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ARTICLE

Association of β_2 adrenergic receptor polymorphisms and related haplotypes with triglyceride and LDL-cholesterol levels

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Adrenergic receptors regulate lipid mobilization, energy expenditure and glycogen breakdown. The β_2 adrenergic receptor (β_2-AR) gene may constitute a potential candidate gene to explain part of the genetic predisposition to human obesity and correlated traits. With regard to the association between β_2-AR gene polymorphisms and obesity-related metabolic disorders, published reports give conflicting results. We investigated the role of three polymorphisms, and related haplotypes of the β_2-AR in the obesity and related traits in a cohort of overweight/obese subjects. We characterized one single nucleotide polymorphism (SNP) in the promoter region (5′LC-Cys19Arg) and two in the coding region (Gly16Arg and Gln27Glu) of the β_2-AR in 642 consecutively recruited overweight/obese subjects in whom extensive clinical and biochemical analysis was performed. The effect of the polymorphisms on quantitative variables was investigated using multiple linear regression analysis. 5′LC-Cys19 homozygous showed higher triglyceride and LDL-cholesterol levels compared to 5′LC-Arg19 homozygous ($P=0.03$ and $P=0.01$, respectively). Similar increase in triglyceride and LDL-cholesterol levels was observed for Arg/Arg genotype compared to Gly/Gly genotype of Gly16Arg polymorphism ($P=0.02$ and $P=0.01$, respectively) and for Gln/Gln genotype compared to Glu/Glu genotype of the Gln27Glu polymorphism ($P=0.01$ and $P=0.03$, respectively). The 5′LC-Cys^{19}Arg^{16}Gln^{27} haplotype determined a significant increase in triglyceride and LDL-cholesterol levels compared to 5′LC-Arg^{19}Gly^{16}Glu^{27} haplotype ($P=0.05$ and $P=0.02$, respectively). Our findings provide additional weight to previous observations on the influence of these three genetic variants on lipid phenotypes; particularly on the increase of triglycerides and LDL-cholesterol levels in overweight/obese subjects carrying the 5′LC-Cys^{19}Arg^{16}Gln^{27} haplotype.
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Introduction

The human β_2 adrenergic receptor gene (β_2-AR) is encoded by a single intronless gene, located on the distal portion of the long arm of chromosome 5 (5q32–q34).

Adrenergic receptors regulate lipid mobilization, energy expenditure and glycogen breakdown through endogenous

catecholamines which are involved in the regulation of adipose tissue lipolysis, nonesterified fatty acid distribution, lipoprotein metabolism, glucose homeostasis, vascular tone and blood pressure.[1] Thus, the β_2-AR gene may constitute a potential candidate gene to explain part of the genetic predisposition to human obesity and related traits.

Genetic association studies have demonstrated an implication of β_2-AR polymorphisms in various disease phenotypes: obesity and type 2 diabetes (T2DM),[2] metabolic syndrome, metabolic disorders[3,4] and hypertension.[5,6]

Several polymorphisms have been found in the coding region of β_2-AR gene (Gly16Arg, Gln27Glu and Thr164Ile) and in the 5′ leader cistron (5′LC-Cys19Arg) each of them leading to amino-acid substitution.[7] When individually investigated, in airway smooth cells and transfected cell lines, each of these single nucleotide polymorphisms (SNPs) induces a variation in β_2-AR function.[8–12] 5′LC-Cys19Arg protein modulates receptor translation[11]; Gly16Arg and Gln27Glu alter cellular trafficking and receptor desensitization.[9,10,12]

However, with regard to the association between these polymorphisms and obesity-related metabolic disorders, published reports give conflicting results including wide interethnic variation, gender-related differences and difference regarding the identity of the allele involved.[13–20]

In light of these considerations, we conducted a study, in a cohort of overweight/obese Italian subjects, to investigate the role of these three polymorphisms: 5′LC-Cys19Arg, Gly16Arg and Gln27Glu of β_2-AR gene and related haplotypes in obesity and related traits.

