Lack of association between the tetranucleotide repeat polymorphism in the 3′-flanking region of the leptin gene and hypertension in severely obese patients

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ABSTRACT. Conflicting data suggest an association between leptin gene polymorphisms and essential hypertension independently of obesity. The aim of this study was to evaluate, in severely obese subjects, the role of one of these polymorphic markers in relation to the development of hypertension. The study included 325 obese patients with mean body mass index (BMI) of 46±6.94 kg/m². One hundred sixty-six were hypertensive and 159 normotensive. In both groups, the presence of a tetranucleotide repeat in the 3′ flanking region of the Ob gene was investigated using polymerase chain reaction (PCR). Due to the genetic variant, in the region studied it is possible to distinguish two alleles with different size distribution: Class I (shorter one) and Class II (longer one). Class I and Class II allele frequencies were not significantly different in obese patients when analyzed according to the presence or absence of hypertension. The results presented herein do not support a significant association of this Ob gene polymorphism with hypertension. These findings are in contrast with that reported in other populations. However, we cannot rule out that different ethnicity and/or phenotypic variability might mask small effects.

INTRODUCTION

About 45% of deaths in the Western world are related to cardiovascular diseases, mainly due to coronary heart disease, heart failure, stroke and renal failure. Among all cardiovascular risk factors, obesity and hypertension emerge today with epidemic proportions and both result from multiple environmental and genetic determinants. These disorders are closely linked in epidemiological studies (1), with high body mass index (BMI) strongly correlated with increased blood pressure (2, 3) and lean individuals with elevated blood pressure showing a predisposition to become obese (3). While environmental determinants certainly account for some of this epidemiological linkage, shared genetic determinants may also contribute to the association between hypertension and obesity. Nevertheless, the pathophysiological mechanisms of the blood pressure rise in obesity are not yet well characterized. Hormonal changes associated with obesity, especially hyperinsulinemia and increased insulin resistance, have been suggested to play a role in the development of hypertension in obese subjects (4, 5).

The recent cloning of the human obese gene and the characterization of its protein products leptin (6) have been major breakthroughs in investigating the etiology of at least some forms of obesity. These studies have shown that leptin is essentially produced by adipose tissue and that plasma leptin levels are strongly and positively correlated to the BMI (7, 8). In addition to its role on appetite and energy expenditure, a direct effect of leptin on blood pressure has been proposed (9, 10) and it seems to be mediated by sympathetic activation (11). Quite recently, the presence of highly polymorphic tetranucleotide repeats in the 3′-flanking region of the leptin gene (12) has been reported, showing a significant association with hypertension (13) independently of obesity.
Indeed, in a Japanese population, the frequency of Class I/Class I genotype was significantly higher in hypertensive subjects than in control normotensive subjects, despite no difference in BMI existed in the two groups. However, several genetic markers at the leptin locus, including the one described by Shintani et al. (12), were not significantly linked to hypertension in another study performed in an African American population (14). These contradictory data prompted us to further investigate the presence of the tetranucleotide repeat in the 3’-flanking region of the leptin gene, in a series of Italian severe obese patients with and without the phenotypic characteristic of essential hypertension.

MATERIALS AND METHODS

Subjects

The present study included 325 obese patients (216 women and 109 men), 43.8±13.9 yr old (mean±SD; range 18-81), with mean BMI of 45.95±7.6 kg/m² (mean±SD; range, 32-85), referred to the Division of General Medicine of the San Giuseppe Hospital, Italian Auxologico Institute (Piacavallo-Verbania), for diagnostic or therapeutic problems related to obesity or its morbidity. Forty-eight, 12 normotensive (8 women and 4 men) and 36 hypertensive (20 women and 16 men), respectively, of the 325 patients had diabetes mellitus controlled by diet alone and/or oral agent. All patients had normal thyroid function and none of them had concomitant severe renal, hepatic or cardiac disease. Body weight was stable for the last 4 weeks before admission. The patients underwent a study protocol including evaluation of BMI, waist-to-hip ratio (WHR), resting energy expenditure (REE), energy intake, fat mass (FM) and free fatty mass (FFM), total cholesterol (TCH), HDL-cholesterol, thyroglobulin (TG), leptin levels, basal glucose, IRI, and thyroid hormone [free T₄ (FT₄) and TSH].

