The Codon 64 Polymorphism of the β_3-Adrenergic Receptor Gene Is Not Associated With Coronary Heart Disease or Insulin Resistance in Nondiabetic Subjects and Non–Insulin-Dependent Diabetic Patients

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Hyperinsulinemia has been shown to predict coronary heart disease (CHD) events in both nondiabetic subjects and patients with non–insulin-dependent diabetes mellitus (NIDDM). Therefore, defects in genes that regulate insulin action could be responsible for an increased risk of CHD. The Trp64Arg polymorphism of the β_3-adrenergic receptor gene has been linked with abdominal obesity, insulin resistance, and early-onset NIDDM. Therefore, we screened for this polymorphism among 185 unrelated nondiabetic subjects (101 men and 84 women; age, 56 ± 1 years [mean ± SEM]; body mass index [BMI], 27.8 ± 0.3 kg/m^2) with angiographically confirmed CHD (stenosis > 50% in ≥ two coronary arteries), among 119 unrelated patients with NIDDM (90 men and 29 women; age, 62 ± 1 years; BMI, 28.7 ± 0.4 kg/m^2; 95 had CHD by the same criteria and 24 had definite myocardial infarction [MI]), and among 82 healthy men (age, 54 ± 1 years; BMI, 26.3 ± 0.4 kg/m^2) from our previous study. The frequency of the Trp64Arg allele of the β_3-adrenergic receptor gene was similar in nondiabetic patients with CHD (8%), NIDDM patients with CHD (7%), and nondiabetic subjects without CHD (7%). No association was found between cardiovascular risk factors and the codon 64 polymorphism of the β_3-adrenergic receptor gene in patients with CHD. Similarly, this polymorphism was not significantly related to insulin resistance in nondiabetic and NIDDM subjects with CHD evaluated by the euglycemic clamp technique. These results indicate that the Trp64Arg allele of the β_3-adrenergic receptor gene does not contribute to the risk of CHD in nondiabetic subjects and NIDDM patients.

SEVERAL POPULATION-BASED studies have indicated that hyperinsulinemia, a compensatory mechanism for insulin resistance, predicts coronary heart disease (CHD) events in nondiabetic subjects.[1-3] The mechanisms by which hyperinsulinemia or insulin resistance exert their effects are largely unknown, but could be direct or indirect through effects on cardiovascular risk factors (low high-density lipoprotein [HDL] cholesterol, high total triglycerides, glucose intolerance, obesity, and central fat distribution).[4,5] Therefore, the genes that modify insulin action could be the key in determining the risk for CHD.

The β_3-adrenergic receptor gene is expressed in brown and white adipose tissues, and it regulates lipolysis and thermogenesis. A common polymorphism in exon 1 of this gene, Trp64Arg substitution, has been related to the increased prevalence of non–insulin-dependent diabetes mellitus (NIDDM) among Pima Indians[6] and other populations.[7] However, no studies have been reported that investigate whether this common polymorphism is associated with CHD. To this aim, we screened nondiabetic and diabetic subjects with CHD for the Trp64Arg polymorphism of the β_3-adrenergic receptor gene and studied its association with insulin resistance by the euglycemic clamp technique.

SUBJECTS AND METHODS

Subjects

All subjects (N = 386, 273 men and 113 women) participating in this study were unrelated, Finnish, and aged 58 ± 0.4 years (mean ± SEM; range, 39 to 77). Finland is a genetic isolate,[8] and the population of Eastern Finland originates from one of the two groups of settlers that were founders of the present population.[9] A coronary angiogram was performed previously at the University Hospital of Kuopio in 1986 to 1996 on 185 nondiabetic subjects (101 men and 84 women) and 95 NIDDM patients (75 men and 20 women) who met World Health Organization (WHO) diagnostic criteria[10] for NIDDM. All of these 280 patients had greater than 50% stenosis in at least two coronary arteries. In addition, we screened for the Trp64Arg polymorphism of the β_3-adrenergic receptor gene 24 patients with NIDDM (15 men and nine women) who had a definite myocardial infarction (MI) according to WHO criteria.[11] The nondiabetic control group consisted of 82 randomly selected men (age, 54 ± 1 years; body mass index [BMI], 26.3 ± 0.4 kg/m^2) with no previous history of MI verified at the hospital, no current symptoms of CHD by the Rose cardiovascular questionnaire, or ischemic electrocardiogram (ECG) changes on an exercise test.[12] All subjects were invited to an outpatient visit including an interview on the smoking history and medication and measurement of the height, weight, waist to hip ratio, and systolic and diastolic blood pressure. All nondiabetic subjects and those whose diabetes diagnosis was uncertain underwent an oral glucose tolerance test. Subjects were classified as nondiabetic or NIDDM according to WHO criteria.[10] Clinical characteristics of the 82 control subjects have been previously reported.[13]

