPAR-γ2* gene was *Pro12Pro* 78.2% (n = 93), *Pro12Ala* 19.3% (n = 23), and *Ala12Ala* 2.5% (n = 3) in nondiabetic subjects, and *Pro12Pro* 80.0% (n = 56), *Pro12Ala* 17.1% (n = 12), and *Ala12Ala* 2.9% (n = 2) in type 2 diabetic patients. The genotype distributions were in Hardy-Weinberg equilibrium. There was no difference between nondiabetic subjects and type 2 diabetic patients in the frequency of the *Ala12* allele (0.122 in nondiabetic subjects *v* 0.114 in type 2 diabetic patients, $P = .871$) or in the distribution of genotypes ($P = .947$).

The characteristics of the study population by the *Pro12Ala* polymorphism of the *PPAR-γ2* gene at the baseline and 10-year examinations are presented in Table 1. At baseline, study weight and BMI did not differ among the genotypes in the nondiabetic subjects or in the type 2 diabetic patients. However, during the 10-year follow-up, nondiabetic subjects having the *Ala12* allele gained more weight than did nondiabetic subjects with the *Pro12Pro* genotype,[40] and at the 10-year examination nondiabetic subjects with the *Ala12* allele had higher BMI than nondiabetic subjects with the *Pro12Pro* genotype *Pro12Ala* genotype.

The levels of ox-LDL autoantibodies and anticardiolipin antibodies showed no significant differences at baseline between those with or without the *Ala12* allele, although those diabetic patients with the *Ala12* allele tended to have higher ox-LDL autoantibody levels than those with the wild type ($P =$

.08). However, at the 10-year examination, the difference in ox-LDL autoantibodies was statistically significant between the genotypes in diabetic patients ($P = .041$). These differences persisted even after adjustment for age, gender, BMI, and hemoglobin A_{1c} (HbA_{1c}) taken at the 5-year follow-up to indicate the degree of hyperglycemia during the follow-up ($P = .043$). No such effects were observed in control subjects nor regarding anticardiolipin antibodies.

DISCUSSION

The novel finding of our study was that the *Ala12* allele was associated with increased ox-LDL antibodies in diabetic subjects only. This association was not seen in nondiabetic subjects nor in terms of anticardiolipin antibodies. This genotype effect may require CD36 receptors and is expressed more evidently in hyperglycemic milieu.

In some studies the *Pro12Ala* polymorphism of the *PPAR-γ2* gene has been associated with protection from type 2 diabetes, low BMI, and insulin levels suggesting an increased insulin sensitivity.[18, 21,22] There is also evidence that this polymorphism is associated with decreased transcriptional activity in vitro.[19] Recently, Stumvoll et al[41] showed that this polymorphism was also associated with increased insulin sensitivity of glucose disposal and suppression of lipolysis. Additionally, the role of CD36 in insulin action and fatty acid metabolism has been observed. Miyaoka et al showed that in a Japanese population CD36 deficiency was associated with many features of insulin resistance, such as increased plasma triglycerides, glucose, and blood pressure levels and lowered high-density lipoprotein (HDL)-cholesterol and insulin action.[42] However, rodents with CD36 deficiency had in fact a marked reduction in atheroma formation as compared to controls.[43] This discrepancy may be explained by that the absence of CD36 results in decreased macrophageal uptake of ox-LDL and reduced foam-cell formation.

The effect of PPAR-γ on ox-LDL uptake is likely to be cell type– and context-dependent.[26] Glucose is an important regulator of macrophage PPAR expression.[44] The association of the *Pro12Ala* polymorphism with antibodies in diabetic patients could be explained by the reduced transcriptional activity of PPAR-γ. This may mean lowered CD36 induction and decreased uptake of ox-LDL by macrophages. PPAR-α inhibits expression of monocyte-recruiting proteins such as vascular cell adhesion molecule (VCAM)-1 and PPAR activation in macrophages and foam cells inhibits the expression of inducible nitric oxide synthase, matrix metalloproteinase-9, and scavenger receptor A. PPAR-γ may also affect the recruitment of monocytes in atherosclerotic lesions, as it is involved in the expression of VCAM-1 and intracellular adhesion molecule-1 in vascular endothelial cells[45] and their increased expression could facilitate the migration of monocytes to the arterial wall. Monocytes are the source of free radicals, which increase the oxidation of LDL in the arterial wall. Thus more circulating ox-LDL may remain as antigens and thus increase the formation of ox-LDL antibodies. As PPAR-γ expression is not required for its ligands to exert their anti-inflammatory effects[27]; this could in turn potentiate the ox-LDL antibody formation. However, in this scenario increased ox-LDL autoantibodies in this hyperglycemic milieu would act as innocent bystanders with respect to the development of atherosclerosis. Despite increased ox-LDL, the reduced expression of CD36 receptors decreases the formation of foam cells, thus stabilizing the atherosclerotic plaques. This effect may in part explain our previous finding from this same population, in which these ox-LDL autoantibodies had no predictive value in diabetic patients with respect to cardiovascular events and showed no association with carotid intima media thickness in diabetic patients.[46]

In our study, the *Ala12* allele frequency was similar as reported in previous studies among Caucasians.[8,11,13,17] The frequency of the *Ala12* allele did not differ between nondiabetic subjects and type 2 diabetic patients. However, the sample size in our study was too small to assess the association between the *Ala12* allele and type 2 diabetes.

In conclusion, the *Pro12Ala* polymorphism of the *PPAR-γ2* gene was associated with increased ox-LDL autoantibodies in type 2 diabetic subjects. Genotype may therefore modulate the oxidative modification of LDL in hyperglycemic milieu.

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