CLINICAL STUDY

A Pro12Ala polymorphism in the human peroxisome proliferator-activated receptor- γ 2 is associated with combined hyperlipidaemia in obesity

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Abstract

Objective: Peroxisome proliferator-activated receptor- γ 2 (PPAR γ 2) is an important regulator of adipose tissue metabolism and insulin sensitivity. The aim of this investigation was to determine whether a PPAR γ 2 Pro12Ala polymorphism was associated with cardiovascular risk factors (obesity, blood pressure, diabetes and blood lipids) in Western Australian Caucasians ($n = 663$).

Design: Subjects were selected from two population studies (the Carotid Ultrasound Disease Assessment Study (CUDAS) and Busselton Population Health Survey) on the basis of body mass index (BMI). 292 obese (BMI ≥ 30 kg/m²) and 371 lean (BMI < 25 kg/m²) subjects were studied.

Methods: Blood pressure and anthropometric measurements were collected from all participants, as well as a fasting venous blood sample. Biochemical measurements (high-density lipoprotein (HDL)- and low-density lipoprotein-cholesterol, triglycerides) and PPAR γ 2 Pro12Ala genotype were also determined.

Results: Obese Pro/Ala and Ala/Ala subjects had lower levels of HDL-cholesterol ($P = 0.032$) and a trend towards higher levels of triglycerides ($P = 0.055$) compared with obese Pro/Pro subjects. In the obese group, the Ala allele was significantly associated with the presence of combined hyperlipidaemia (odds ratio = 2.33, $P = 0.042$). There was no significant difference in the frequency of the polymorphism between lean and obese groups ($P = 0.069$). No association was observed between Pro12Ala genotype and obesity, blood pressure or diabetes in either group.

Conclusions: Obese carriers of the Pro12Ala polymorphism have a greater risk of developing combined hyperlipidaemia, possibly due to impaired activation of PPAR γ target genes. The Pro12Ala polymorphism is not directly associated with obesity, hypertension or diabetes in this population.

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Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily. There are three types of PPAR and, of these, PPAR γ has been shown to regulate adipocyte differentiation and lipid metabolism through the activation of adipocyte-specific genes (1). PPAR γ controls the transcription of target genes by the formation of a heterodimeric complex with retinoid X receptors, and this complex binds to specific PPAR γ response elements in the promoter regions of these genes (2). Forced expression of PPAR γ in fibroblasts stimulates differentiation into adipocytes (3).

Rare gain-of-function (mutant alleles directing over-expression of normal gene products) and dominant

negative mutations in PPAR γ have provided insight into its biological function *in vivo*. A Pro115Gln PPAR γ mutant is associated with excessive adipose tissue accumulation through an intrinsic increase in adipogenic activity (4). Dominant negative PPAR γ mutations are associated with severe insulin resistance, hypertension and alterations in lipid profile (low high-density lipoprotein (HDL), high triglycerides) (5).

Alternative promoter regions within the PPAR γ gene allow the formation of three PPAR γ isoforms: PPAR γ 1, γ 2 and γ 3. While PPAR γ 1 mRNA is found in a wide range of tissues (including heart, liver, skeletal muscle and adipose tissue), PPAR γ 2 is most abundant in adipose tissue (6). PPAR γ 3 is expressed in macrophages, colon epithelium and adipose tissue (7). PPAR γ 2 contains 30 additional amino acids at its N-terminus, which may

confer a greater ligand-independent activation function (augmented by insulin) upon this isoform (8). PPAR γ 2 expression is increased in adipose tissue taken from obese subjects compared with lean subjects, and is reduced during weight loss (6).

A common polymorphism (frequency approximately 0.12 in Caucasians) occurs in the PPAR γ 2-specific exon B (9). At position 34, a C to G substitution causes an amino acid change from a proline to an alanine at codon 12 (Pro12Ala). Functionally, this Ala variant has been shown to have a reduced affinity for the response element and a lower capacity for activating target genes (10).

This polymorphism has been previously studied for association with obesity and non-insulin-dependent diabetes mellitus. Results have been inconsistent with respect to body mass index (BMI) (10–15), blood lipid profile (10, 11, 14) and insulin sensitivity (10, 13, 14, 16–18).