Determination of Trp64Arg Polymorphism in Exon 1 of the β_3-Adrenergic Receptor Gene

DNA was prepared from peripheral blood leukocytes by a proteinase K-phenol-chloroform extraction method. Exon 1 was amplified with the polymerase chain reaction (PCR) with the primers BSTNUP 5′-CGCCCAATACCGCCAACAC-3′ and BSTNDOWN 5′-CCACCG-GAGTCCCATCACC-3′ (product size, 210 base pairs [bp]). PCR amplification was performed in a 15-μL vol containing 50 ng genomic DNA, 5 pmol of each primer, 10 mmol/L Tris hydrochloride (pH 8.8), 50 mmol/L KCl, 1.5 mmol/L MgCl and 0.1% Triton X-100, 0.25 U DNA polymerase (DynaZyme DNA Polymerase; Finnzymes, Espoo, Finland), and 200 μmol/L dNTP. PCR conditions were as follows:

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denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 63°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 4 minutes. The single-base replacement of T by C in codon 64 in exon 1 predicts an amino acid change from tryptophan (TGG) to arginine (CGG). Therefore, amplified exon 1 fragments were digested with *Bst*N1, a restriction enzyme specific for the sequence CC(A/T)GG, in a 15-μL vol containing 5 μL PCR product. The mixture was incubated at 60°C for 1 hour. Digested samples were separated on a 3% agarose gel (NuSieve GTG; FMC Bioproducts, Rockland, ME). Digestion of the normal sequence yields fragments of 15, 34, 61, 66, 94, and 97 bp in length, whereas the Trp64Arg substitution eliminates one of the *Bst*N1 sites, yielding a novel 158-bp product. The other fragment sizes in individuals with the Trp64Arg polymorphism are 15, 34, 66, and 94 bp.

Hyperinsulinemic-Euglycemic Clamp

The degree of insulin resistance was evaluated with the euglycemic clamp technique after a 12-hour fast as previously described.[12] After baseline blood sampling, a priming dose of insulin (Actrapid 100 IU/mL; Novo Nordisk, Gentofte, Denmark) was administered in nondiabetic subjects during the initial 10 minutes to increase insulin quickly to the desired level, where it was maintained by a continuous insulin infusion of 480 pmol (80 mU)/m^2/min. Under these study conditions, hepatic glucose production is completely suppressed in normoglycemic subjects according to our experience[14] and the findings of other investigators.[15] Blood glucose was clamped at 5.0 mmol/L for the next 180 minutes by infusion of 20% glucose at varying rates according to blood glucose measurements performed at 5-minute intervals. The mean value for the last hour was used to calculate the rate of whole-body glucose uptake (WBGU). In patients with NIDDM, [3-^{3}H]glucose was infused as a primed (40 μCi)-constant (0.40 μCi/min) infusion for 180 minutes before initiating the insulin infusion (120 mU [480 pmol/L]/m^2/min). The rates of glucose appearance (R_a) and disappearance (R_d) during the euglycemic-hyperinsulinemic clamp were quantified from serum [3-^{3}H]glucose specific activity and calculated using Steele's equations in their modified derivative form, because the tracer exhibits non–steady-state kinetics under these conditions.[16] The rate of hepatic glucose output during the euglycemic clamp was calculated as the difference between the R_a and exogenous glucose infusion rate. Negative numbers for hepatic glucose output, largely due to a model error emerging at high rates of glucose metabolism,[17] were taken to indicate completely suppressed hepatic glucose output. The data were calculated for each 20-minute interval, and the mean value for the period from 120 to 180 minutes was used in the calculation of WBGU rates. In subjects with normal glucose tolerance, [3-^{3}H]glucose was not infused, and the rate of WBGU therefore equals the glucose infusion rate.

The protocol was approved by the Ethics Committee of the University of Kuopio and was in accordance with the Helsinki Declaration.

Analytical Methods

Plasma glucose levels in the fasting state and blood glucose levels during the euglycemic clamp were measured by the glucose oxidase method (2300 Stat Plus; Yellow Springs Instrument, Yellow Springs, OH). For the determination of plasma insulin, blood was collected in EDTA-containing tubes, and after centrifugation, the plasma was stored at −20°C until analysis. The plasma insulin concentration was determined by a commercial double-antibody solid-phase radioimmunoassay (Phadeseph Insulin RIA 100; Pharmacia Diagnostics, Uppsala, Sweden). Lipoprotein fractionation was performed by ultracentrifugation and selective precipitation[18] as previously described.[19] Cholesterol and triglyceride from whole serum and from lipoprotein fractions were assayed by automated enzymatic methods (Boehringer, Mannheim, Germany).