Subjects and methods

Subjects

We studied 642 overweight/obese subjects (body mass index (BMI) $>25\,kg/m^2$), who were consecutively recruited from the metabolic Day Hospital of the Department of Clinical Sciences of University of Rome 'La Sapienza'. Exclusion criteria were: previous diagnosis of diabetes according to ADA criteria,[21] chronic liver diseases and any treatment known to interfere with insulin sensitivity and metabolic syndrome-related parameters. All subjects signed a written consent to participate in the study. The study protocol was approved by the Ethical Committee of the University of Rome 'La Sapienza'.

In all subjects BMI, blood pressure, waist circumference (measured midway between the lowest rib margin and the iliac crest) and 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).

Methods

Cholesterol and triglycerides concentrations were determined in plasma by Technicon RA-1000 Autoanalyzer; HDL was measured after precipitation of ApoB-containing lipoproteins with photungstic acid/$MgCl_2$. LDL-cholesterol was determined by the Friedewald formula.[22] Glucose levels were calculated by the glucose oxidase method (Autoanalyzer, Beckman Coulter, USA). Plasma insulin was measured in frozen samples by radioimmunoassay (Adaltis insulin kit, Bologna, Italy), according to the manufacturer's instruction, with a limit of detection of $<2.0\,\mu U/ml$.[23]

Insulin resistance status was estimated according to Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) following the formula previously described[24]: fasting insulin (mcU/ml) × fasting glucose (mmol/l)/22.5.

Nomenclature

The β_2-AR transcription start site is 5′ to a small ORF (termed the β_2-AR 5′-Leader Cistron or 5′-LC) which encodes a 19-aa peptide and denotes the β_2-AR upstream peptide (BUP). In this peptide it was found a polymorphism at amino-acid 19 (5′LC-Cys19Arg). N-terminal polymorphisms consist of substitutions of glycine for arginine at amino-acid position 16 (Cys16Arg) and glutamine for glutamic acid at position 27 (Gln27Glu).

Genotype analysis

The missense mutations 5′LC-Cys19Arg, Gly16Arg and Gln27Glu of β_2-AR gene, were genotyped using the flurogenic 5′ nuclease assay application of the ABI PRISM 7900 HT Sequence Detection System (ABI, Foster City, USA).

Genotyping of the 5′LC-Cys19Arg was performed using primers (0.9 μmol/l each) Forward 5′-CCGCTGAATGAGG CTTCCA-3′ and Reverse 5′-CCATGGCGCGCAGTCT-3′ and the TaqMan MGB probes Fam TCAGCAGGCGGAC and Vic TCAGCGGGCGGAC.

Genotyping of the Gly16Arg was performed using primers (0.9 μmol/l each) Forward 5′-GGCAGCGCCTTC TTGCT-3′ and Reverse 5′-ACCCACACCTCGTCCCTTT-3′ and the MGB probes Fam CCCAATGGAAGCCA and Vic CCCAATAGAAGCCATG.

Genotyping of the Gln27Glu was performed using primers (0.9 μmol/l each) Forward 5′-GCGCCGGACCAC GAC-3′ and Reverse 5′-CCACCACCCACACCTCGT-3′ and the MGB probes Fam TCACGCAGGAAAG and Vic TCACG CAGCAAAG.

Of the 10 ng/μl stok of DNA 4 ml were dispensed into 384-well PCR plates using a Biomek FX robot (Beckman Coulter, Fullerton, USA) to which 6 μl of a mix containing primers, MGB probes and TaqMan Universal PCR Master Mix (ABI, Foster City, CA, USA) were added as per the manufacturers' instructions. These were sealed with optical seals (ABI, Foster City, CA, USA) and incubated for 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).

Statistical analysis

Statistical analysis was performed using SPSS statistical software, version 12 (SPSS, Illinois, USA). Genotypic and allelic distributions were compared using the Pearson χ^2 test. The effect of the polymorphisms on quantitative variables was investigated using multiple linear regression analysis.

We created two dummy variables to regress the three genotypes of each SNP; in order to incorporate genotypes as predictors in the multiple regression analysis and we decided to base the reference category on homozygote wild-type subjects. We adjusted the crude effect of the polymorphisms taking into account gender, age and BMI and we included triglycerides in the model as predictor of LDL-cholesterol (or vice-versa).

