The Leu7Pro Polymorphism of the Neuropeptide Y Gene Regulates Free Fatty Acid Metabolism

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The Leu7Pro polymorphism in the signal peptide of the preproneuropeptide Y (NPY) has been associated with dyslipidemias and free fatty acid (FFA) levels during exercise. The association of this polymorphism with insulin sensitivity has not been studied. In this study, the Leu7Pro polymorphism was determined in 2 groups of nondiabetic middle-aged subjects (n = 266 and n = 295). Insulin sensitivity was measured with the hyperinsulinemic euglycemic clamp (n = 266) or with an intravenous glucose tolerance test (IVGTT, n = 295). First-phase insulin secretion was determined as insulin area under the curve (AUC) during the first 10 minutes of the IVGTT. FFAs were measured both in the fasting state and during the hyperinsulinemic clamp. The Leu7Pro polymorphism of the NPY gene was not associated with the rates of whole body glucose uptake, insulin sensitivity index, insulin secretion during the IVGTT, or insulin AUC during the oral glucose tolerance test. However, the Pro7 allele was associated with low FFA levels both in the fasting state (*P* = .043) and during the hyperinsulinemic clamp (*P* = .003). In conclusion, the Leu7Pro polymorphism of the NPY gene associates with alterations in FFA metabolism but does not have an impact on insulin sensitivity, insulin secretion, or glucose metabolism.

NEUROPEPTIDE Y (NPY) is widely expressed in the central and peripheral nervous system.[1] In humans, the Leu7Pro polymorphism in the signal peptide of the preproNPY has been associated with high serum total and low-density lipoprotein (LDL) cholesterol,[2,3] and with high birth weight and high serum triglycerides in young children.[4] Moreover, in elderly nondiabetic and diabetic subjects, the Pro7 allele has been related to enhanced atherosclerosis.[5] However, the mechanisms that mediate the effects of the preproNPY polymorphism on lipid metabolism and accelerated atherosclerosis are unknown. Because free fatty acid (FFA) levels regulate insulin sensitivity,[6] an impairment in NPY's antilipolytic action[7,8] could lead to high risk of atherosclerosis. Therefore, we investigated the association of the Leu7Pro polymorphism with FFA levels and insulin sensitivity in middle-aged subjects (n = 266) using the hyperinsulinemic euglycemic clamp technique. In addition, insulin sensitivity and the first-phase insulin secretion were studied in a second group of middle-aged subjects (n = 295) using the intravenous glucose tolerance test (IVGTT).

MATERIALS AND METHODS

Subjects

The study population consisted of 2 groups of middle-aged subjects from our earlier studies. The first group consisted of 110 healthy unrelated subjects (82 men, 28 women; age, 50 ± 8 years; body mass index [BMI], 26.1 ± 3.6 kg/m^2), 105 members (66 men, 39 women; age, 50 ± 12 years; BMI, 26.0 ± 4.2 kg/m^2) of families with familial combined hyperlipidemia (FCHL),[9] and 51 nondiabetic subjects (14 men, 37 women; age, 56 ± 7 years; BMI, 27.1 ± 3.0) from our coronary heart disease sibpair study[10] who had undergone the hyperinsulinemic clamp. The second middle-aged group consisted of 295 normoglycemic subjects (150 men, 145 women; age, 44 ± 11 years; BMI, 25.6 ± 3.7) from our previous population-based study in which an IVGTT had been performed.[11] All study subjects had a normal glucose tolerance according to the World Health Organization criteria,[12] and normal liver, kidney, and thyroid function tests; none had a history of an excessive alcohol intake. Informed consent was obtained from all subjects after the purpose and potential risks of the study had been explained. The protocol was approved by the Ethics Committee of the Kuopio University Hospital and was in accordance with the Helsinki Declaration.