Statistical Analysis

All calculations were performed using the SPSS/WIN program (SPSS, Chicago, IL). Data are presented as the mean ± SEM. Statistical significance between the two groups was evaluated with the chi-square test or Student's *t* test when appropriate. In a comparison of two groups, adjustment for confounding factors was made with analysis of covariance. Before statistical testing, fasting plasma glucose, insulin, and serum triglycerides were logarithmically transformed to achieve a normal distribution.

RESULTS

Table 1 lists the clinical characteristics of subjects with CHD. There were fewer smokers among subjects with NIDDM versus subjects with normal glucose tolerance. Subjects with NIDDM were more obese and had higher levels of systolic blood pressure, glucose, insulin, and total triglycerides and lower levels of HDL cholesterol than nondiabetic subjects.

The allele frequency of Trp64Arg polymorphism of the β_3-adrenergic receptor gene was not different in nondiabetic (8%) and diabetic (7%) patients with CHD compared with nondiabetic subjects without CHD (7%) from our previous study.[13] No homozygous patients for the Trp64Arg genotype were found in any of the study groups.

In normoglycemic subjects with CHD, there was a trend for a higher BMI and lower serum triglyceride levels in subjects heterozygous for the Trp64Arg polymorphism compared with subjects with the Trp64Trp genotype, but these differences were not statistically significant. The waist to hip ratio, fasting serum total and HDL cholesterol, and the rate of WBGU during the last hour of the euglycemic clamp did not differ between subjects with and without the Trp64Arg polymorphism of the β_3-adrenergic gene (Table 2). Analyzing males and females separately had no influence on the results.

Table 1. Clinical Characteristics of Subjects With CHD

Characteristic	Normal Glucose Tolerance (n = 185)	NIDDM (n = 119)
Age (yr)	56 ± 1	62 ± 1*
BMI (kg/m^2)	27.8 ± 0.3	28.7 ± 0.4*
Waist to hip ratio	0.95 ± 0.01	0.97 ± 0.01
Smokers (%)	16	4
Use of β-blockers (%)	78	77
Systolic blood pressure (mm Hg)	136 ± 1	146 ± 2*
Diastolic blood pressure (mm Hg)	81 ± 1	83 ± 1
Fasting plasma glucose (mmol/L)	5.7 ± 0.1	8.9 ± 0.3*
Fasting plasma insulin (pmol/L)	78.4 ± 3.2	97.6 ± 5.9*
Total serum cholesterol (mmol/L)	5.98 ± 0.07	6.04 ± 0.12
Serum HDL cholesterol (mmol/L)	1.23 ± 0.02	1.09 ± 0.03*
Total serum triglycerides (mmol/L)	1.85 ± 0.06	2.59 ± 0.23*

NOTE. Values are the mean ± SEM.
*$P < .01$.

Table 2. Clinical and Biochemical Characteristics and WBGU Rate in Nondiabetic and NIDDM Subjects With CHD According to Codon 64 Polymorphism of the β_3-Adrenergic Receptor Gene

Parameter	Normal Glucose Tolerance			NIDDM		
	Trp64Trp (n = 156)	Trp64Arg (n = 29)	P	Trp64Trp (n = 103)	Trp64Arg (n = 16)	P
BMI (kg/m^2)	27.6 ± 0.3	28.7 ± 0.9	.167	28.8 ± 0.4	28.3 ± 1.1	.657
Waist to hip ratio	0.96 ± 0.01	0.95 ± 0.01	.731	0.98 ± 0.01	0.96 ± 0.03	.491
Systolic blood pressure (mm Hg)	136 ± 2	140 ± 3	.302	146 ± 2	147 ± 5	.716
Diastolic blood pressure (mm Hg)	81 ± 1	82 ± 2	.420	83 ± 1	83 ± 3	.692
Fasting plasma glucose (mmol/L)	5.7 ± 0.1	5.6 ± 0.1	.824	8.9 ± 0.3	9.2 ± 1.0	.620
Fasting plasma insulin (pmol/L)	76.5 ± 3.1	88.9 ± 13.5	.376	98.7 ± 6.7	93.3 ± 9.2	.684
Total serum cholesterol (mmol/L)	6.02 ± 0.08	5.70 ± 0.15	.281	6.01 ± 0.13	6.22 ± 0.34	.571
Serum HDL cholesterol (mmol/L)	1.24 ± 0.02	1.22 ± 0.04	.328	1.10 ± 0.03	1.09 ± 0.05	.955
Total serum triglycerides (mmol/L)	1.89 ± 0.07	1.64 ± 0.12	.153	2.54 ± 0.26	2.93 ± 0.37	.552
WBGU (μmol/kg/min)	47.9 ± 2.8 (n = 14)	42.1 ± 3.8 (n = 9)	.242	42.2 ± 4.1 (n = 17)	35.8 ± 1.3 (n = 4)	.464

NOTE. Values are the mean ± SEM. For WBGU, the number of subjects examined in each group is shown in parentheses.