Data for insulin, triglycerides, HOMA-IR, were $\log_{10}$ transformed to normalize their distribution. The frequencies haplotypes and the linkage disequilibrium matrix (LD) based on the D′ parameter, were estimated using THESIAS *(Testing Haplotype Effects in Association Studies)* software. The objective of this program is to perform haplotype-based association analysis in unrelated individuals. THESIAS software is based on the maximum likelihood model described in Tregouet *et al*[25] and is linked to the SEM algorithm.[26] THESIAS also allowed the simultaneous estimation of haplotype frequencies and of their associated effects on the phenotype of interest. The estimate haplotype effects to the levels of biochemical variables are approximately half of the overall phenotypic mean. We adjusted the crude effect of the haplotype taking into account gender, age and BMI. We adjusted the *P*-values using Bootstrap method performing 1000 runs under the null hypothesis that no variant or haplotypes influence any trait tested. The bootstrap method creates pseudo-data sets by sampling observations with replacement from each within-stratum pool of observations. An entire data set is thus created, and *P*-values for all tests are computed on this pseudo-data set. A counter records whether the minimum *P*-value from the pseudo-data set is less than or equal to the actual *P*-value for each base test. This process is repeated a large number of times, and the proportion of resampled data sets where the minimum pseudo-*P*-value is less than or equal to an actual *P*-value is the adjusted *P*-value. Bootstrap analysis of the minimum *P*-values across all tests performed revealed that 20 of 1000 replicates (0.02) were less than the best nominal *P*-value observed in our studies. Therefore, we can reject the global null hypothesis: no SNP or haplotype are associated with any traits investigated.

Results

The genotype frequencies of 5′LC-Cys19Arg, Gly16Arg and Gln27Glu polymorphisms were in agreement with Hardy–Weinberg equilibrium (0.23, 0.4 and 0.5, respectively).

The allele frequencies of the three polymorphisms were: 0.33 for the 5′LC-Arg19, 0.4 for the Arg allele of Gly16Arg and 0.31 for the Glu allele of the Gln27Glu.

The 5′LC-Cys19 homozygous carriers showed higher levels of triglyceride and LDL-cholesterol compared to 5′LC-Arg19 homozygous ($P=0.03$ and $P=0.01$, respectively) (Table 1). A similar increase in triglyceride and LDL-cholesterol levels was observed for Arg/Arg genotype compared to Gly/Gly genotype of Gly16Arg ($P=0.02$ and $P=0.01$, respectively) (Table 1) and for Gln/Gln genotype compared to Glu/Glu genotype of the Gln27Glu ($P=0.01$ and $P=0.03$, respectively) (Table 1). A strong LD was observed between the three polymorphisms (5′LC-Cys19 allele is in LD with either Arg16 and Gln27, $D'=0.84$ and $D'=0.94$, respectively; the Arg16 allele is in LD with Gln, $D'=0.83$); we used THESIAS software to estimate the frequencies of all different haplotypes (only the ones with a percentage higher than 10% are shown in Table 3), and we observed that the 5′LC-Cys^{19}Arg^{16}Gln^{27} haplotype determined an increase in triglyceride and LDL-cholesterol levels compared to 5′LC-Arg^{19}Gly^{16}Glu^{27} haplotype ($P=0.05$ and $P=0.02$, respectively) (Table 3). Moreover, the associated SNPs/haplotypes have independent influences on LDL-cholesterol and triglyceride levels.

The analysis of each SNP (Table 2) and related haplotype showed no statistically significant association with the other parameters investigated (data not shown).

Discussion

The present study shows a significant association between the three polymorphisms of β_2-AR gene, 5′LC-Cys19Arg, Gly16Arg and Gln27Glu, and triglyceride and LDL-cholesterol plasma levels.

Results concerning the association of Gly16Arg and Gln27Glu polymorphisms of β_2-AR gene and obesity-related traits are conflicting; some studies found an association in the whole population,[3,18,30] others only in male[18,27–29] or only in female populations.[14]

Also the results on the identity of the allele involved in the association of Gly16Arg and Gln27Glu polymorphisms with lipid profile appear contradictory.