Measurement of Insulin Sensitivity and Secretion

Hyperinsulinemic euglycemic clamp was performed after a 12-hour fast as previously described in detail.[13] After baseline blood drawing a priming dose of insulin (Actrapid 100 IU/mL, Novo Nordisk, Gentofte, Denmark) was administered during the initial 10 minutes to raise insulin concentration quickly to the desired level, where it was maintained by a continuous insulin infusion of 480 pmol/m^2/min. Blood glucose was clamped at 5.0 mmol/L for the next 180 minutes by the infusion of 20% glucose at varying rates according to blood glucose measurements performed at 5-minute intervals. The mean value of the glucose infusion rate during the last hour was used to calculate the rates of insulin-stimulated whole body glucose uptake (WBGU). Indirect calorimetry with a computerized flow-through canopy gas analyzer system (Deltatrac, Datex, Helsinki, Finland) was combined with the clamp study as previously described.[14] Gas exchange was measured during the last 30 minutes of the euglycemic clamp. The first 10 minutes of each measurement were discarded and the mean value of the last 20 minutes was used in calculations. The rates of glucose oxidation were calculated according to Ferrannini (determined by indirect calorimetry in the last 20 minutes of the euglycemic clamp).[15] The rates of nonoxidative glucose disposal during the euglycemic clamp were estimated by subtracting the carbohydrate oxidation rate from the rates of WBGU.

IVGTT was performed to determine the first phase insulin secretion capacity as the area under the insulin concentration curve (AUC) during the first 10 minutes of the IVGTT and insulin sensitivity index (Si) using Bergman's minimal model.[16] In addition At 8 AM after a 12-hour overnight fast, an intravenous catheter was placed in the

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Table 1. Sex Distribution, Age, BMI, Waist-to-Hip Ratio, Fasting Plasma Glucose, Insulin, and Insulin AUC After an Oral Glucose Load and Fasting Serum FFAs According to the Leu7Pro Polymorphism of the NPY Gene in the Two Middle-Aged Study Groups

	Leu7Pro Polymorphism of the NPY Gene		
	Leu7Leu	Leu7Pro	Pro7Pro
Middle-aged group I (clamp study)			
Sex (men/women)	165/73	18/8	2/0
Age (yr)	52 ± 10	50 ± 8	53 ± 5
BMI (kg/m^2)	26.6 ± 3.9	27.3 ± 5.4	31.2 ± 3.1
Waist-to-hip ratio	0.94 ± 0.08	0.95 ± 0.07	0.98 ± 0.04
Fasting glucose (mmol/L)	5.5 ± 0.6	5.4 ± 0.6	5.8 ± 0.1
Fasting insulin (pmol/L)	66.7 ± 38.1	56.5 ± 25.9	109.2 ± 103.5
Insulin AUC (pmol · L^{-1} · min)	38,180 ± 28,739	33,246 ± 22,893	88,749 ± 10,659
Middle-aged group II (IVGTT study)			
Sex (men/women)	129/131	18/14	3/0
Age (yr)	45 ± 12	40 ± 11	41 ± 12
BMI (kg/m^2)	25.8 ± 3.7	24.1 ± 3.4	26.0 ± 2.5
Waist-to-hip ratio	0.90 ± 0.08	0.88 ± 0.08	0.95 ± 0.06
Fasting glucose (mmol/L)	5.2 ± 0.6	5.0 ± 0.5	5.9 ± 1.1
Fasting insulin (pmol/L)	56.8 ± 35.0	50.1 ± 21.4	67.2 ± 14.9
Insulin AUC (pmol · L^{-1} · min)	32,804 ± 22,357	28,701 ± 15,457	43,704 ± 18,141

NOTE. Values are means ± SD. No statistically significant differences were observed.

antecubital vein for the infusion of glucose. Another cannula for blood sampling was inserted into a wrist vein surrounded by a heated box (40°C). After baseline blood collection, a bolus of glucose (300 mg/kg in a 50% solution) was given into the antecubital vein to quickly increase the blood glucose level. Samples for the measurement of blood glucose and plasma insulin were drawn at 0, 4, 6, 8, 10, 19, 29, 37, 67, 90, and 180 minutes. At 20 minutes an intravenous injection of regular insulin (dose 0.03U/kg) was administered to increase the accuracy of the modeling analyses.

Analytical Methods

Plasma glucose in the fasting state and after an oral glucose load (75 g) was measured by the glucose oxidase method (2300 Stat Plus, Yellow Springs Instrument Co, Yellow Springs, OH). For the determination of plasma insulin, blood was collected in EDTA-containing tubes and after centrifugation the plasma was stored at −70°C until the analysis. Plasma insulin concentration was determined by a commercial double-antibody solid-phase radioimmunoassay (Phadeseph Insulin RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden) and serum FFAs from freshly frozen samples by an enzymatic method (Wako Chemicals GmbH, Neuss, Germany).