It was the aim of this study to clarify whether the PPAR γ 2 Ala allele is associated with obesity or altered cardiovascular risk profile (visceral adiposity, blood pressure, diabetes and blood lipids) in a large group of Caucasian Western Australians.

Subjects and methods

Subjects were selected from two populations: the Carotid Ultrasound Disease Assessment Study (CUDAS) (19) and the Busselton Population Health Survey (20). These two populations were predominantly Caucasian and were collected from the same region of Western Australia.

The CUDAS group was composed of original participants in the 1989 Australian National Heart Foundation Perth Risk Factor Prevalence Survey, a random electoral survey from the Perth metropolitan area. From this survey, all subjects classified as obese (BMI ≥ 30 kg/m²; $n = 188$) were genotyped for the Pro12Ala polymorphism, as were a randomly selected group of lean controls (BMI < 25 kg/m²; $n = 271$). BMI was the only selection criterion for genotyping.

Subjects from the Busselton Population Health Survey were studied as part of a cross-sectional community study in 1994. From 104 families with two or more overweight siblings (BMI > 25 kg/m²), one obese (BMI ≥ 30 kg/m²) sibling was randomly selected for genotyping. A group of 100 lean controls with similar age and sex distribution was also studied.

CUDAS and Busselton groups were both selected on the basis of BMI and did not differ significantly with respect to age, sex or allele frequency (data not shown). The obese and lean groups from each population were combined to form larger groups of 292 obese and 371 lean subjects. All subjects gave written informed consent. The study protocol was approved by the Institutional Ethics Committee of the University of Western Australia, the Sir Charles Gairdner Research

Institutional Ethics Committee and the Busselton Population Medical Research Foundation.

A self-administered questionnaire was used to report the prevalence of smoking and diabetes. Resting blood pressures and anthropometric measurements (height, weight, waist and hip circumferences) were taken from all subjects. A fasting venous blood sample was collected for lipid measurements. Triglycerides, HDL-cholesterol and total cholesterol were determined using a Hitachi 747 autoanalyser. Low-density lipoprotein (LDL)-cholesterol was calculated by the method of Friedewald *et al.* (21).

A salt/phenol/chloroform method was used to isolate the genomic DNA from leukocytes. Mutagenically separated PCR was performed using two different length allele-specific downstream primers (P1 and P2), and a common upstream primer (P3) (22). P1 (5' GTGTATCAGTGAAGGAATCGCTTTCTTG-3') was specific for the C allele (Pro); and primer P2 (5'-TTGTGATATGTTTGCAGACAAGGTATCAGTG AAGGAATCGCTTTGTGC-3') bound to the G allele (Ala). The upstream primer (P3) was 5'-TTTCTGTGTTTATTCCCATCTCTCCC-3'. Bases underlined and in bold type indicate the location of mismatches to maintain the specificity of the two separate amplification reactions (22).

DNA (40 ng) was added to a 25 μ l reaction mix containing 3 mM MgCl₂, 10 pmol P1, 5 pmol P2 and 10 pmol P3. The PCR conditions were: an initial denaturation of 3 min at 94 °C; followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 62 °C for 45 s, and extension at 72 °C for 45 s; then a final extension step of 3 min at 72 °C. A 230 bp product identified the Pro allele, and the Ala-specific product was 250 bp. One example of each genotype was sequenced to check for correct detection of the polymorphism.

Statistical analysis was performed using MINITAB (version 12.1) and InStat (version 3.00) software. Chi-square and Fisher's exact test were used to compare the categorical variables of sex, Pro12Ala genotype, smoking and diabetes between groups. Continuous variables were tested for normality according to the Kolmogorov–Smirnov test, and data sets not normally distributed (age, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, systolic blood pressure, diastolic blood pressure, BMI and waist-to-hip ratio) were log₁₀-transformed. Variables normalised by log₁₀-transformation (total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides) were then compared using unpaired two-tailed Student's *t*-tests, and the significance level was $P < 0.05$. Data sets not normally distributed after log₁₀-transformation (age, systolic blood pressure, diastolic blood pressure, BMI and waist-to-hip ratio) were compared using the Mann–Whitney U test. A general linear model was used to control for the variables of age, sex, BMI, smoking and diabetes where appropriate.