DISCUSSION

Several studies have shown that a family history of CHD increases the risk of CHD by twofold to sevenfold, implying the role of genetic factors in the etiology of CHD.[20] Genetic factors are particularly important with respect to the risk of CHD in subjects under the age of 50 to 60 years, with heritability accounting for about half of the morbidity.[21,22]

Hyperinsulinemia has been shown to be an independent risk factor for the development of CHD in several population-based studies.[3,23] Furthermore, cross-sectional studies applying the euglycemic clamp technique have demonstrated that insulin resistance is associated with an increased occurrence of atherosclerotic manifestations in the femoral and carotid arteries.[24,25] Since resistance to insulin action in cross-sectional studies is linked with different manifestations of atherosclerosis, the defects in genes that regulate insulin action are promising candidate genes for CHD. The β_3-adrenergic receptor gene regulates lipolysis, and, the Trp64Arg allele of the β_3-adrenergic receptor gene has indeed been linked with features of insulin resistance.[26,27] In Pima Indians, the missense mutation in codon 64 of the β_3-adrenergic receptor gene, Trp64Arg, has been shown to be related to an earlier onset of NIDDM,[6] and in Finnish nondiabetic subjects, it was also associated with clinical features of the insulin resistance syndrome, abdominal obesity, and hypertension.[26] In contrast, codon 64 amino acid polymorphism of the β_3-adrenergic receptor gene in the heterozygous form was not associated with decreased insulin sensitivity in healthy Danes.[28] In addition, the Trp64Arg polymorphism in the heterozygous form was not a major determinant of β_3-adrenergic receptor function as assessed by lipolysis in white adipose tissue.[29]

We investigated the association of codon 64 polymorphism of the β_3-adrenergic receptor gene with CHD in nondiabetic and NIDDM subjects. The allele frequency of this polymorphism was similar in subjects with and without CHD, indicating that it is very unlikely that the Trp64Arg polymorphism could contribute to coronary atherosclerosis in our subjects. There was a trend for lower serum triglyceride levels in normoglycemic subjects heterozygous for the Trp64Arg polymorphism, indicating that the Trp64Arg polymorphism of the β_3-adrenergic receptor gene might contribute to decreased lipolysis, leading to increased visceral obesity. This finding is in agreement with previous studies in which the Trp64Arg polymorphism of the β_3-adrenergic receptor gene has been associated with visceral obesity and lower serum triglyceride levels[30] and a lower basal metabolic rate.[31] In the present study, the Trp64Arg polymorphism in the heterozygous form was not associated with the waist to hip ratio, insulin resistance, or hyperinsulinemia in subjects with CHD regardless of gender, and we have previously reported similar findings on normoglycemic subjects without CHD.[13] Thus, our study does not support the hypothesis that the β_3-adrenergic receptor gene could be a major gene contributing to insulin resistance. In the present study, we found no homozygous subjects for the Trp64Arg allele of the β_3-adrenergic receptor gene. In addition, the BMI values for the subjects were not in the morbidly obese category, and perhaps it would be only in the homozygous condition of the Trp64Arg allele that a significant association could be made. Since heterogeneity in the prevalence of the Trp64Arg allele of the β_3-adrenergic receptor gene has been reported in different populations, the ethnic stratification could explain the discordant findings of obesity with this marker in some populations.

All CHD patients selected for this study had stenosis greater than 50% on a coronary angiogram in at least two main main coronary arteries or definite MI according to WHO criteria. Control subjects from our previous study[13] were nondiabetic and had no history of angina pectoris or ischemic ECG changes on a exercise test. Because the clinical symptoms and ischemic ECG changes are late manifestations of CHD, these findings do not completely exclude the possibility of CHD in our control group. However, our control subjects are likely a representative group of subjects without CHD, since the prevalence of codon 64 polymorphism was similar compared with an other study on the Finnish population.[26]

In conclusion, the Trp64Arg polymorphism of the β_3-adrenergic receptor gene was not associated with CHD or insulin resistance. These results imply that the Trp64Arg polymorphism of the β_3-adrenergic receptor gene in the heterozygous form is not likely to contribute to the risk of atherosclerosis in the Finnish population.

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