Determination of the Leu7Pro Polymorphism of the NPY Gene

DNA was prepared from peripheral blood leukocytes by proteinaseK-phenol-chloroform extraction method. Genotyping was done using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. RFLP was based on the selective recognition of the cytosine1128 allele, corresponding to the Pro7 allele, by BsiEI enzyme as previously described.[2]

Statistical Analysis

All statistical calculations were performed with the SPSS/WIN programs (version 10.0, SPSS Inc, Chicago, IL). The frequencies between the study groups were compared with the chi-square test. The effect of the variants on continuous variables was tested with analysis of covariance (ANCOVA) using age and sex as covariates. Insulin and FFA values were logarithmically transformed for statistical analysis to achieve normal distribution. All data are presented as mean ± SD. *P* values less than .05 were considered statistically significant.

Table 2. Insulin Sensitivity Measured Using Either the Rates of WBGU During the Hyperinsulinemic Euglycemic Clamp or S_i During the IVGTT and First-Phase Insulin Secretion at 4 Minutes and During the First 10 Minutes of the IVGTT According to the Leu7Pro Polymorphism of the NPY Gene

	Leu7Pro Polymorphism of the NPY Gene		
	Leu7Leu	Leu7Pro	Pro7Pro
Middle-aged group I (clamp study)	(n = 238)	(n = 26)	(n = 2)
WBGU (μmol/kg/min)	52.7 ± 15.0	52.0 ± 14.7	40.9 ± 26.4
Glucose oxidation	17.7 ± 3.6	17.6 ± 3.6	14.5 ± 1.1
Nonoxidative glucose disposal	35.0 ± 13.3	34.4 ± 12.9	26.4 ± 25.5
Lipid oxidation (mg/kg/min)	0.04 ± 0.25	0.02 ± 0.28	0.09 ± 0.16
Middle-aged group II (IVGTT study)	(n = 260)	(n = 32)	(n = 3)
$S_i \times 10^{-4}$ (min^{-1} · μU^{-1} · mL^{-1})	4.24 ± 2.41	4.70 ± 2.35	2.23 ± 1.47
4-min insulin (pmol/L)	60.4 ± 41.7	59.7 ± 24.2	50.8 ± 29.1
Insulin AUC 10 min (pmol/L · min)	2,585 ± 1,694	2,544 ± 1,130	2,213 ± 850

NOTE. Values are means ± SD. No statistically significant differences were observed.

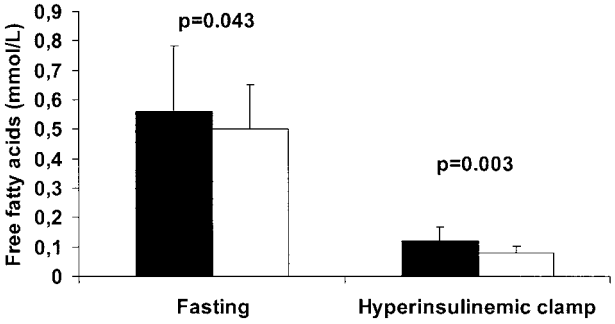

Fig 1. FFA levels (mean ± SD) in the fasting state and during the hyperinsulinemic clamp in subjects with (□, n = 28) and without (■, n = 238) the Pro7 allele of the NPY gene.

RESULTS

The frequency of the Leu7Pro variant of the NPY gene did not differ between the study groups (0.05 in the first middle-aged group and 0.06 in the second middle-aged group) and genotypes were in Hardy-Weinberg equilibrium. Table 1 shows age, BMI, waist-to-hip ratio, fasting plasma glucose, and insulin values and insulin area under the curve (AUC) during the oral glucose tolerance test. No difference between the groups was seen in these variables according to the Leu7Pro polymorphism.

No association was seen between the Leu7Pro polymorphism and insulin sensitivity measured as the rates of WBGU (n = 266) or as Si (n = 295) in middle-aged subjects (Table 2). The rates of glucose oxidation and nonoxidative glucose disposal and lipid oxidation were unaffected by this polymorphism during the clamp. In addition, this polymorphism was not related to the first-phase insulin secretion determined as 4-minute insulin or insulin AUC during the first 10 minutes of the IVGTT (Table 2).