Table 1 Biochemical and anthropometric data for obese and lean groups. Data are presented as means $\pm$ s.d.

	Obese (n=292)	Lean (n=371)	P
Men	145	180	
Women	147	191	
Age (years)	54.8 $\pm$ 12.0	51.6 $\pm$ 13.3	0.0011
Total cholesterol (mmol/l)	5.76 $\pm$ 0.98	5.43 $\pm$ 0.99	<0.0001
HDL-cholesterol (mmol/l)	1.15 $\pm$ 0.29	1.50 $\pm$ 0.39	<0.0001
LDL-cholesterol (mmol/l)	3.82 $\pm$ 0.87	3.47 $\pm$ 0.89	<0.0001
Triglycerides (mmol/l)	1.72 $\pm$ 0.87	1.01 $\pm$ 0.54	<0.0001
Systolic blood pressure (mmHg)	136 $\pm$ 19	122 $\pm$ 17	<0.0001
Diastolic blood pressure (mmHg)	84 $\pm$ 10	76 $\pm$ 10	<0.0001
BMI (kg/m ²)	32.9 $\pm$ 2.6	22.0 $\pm$ 1.9	<0.0001
BMI range (kg/m ²)	30.0–43.4	16.4–25.0	
Waist-to-hip ratio	0.89 $\pm$ 0.09	0.81 $\pm$ 0.08	<0.0001
Smoking history (n) (%)	154 (53%)	171 (46%)	0.089
Diabetes (n) (%)	11 (4%)	3 (1%)	0.009

A binary logistic regression model was used to test for the association of Pro12Ala genotype and combined hyperlipidaemia, controlling for the risk factors of male gender, age, smoking, diabetes, systolic blood pressure and BMI.

Results

The biochemical and anthropometric results for the 292 obese (BMI $\geq$ 30 kg/m²) and 371 lean controls (BMI <25 kg/m²) are shown in Table 1. The obese group had significantly higher total plasma cholesterol, LDL-cholesterol, triglycerides, blood pressure, waist-to-hip ratio and lower HDL-cholesterol than the group of lean controls (all P < 0.0001). The obese subjects were significantly older than the lean controls (54.8 $\pm$ 12.0 vs 51.6 $\pm$ 13.3 years (mean age $\pm$ s.d.), P = 0.0011) and reported a higher prevalence of diabetes (4% vs 1%, χ^2 = 6.919, df = 1, P = 0.009).

All genotype groups obeyed the Hardy–Weinberg equilibrium (Table 2). The frequency of the Pro12Ala polymorphism ($f(\text{Ala})$) did not differ significantly between the obese group and the lean controls ($f(\text{Ala})_{\text{obese}}$ = 0.145 vs $f(\text{Ala})_{\text{lean}}$ = 0.129, χ^2 = 5.362, df = 2, P = 0.069).

Table 2 Prevalence of the Pro12Ala polymorphism in CUDAS and Busselton populations.

Population	Group	n	Genotype			f(Ala)
			Pro/Pro	Pro/Ala	Ala/Ala	
CUDAS	Obese	188	136	45	7	0.157
	Lean	271	205	64	2	0.125
Busselton	Obese	104	79	24	1	0.126
	Lean	100	72	28	0	0.140
Total	Obese	292	215	69	8	0.145
	Lean	371	277	92	2	0.129*

* No significant difference for Ala allele frequency between obese and lean groups (χ^2 = 5.362, df = 2, P = 0.069).

Due to the small number of homozygotes for the polymorphism, Ala/Ala individuals were included with the Pro/Ala group so that genotype was examined as a dichotomous variable (presence or absence of the polymorphism) (Table 3). In the lean group, the presence of the Ala allele was not associated with differences in BMI, waist-to-hip ratio, blood lipids, blood pressure or prevalence of diabetes. Obese Pro/Ala and Ala/Ala subjects had higher triglycerides and lower HDL-cholesterol compared with obese Pro/Pro subjects (triglycerides: 1.76 $\pm$ 0.34 vs 1.54 $\pm$ 0.33 mmol/l, P = 0.039; HDL-cholesterol: 1.07 $\pm$ 0.13 vs 1.16 $\pm$ 0.14 mmol/l, P = 0.011). When these results were adjusted for covariates (age, sex, BMI, smoking and diabetes) using a general linear model, the association between the Ala allele and HDL-cholesterol levels remained significant (P = 0.032), while the difference in triglyceride levels between genotypes was of borderline significance (P = 0.055).