Meirhaeghe A *et al*[27] observed that the Glu allele determines lower triglyceride levels while they did not find any significant association with Gly16Arg polymorphism.

On the other hand, other studies pointed out that Gly and Glu variants are associated with obesity and related traits.[3,18,29–31] In details, three previous studies found the Glu27 variant to be associated with higher triglyceride levels.[3,30,31] Two other studies found a similar association with higher LDL-cholesterol levels but did not observe any association with triglycerides.[18,29] Ukkola *et al*[18] observed that Gly16 variant determines higher LDL-cholesterol

Table 1 Biochemical parameters of subjects according to the three SNPs of β_2-AR gene

	5′LC-Cys19Arg				*Gly16Arg*				*Gln27Glu*			
	Cys/Cys Mean ± SD	*Cys/Arg* Mean ± SD	*Arg/Arg* Mean ± SD	*P**	*Gly/Gly* Mean ± SD	*Arg/Gly* Mean ± SD	*Arg/Arg* Mean ± SD	*P°*	*Gln/Gln* Mean ± SD	*Gln/Glu* Mean ± SD	*Glu/Glu* Mean ± SD	*P§*
n (men/woman)	285 (84/201)	295 (60/235)	62 (18/44)		230 (47/184)	312 (81/230)	100 (34/66)		301 (78/223)	280 (64/216)	61 (20/41)	
Total cholesterol (mmol/l)	5.38 ± 1.03	5.25 ± 1.04	5.02 ± 0.92	NS	5.34 ± 1.06	5.27 ± 1.04	5.42 ± 1.04	NS	5.3 ± 1.0	5.36 ± 1.04	5.11 ± 1.03	NS
HDL cholesterol (mmol/l)	1.32 ± 0.31	1.34 ± 0.34	1.35 ± 0.33	NS	1.34 ± 0.28	1.34 ± 0.37	1.32 ± 0.37	NS	1.33 ± 0.32	1.32 ± 0.34	1.32 ± 0.34	NS
LDL cholesterol (mmol/l)[a]	3.41 ± 0.83	3.24 ± 0.93	2.90 ± 0.70	0.01	3.13 ± 0.88	3.21 ± 0.87	3.46 ± 0.87	0.01	3.40 ± 0.86	3.25 ± 0.9	3.06 ± 0.74	0.03
Triglycerides (mmol/l)[b]	1.52 ± 0.98	1.26 ± 0.68	1.17 ± 0.74	0.03	1.25 ± 0.7	1.31 ± 0.88	1.49 ± 0.88	0.02	1.57 ± 0.92	1.37 ± 0.78	1.2 ± 0.78	0.01
Fasting glucose (mmol/l)	4.89 ± 0.73	4.87 ± 0.58	4.83 ± 0.55	NS	4.92 ± 0.61	4.85 ± 0.65	4.94 ± 0.65	NS	4.88 ± 0.70	5.04 ± 0.82	4.91 ± 0.62	NS
Fasting insulin (pmol/l)	180.82 ± 110.5	179.97 ± 115.7	181.19 ± 118.40	NS	179.4 ± 109.8	179.2 ± 116.48	180.82 ± 112.3	NS	181.5 ± 112.7	180.62 ± 114.8	180.8 ± 112.97	NS
$HOMA_{IR\ (arbitrary\ units)}$	5 ± 3.15	5.1 ± 3.2	5.07 ± 3.3	NS	5 ± 3.25	5.2 ± 3.38	5.1 ± 3.2	NS	5.07 ± 3.2	5.33 ± 3.36	5.1 ± 3.3	NS

Data are given as means and SD. All comparisons are adjusted for age, BMI and gender.
P^* = Cys/Cys *vs* ArgArg; P° = Gly/Gly *vs* Arg/Arg; $P^\S$ = Gln/Gln *vs* Glu/Glu; *P*-values are adjusted using bootstrap method.
[a]*P*-values are adjusted also for Triglycerides.
[b]*P*-values are adjusted also for LDL-cholesterol.