FFAs were lower in middle-aged subjects with the Pro7 allele both in the fasting state (0.50 ± 0.22 *v* 0.56 ± 0.23 mmol/L, $P = .043$, adjusted for age, BMI, and sex) and during the hyperinsulinemic clamp (0.08 ± 0.09 *v* 0.12 ± 0.10, $P = .003$) compared to subjects with the Leu7Leu genotype (Fig 1). If unrelated nondiabetic subjects and FCHL family members were analyzed separately, no statistically significant difference in FFAs were observed, but values tended to be lower in subjects with the Pro7 allele than in subjects with the Leu7Leu genotype in the fasting state ($P = .092$ and $P = .252$) and during the clamp ($P = .066$ and $P = .147$) in both groups.

DISCUSSION

NPY is a neural transmitter that regulates food intake and energy storage.[1] Because of its antilipolytic effect[7] NPY may also regulate serum FFA levels and insulin sensitivity. The main finding of this study was a confirmation of an association of the Pro7 allele of the preproNPY with lower FFA levels both in the fasting state and during hyperinsulinemia as previously reported during exercise.[8] However, no change in insulin-stimulated glucose uptake and glucose levels in the fasting state and during the oral glucose tolerance test was observed.

In our earlier study we have shown that the Pro7 allele of the NPY gene, which is associated with high NPY levels, is related to lower exercise-induced lipolysis.[8] In this study, we extend this finding to two states without physical activity, ie, the fasting state and hyperinsulinemia induced by insulin infusion during the hyperinsulinemic clamp. Although hyperinsulinemia may cause low adrenergic stimulation, our findings imply that the inhibitory effect of the Pro7 allele on lipolysis can be observed without strong adrenergic stimulation.

Low levels of FFAs in subjects with the Pro7 allele of preproNPY was observed in the presence of both low and high insulin levels, which indicates that this effect is independent of insulin's antilipolytic action. This notion is also supported by our novel observations that the activities of lipoprotein and hepatic lipases, which are regulated by insulin, are not changed in subjects with the Pro7 allele.[17] Most likely, insulin and NPY independent of each other lower cyclic adenosine monophosphate (cAMP) levels, which in turn leads to inactivation of hormone-sensitive lipase and lower serum FFAs.[18]

High insulin sensitivity is associated with low FFA levels[6] but no association of the Leu7Pro polymorphism with insulin sensitivity measured directly using either the rates of WBGU or the Si index in healthy middle-aged subjects was observed. Although middle-aged subjects with the Pro7Pro genotype (n = 5, all men) tended to have low insulin sensitivity, no additive effect of an increasing number of Pro7 alleles on insulin resistance was observed (Table 2). Because this polymorphism had no effect on insulin secretion in the IVGTT, it is unlikely that this allele impairs significantly glucose metabolism. In addition, these findings are not likely to explain the high risk of atherosclerosis in subjects with the Pro7 allele[5] because the Pro7 allele is associated with low, not high, levels of FFAs.

The Leu7Pro polymorphism lies in the signal peptide part of preproNPY and the Pro7 allele is associated with enhanced processing of NPY from the preproNPY.[8] Therefore, this variant probably does not lead to changes in the structure of mature NPY and the interaction with NPY receptors is likely to remain unaltered. In cell lines the antilipolytic action has been proposed to be mediated via the Y5 NPY receptor.[7] However, serum FFA levels of Y5 receptor null mice,[19] Y1 receptor null mice,[20] or NPY null mice[21] have not been reported. In humans, some variants in the noncoding region of NPY Y5 receptor gene have been associated with obesity in Pima Indians,[22] but no significant variants have been found in the Y1 and Y5 receptor genes in other studies.[23,24] No information on serum FFA levels was reported in these studies and therefore the role of NPY receptors in mediating the antilipolytic action of NPY in humans remains unknown.

We conclude that the Pro7 allele of the NPY gene is associated with low FFA levels in the fasting state and during hyperinsulinemia independently of insulin's antilipolytic effect. The physiological significance of this finding remains to be determined.

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