The obese group was further examined by stratification according to combined hyperlipidaemia status. An individual was classed as hyperlipidaemic according to the following criteria: total cholesterol >5.2 mmol/l, LDL-cholesterol >3.5 mmol/l and triglycerides >2.3 mmol/l (suggested clinical thresholds for combined hyperlipidaemia according to the European Atherosclerosis Society International Task Force for Prevention of Coronary Heart Disease) (23). This hyperlipidaemic group comprised 28 individuals (16 Pro/Pro and 12 Pro/Ala – none were Ala/Ala). The unadjusted odds ratio for association of the Ala allele with combined hyperlipidaemia was 2.30 (95% confidence interval (CI), 1.03–5.11; P = 0.044, Fisher's exact test).

A logistic regression model (Table 4) was used to adjust the odds ratio for combined hyperlipidaemia and the presence of the Ala allele, while controlling for other known risk factors (23). Male gender, smoking and diabetes were analysed as categorical variables, while age, systolic blood pressure and BMI were included in the model as continuous variables. The

Table 3 Biochemical and anthropometric data for obese and lean subjects according to Pro12Ala genotype. Data are presented as means $\pm$ s.d.

	Obese subjects (n=292)			Lean subjects (n=371)		
	Pro/Pro (n = 215)	Pro/Ala and Ala/Ala (n = 77)	P	Pro/Pro (n = 277)	Pro/Ala and Ala/Ala (n = 94)	P
Men/women	102/113	43/34		132/145	48/46	
Age (years)	54.9 $\pm$ 12.2	54.5 $\pm$ 11.6	0.78	51.9 $\pm$ 13.1	50.5 $\pm$ 14.1	0.32
Total cholesterol (mmol/l)	5.75 $\pm$ 0.97	5.79 $\pm$ 1.04	0.76	5.38 $\pm$ 1.21	5.24 $\pm$ 1.19	0.22
HDL-cholesterol (mmol/l)	1.16 $\pm$ 0.14	1.07 $\pm$ 0.13	0.011*	1.50 $\pm$ 0.38	1.50 $\pm$ 0.44	0.41
LDL-cholesterol (mmol/l)	3.72 $\pm$ 1.25	3.71 $\pm$ 1.31	0.98	3.50 $\pm$ 0.91	3.37 $\pm$ 0.81	0.13
Triglycerides (mmol/l)	1.54 $\pm$ 0.33	1.76 $\pm$ 0.34	0.039*	1.02 $\pm$ 0.57	0.98 $\pm$ 0.46	0.77
Systolic blood pressure (mmHg)	137 $\pm$ 20	135 $\pm$ 19	0.45	122 $\pm$ 16	124 $\pm$ 18	0.40
Diastolic blood pressure (mmHg)	84 $\pm$ 10	86 $\pm$ 10	0.20	76 $\pm$ 10	76 $\pm$ 10	0.88
BMI (kg/m ²)	32.9 $\pm$ 2.6	32.9 $\pm$ 2.6	0.89	22.0 $\pm$ 1.8	22.1 $\pm$ 2.0	0.47
Waist-to-hip ratio	0.89 $\pm$ 0.89	0.90 $\pm$ 0.08	0.25	0.81 $\pm$ 0.08	0.81 $\pm$ 0.08	0.73
Diabetes (n) (%)	8 (4%)	3 (4%)	0.99	2 (1%)	1 (1%)	0.99

* $P < 0.05$.

adjusted odds ratio for association of the Ala allele and hyperlipidaemia in the obese group was 2.33 (95% CI, 1.03–5.29; $P = 0.042$).

Discussion

The main finding of this study is that obese carriers of the PPAR γ 2 Ala allele were found to have a more atherogenic lipid profile (characterised by lower HDL-cholesterol levels, and a trend towards higher triglycerides) compared with obese Pro/Pro individuals. Both lower HDL and higher triglycerides are known risk factors for coronary artery disease (24, 25), suggesting that the presence of the Ala allele may exacerbate the altered lipid profile common in obesity. The presence of the Ala allele was the only significant predictor of combined hyperlipidaemia in obese subjects when other risk factors such as blood pressure, smoking, BMI, male gender, age and diabetes were taken into account. These results were not seen in the lean group.