Table 2 Clinical features of subjects according to the three SNPs of β_2-AR gene

	5′LC-Cys19Arg				*Gly16Arg*				*Gln27Glu*			
	Cys/Cys Mean ± SD	*Cys/Arg* Mean ± SD	*Arg/Arg* Mean ± SD	*P**	*Gly/Gly* Mean ± SD	*Arg/Gly* Mean ± SD	*Arg/Arg* Mean ± SD	*P°*	*Gln/Gln* Mean ± SD	*Gln/Glu* Mean ± SD	*Glu/Glu* Mean ± SD	*P§*
n	285	295	62		230	312	100		301	280	61	
Age (years)	38.76 ± 13.61	40.40 ± 13.63	38.23 ± 12.46	NS	39.58 ± 13.44	39.03 ± 13.55	39.76 ± 14.68	NS	39.25 ± 13.64	39.71 ± 13.77	38.41 ± 13.46	NS
BMI (Kg/m^2)	39.63 ± 8.58	38.12 ± 7.52	39.80 ± 10.99	NS	39.15 ± 8.26	39.30 ± 8.30	38.59 ± 10.26	NS	38.88 ± 8.56	39.1 ± 7.79	38.54 ± 10.19	NS
Systolic BP (mmHg)	130.31 ± 17.54	131.34 ± 17.76	128.91 ± 15.13	NS	130.09 ± 17.41	130.63 ± 16.78	129.38 ± 19.11	NS	129.78 ± 17.66	132.67 ± 17.19	128.88 ± 16.81	NS
Diastolic BP (mmHg)	84.55 ± 11.11	84.48 ± 12.12	83.02 ± 10.14	NS	83.78 ± 10.65	84.16 ± 12.09	84.80 ± 12.07	NS	83.89 ± 11.40	84.83 ± 11.80	83.67 ± 11.97	NS
WAIST/HIP	0.88 ± 0.09	0.88 ± 0.09	0.86 ± 0.05	NS	0.87 ± 0.08	0.88 ± 0.10	0.89 ± 0.07	NS	0.87 ± 0.09	0.88 ± 0.09	0.86 ± 0.12	NS

Data are given as means and SD. All comparisons are adjusted for age, BMI and gender.
P^* = Cys/Cys *vs* ArgArg; P° = Gly/Gly *vs* Arg/Arg; $P^\S$ = Gln/Gln *vs* Glu/Glu; *P*-values are adjusted using bootstrap method.

Table 3 Frequency haplotypes and lipid profile according to the aploid configuration relative to the three most frequent haplotypes of β_2-AR gene in overweight/obese subjects

Aploid configuration	*Frequency*	*Total cholesterol (mmol/l)*	*CI*	*HDL cholesterol (mmol/l)*	*CI*	*LDL cholesterol*[§] *(mmol/l)*	*CI*	*Triglycerides*[*] *(mmol/l)*	*CI*
5′LC-Cys^{19}Arg^{16}Gln^{27}	0.38	1.99	1.80–2.21	0.53	0.46–0.60	1.27	1.11–1.44	0.65	0.49–0.81
5′LC-Cys^{19}Gly^{16}Gln^{27}	0.26	2.07	1.90–2.30	0.53	0.45–0.59	1.22	1.06–1.39	0.60	0.43–0.77
5′LC-Arg^{19}Gly^{16}Glu^{27}	0.28	1.94	1.70–2.20	0.58	0.47–0.70	1.10	0.93–1.27	0.57	0.38–0.74

[§]$P = 0.02$ 5′LC-Cys^{19}Arg^{16}Gln^{27} *vs* 5′LC-Arg^{19}Gly^{16}Glu^{27} adjusted for age, BMI, gender and triglycerides; *P*-value is adjusted using bootstrap method.
[*]$P = 0.05$ 5′LC-Cys^{19}Arg^{16}Gln^{27} *vs* 5′LC-Arg^{19}Gly^{16}Glu^{27} adjusted for age, BMI, gender and LDL-cholesterol; *P*-value is adjusted using bootstrap method.

levels. It is, however, interesting to notice that other authors did not observe any association at all.[13,15,16,20]

These discrepancies and heterogeneity of the results can be explained by various factors such as: different genetic backgrounds, environmental factors and sex-related variables. Other influencing factors may be insufficient power or heterogeneity of the population studied in term of clinical phenotype (lean, obese) and different type of study (population based, clinical based).