Fasting blood samples were obtained to determine all biochemical parameters and to isolate DNA, assessment of REE and body composition were performed after 12-h fast and before beginning any other treatment. WHR was calculated on the basis of the anthropometric measurements taken at patients’ admission. Patients also underwent a 7-day dietary recall to estimate their usual daily energy intake as described by Wadden et al. (15).

To estimate the frequency of the polymorphism studied in the normal Italian population, we also genotyped 90 normal weight (BMI 23.9±1.65 kg/m²) subjects.

The study protocol was approved by the Institution Ethics Committee; the aim and the design of the study were explained to the patients who gave their informed consent.

Measurement

REE was measured at 08:00 h, after a 12-h fast, in a comfortable and thermo-regulated (22.24 °C) room where only the investigator and the patient were present. After a 10-min steady-state period, values were recorded every minute for 30 min; the mean value was then expressed as kcal/24 h. REE was assessed by a computerized, open-circuit, indirect calorimetric system that measured resting oxygen uptake and resting carbon dioxide production using a ventilated canopy (Sensormedics, Milano, Italy). Body fat distribution was estimated using WHR. The waist circumference was taken to the smallest standing horizontal circumference between ribs and the top of the iliac crest, the hip circumference was taken as the largest standing horizontal circumference of the buttocks.

The percentage of total body FM, and FFM was determined by the bioelectrical impedance analysis method (BIA 101/S Akern Firenze, Italy) in the morning, after an overnight fast and after voiding.

Glucose, TCH, HDL cholesterol and TG were measured by enzymatic methods (Boehringer-Mannheim, Mannheim, Germany); FT₄, TSH and insulin were determined by chemiluminescent methods (Immulite DPC Euro/DPC Ltd, Lamberis, UK). Serum leptin levels were measured by RIA using reagents supplied by Linco Research Inc (St Louis, MO, USA).

Insulin sensitivity was evaluated by the quantitative insulin sensitivity check index [homeostasis model assessment-insulin resistance (HOMA-IR)] (16). This index derives from a mathematical model \( \frac{\text{I} \times \text{G}}{\text{G} \times \text{I}} \times \text{G} \) (mmol/l/22.5), that takes into account the fasting insulin and glucose levels to evaluate insulin sensitivity and has a very good linear correlation with the gold standard clamp measurement. The cut-off limit was set at 2.88 that was the value determined in normal controls.

Polymorphism screening

Genomic DNA was extracted from the whole blood using a commercial kit (Nucleon BACC 2/3; Amersham, USA). Genotyping of the tetranucleotide repeats polymorphism in the 3’-flanking region of the human leptin gene was detected by polymerise chain reaction (PCR), using a sense primer (HOBF) containing a fluorochrome group (5'-*AGT CTA AAT AGA GGT CCA AAT CA-3') and an antisense primer (HOBRI) (5' TTC TGA GGT TGT GTC ACT GCC A 3') that flanks the region containing the base-pair (bp) repeats, as previously described (13). PCR contained 100 ng genomic DNA template, 10 µM of each primer, 25 mM Mg²⁺, 25 mM of dNTPs mix, 5 U/µl Taq Polymerase (Promega, Italy) and reaction buffer in a total volume of 15 µl. PCR was performed at 94 C for 3 min, followed by 10 cycles of denaturation at 94 C for 30 sec, annealing at 54 C for 30 sec, and elongation at 72 C for 30 sec, followed by 20 cycles at 89 C for 15 sec, 55 C for 15 sec and 72 C for 30 sec, with final extension step of 72 C for 10 min. The PCR products labelled with FAM, were diluted 1:20 with H₂O in a final volume of 50 µl, and a ladder containing fragments of known length labelled with a different fluorochrome (rhodamine: ROX) was added. The allelic-specific DNA fragments were separated by means of capillary electrophoresis, on an automated sequencer (ABI PRISM 310) capable of discriminating the different wavelengths emitted by the specific fluorochromes. The data collected during the run were automatically processed by specific Gene Scan Analysis software (ABI), and each fluorescent peak was quantified in terms of size (base pairs).

Statistics

Data are expressed as mean±SD. Considering the reported 23-29% prevalence of the short allele (13), the sample size used allows us to detect, with an 80% statistical power of avoiding a type II error and 5% level of significance, at least a 1.5-fold increase in the risk of developing hypertension. Statistical analysis was carried out using the Statview SAS statistical package Allele, and genotypes frequencies were compared between groups using the χ² test.