In this study, there was no evidence to suggest that the Pro12Ala polymorphism was a predisposing factor for obesity. Allele frequencies were similar between the obese group and lean controls, and the

Ala variant was not associated with higher BMI or waist-to-hip ratio within either group. Also, no differences between genotypes were observed for total cholesterol, LDL-cholesterol, blood pressure or frequency of diabetes in obese or lean subjects.

The association between Pro12Ala genotype, HDL-cholesterol and triglycerides in a large group of subjects has not been described previously. In a group of 57 extremely obese men, Beamer *et al.* (11) found that subjects with the Ala allele had lower HDL and higher triglyceride levels compared with Pro/Pro subjects. This effect was not observed in 112 extremely obese women or in a cohort of 517 lean to moderately obese individuals (BMI 18.6–43.2 kg/m²). Our positive observations were seen in a much larger group of obese subjects ($n = 292$).

A population study of 973 elderly Finnish subjects (10) found that Ala/Ala individuals ($n = 20$) had significantly higher HDL-cholesterol and lower triglycerides at follow-up compared with Pro/Pro and Pro/Ala subjects. However, these subjects were of lower BMI than the obese subjects studied here (mean BMI 25–27 vs 32.9 $\pm$ 2.6 kg/m²), and therefore they may be more equivalent to our lean group. Furthermore, in these subjects there appeared to be no difference in HDL-cholesterol between Pro/Pro and Pro/Ala subjects (1.27 $\pm$ 0.01 vs 1.24 $\pm$ 0.02 mmol/l), which is concordant with our findings in subjects of a similar BMI.

Another large population-based study (14) observed higher levels of total cholesterol, LDL-cholesterol and apolipoprotein B in carriers of the Ala allele compared with Pro/Pro individuals. Again, the mean BMI for each of the genotypes was much lower than that of our obese group (mean BMI 25–26 kg/m²), so these results are not directly comparable with the results seen in obese individuals.

The higher rate of combined hyperlipidaemia seen in the obese subjects with the polymorphism may be due to an intrinsic reduction in PPAR γ 2 activity. *In vitro*,

Table 4 Logistic regression model for association of PPAR γ 2 Ala allele with combined hyperlipidaemia* in obese subjects.

Predictor variables	Odds ratio	95% CI	P
Ala allele	2.33	1.03–5.29	0.042
Systolic blood pressure	1.02	1.00–1.04	0.087
Smoking history	1.67	0.70–3.99	0.25
BMI	1.09	0.93–1.28	0.27
Male gender	1.60	0.66–3.87	0.30
Age	0.99	0.96–1.03	0.77
Diabetes	0.91	0.11–7.84	0.93

* Combined hyperlipidaemia is defined as: total cholesterol >5.2 mmol/l, LDL-cholesterol >3.5 mmol/l and triglycerides >2.3 mmol/l (23). The PPAR γ 2 Ala allele, smoking history, male gender and diabetes are included in the model as categorical variables. Systolic blood pressure, BMI and age are given as continuous variables.

the Ala isoform of PPAR γ 2 has been shown to be less effective at activating target genes (10, 26). One of these target genes is lipoprotein lipase (LPL) (27). LPL hydrolyses triglycerides in circulating chylomicrons and very low-density lipoproteins, releasing fatty acids, chylomicron remnants and LDL-cholesterol. LPL activity has also been shown to correlate strongly with plasma HDL levels (28). Similar alterations in blood lipid profile (increased triglycerides, decreased HDL) are seen in heterozygous LPL deficiency (29), an effect which is more pronounced in obese individuals (30). The effect of the Ala allele on blood lipid profile might only be revealed in the obese state due to the larger adipose tissue mass and therefore relative abundance of the fat-specific PPAR γ 2 isoform.

In conclusion, the PPAR γ 2 Pro12Ala polymorphism was found to be significantly associated with a change in lipid profile in obese carriers. For these subjects, this common polymorphism may constitute a novel genetic risk factor for combined hyperlipidaemia and cardiovascular disease.

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