The haplotype analysis coincides with the results obtained by the evaluation of each single SNP showing an increase of triglyceride and LDL-cholesterol levels in 5′LC-Cys^{19}Arg^{16}Gln^{27} compared to 5′LC-Arg^{19}Gly^{16}Glu^{27} haplotype. In order to distinguish the effect of each SNP independently, due to a strong LD, we would need a larger sample size. Consistently with our findings, a recent study revealed an association between LDL-cholesterol and Arg^{16}Gln^{27} haplotype.[32] The increase in triglyceride levels could be explained by the fact that the 5′LC-Cys^{19}Arg^{16}Gln^{27}/5′LC-Cys^{19}Arg^{16}Gln^{27} genotype modulates lipolysis (noradrenaline induced) rate if compared to 5′LC-Arg^{19}Gly^{16}Glu^{27}/5′LC-Arg^{19}Gly^{16}Glu^{27} as it was demonstrated in a recent study.[33] Furthermore, Drysdale *et al*[34] showed that subjects carrying the 5′LC-Cys^{19}Arg^{16}Gln^{27} haplotype have a 50% lower response to agonist than those carrying the second haplotype. These authors were the first to explore the combination of the entire haplotype of β_2-AR gene; their study showed different results from those previously obtained with individual SNPs.[9,10,35,11,13]

Regarding the Gly16Arg polymorphism, Large *et al*[36] found that terbutaline evoked a lipolytic response that was higher in Gly16 allele carriers compared to Arg16 homozygotes. Additionally, the Glu isoform of the Gln27Glu polymorphism, may be responsible for the altered ability of the receptor to be degraded by not being able to reach the mature wild-type conformation.[9] Furthermore, Arner *et al*[37] observed an inverse relationship between sensitivity and plasma triglyceride levels. Accordingly, Meirhaeghe *et al*[27] postulated that an accumulation of triglyceride levels in Gln27 carriers may be due to a less efficient lipolysis compared to Glu27 carriers; all the above-mentioned observations seem to lead to the same direction of our results.

A previous *in vitro* study showed that Gly16Arg and Gln27Glu polymorphisms have different properties of agonist-promoted downregulation. This downregulation appears to be significantly enhanced with the Gly16 variant; while the Glu27 variant shows to be strongly resistant.[9] However, the combination of the two variants displays enhanced downregulation showing that the Gly16 effects dominate the phenotype.[9]

Rather conflicting results have been published on the *ex vivo* agonist promoted desensitization of these β_2-AR isoforms. Chong *et al*[38] found that both mutant forms

(Gly16 and Glu27) of the β_2-AR were resistant to isoprenaline-induced desensitization compared to wild-type forms (Arg16 and Gln27), while an opposite result was also reported.[12] These alterations may be due to a different mechanism considering that ligand binding and functional activation of these receptors were not affected. It is not clear whether the different properties of agonist-promoted down-regulation, due to the two polymorphisms, could influence the lipolysis rate.

Finally, it has been observed that the 5′LC-Cys19 variant determines consistently greater β_2-AR expression levels as compared to cells expressing 5′LC-Arg19.[11]

In conclusion, despite a considerable amount of research on β_2-AR coding SNPs, the results obtained by the different studies are often divergent. This inconsistency has more than one explanation. First of all, the findings are complicated by the unclear relation between the expression of the receptor, lipolysis and plasma triglyceride values. Functional studies showed the pharmacologic stimulation of the β_2-AR does not change or even decrease plasma lipid levels[39] and low β_2-AR sensitivity is linked to hypertriglyceridemia.[37] Second, because the presence of LD between SNPs and the different functional effects of the haplotypes created by these SNPs are potential sources of bias when studying different SNPs of the same gene, especially when all of them can be functionally relevant.

Our findings provide additional weight to previous observations on the influence of these three genetic variants on lipid phenotypes, particularly on the increase of triglycerides and LDL-cholesterol levels in overweight/obese subjects carrying the 5′LC-Cys^{19}Arg^{16}Gln^{27} haplotype.

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