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Leptin Receptor Gene Polymorphisms Are Associated with Insulin in Obese Women with Impaired Glucose Tolerance*

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ABSTRACT

Leptin receptors are present on β-cells as well as on muscle and fat cells, thus enabling leptin to modulate both insulin secretion and insulin action. Leptin inhibits especially the glucose-stimulated insulin secretion from pancreatic cells. The leptin receptor (LEPR) gene could thus play a role in the regulation of glucose and insulin after an oral glucose load. Therefore, the relationship between LEPR polymorphisms and glucose and insulin response to an oral glucose tolerance test (OGTT) was investigated.

Three LEPR polymorphisms (*Lys*^{109}*Arg*, *Gln*^{223}*Arg*, and *Lys*^{656}*Asn*) were typed on genomic DNA of 358 overweight and obese women, aged 18–60 yr. Based on an OGTT, 269 subjects were defined with normal glucose tolerance, and 89 with impaired glucose tolerance (IGT). Associations between genotypes and glucose metabolism were analyzed with a general linear models procedure in pre- and postmenopausal women separately, after adjusting the data for age and fat mass.

In postmenopausal women with IGT (n = 24), associations were found with *Lys*^{109}*Arg* and *Lys*^{656}*Asn* for fasting insulin ($P = 0.05$) and with *Lys*^{109}*Arg* and *Gln*^{223}*Arg* for the insulin response to an OGTT ($P < 0.02$). In the same group, trends were found with *Lys*^{656}*Asn* for fasting glucose as well as in response to the OGTT. In premenopausal women with IGT (n = 65), associations were found with *Lys*^{109}*Arg* and *Lys*^{656}*Asn* for overall glucose response to the glucose load. In contrast, no associations with insulin or glucose were found in women with normal glucose tolerance.

In conclusion, these data indicate that LEPR polymorphisms are associated with insulin and glucose metabolism in women with impaired glucose homeostasis. (*J Clin Endocrinol Metab* **86:** 3227–3232, 2001)

OBESITY IS FREQUENTLY associated with insulin resistance and the development of type 2 diabetes (1). Both obesity and diabetes are multifactorial diseases, determined largely by environmental factors, such as food intake and physical activity, but also by genetic factors (2). Although obesity runs in families, it is not, in most cases, simply inherited as a Mendelian trait. It is a polygenic disease for which most of the genes involved are not yet known. Indeed, each year new candidate genes are tested for their possible contributions to the development of obesity and the determination of body fat distribution [for last update see Human Obesity Gene map 2000 (3)].

Two such candidate genes are leptin and its receptor (LEPR). Leptin is a hormone known to be important in body weight regulation. When absent or deficient, it causes a syndrome of severe obesity and diabetes in rodents as well as in humans, although this is extremely rare (4–6). Leptin is synthesized and secreted by adipocytes, is present in the bloodstream in amounts related to the amount of fat in the body, and acts primarily on the brain to regulate food intake. Leptin receptors have been identified in hypothalamic regions known to be important in food intake regulation (7, 8). Furthermore, these receptors are also present in peripheral tissues such as pancreatic β-cells (9), hepatocytes, muscle, and adipose tissue. Leptin has been shown to be able to modulate insulin secretion and action through these receptors. *In vitro*, a direct leptin-induced inhibition of basal and glucose-stimulated insulin secretion was shown in pancreatic islets under conditions of high concentrations or prolonged exposure (10–12). Administration of leptin to rodents decreases insulin levels (12–14). Leptin was also shown to be able to modulate insulin-induced activities in hepatocytes (15–17), adipocytes (17–20), and muscle cells (20, 21). These effects appear to be mediated by both central nervous system pathways, probably through the autonomic nervous system, and peripheral mechanisms related to leptin receptors in peripheral organs (22).

The LEPR in humans has several, alternatively spliced, variants, of which the longest isoform, LEPR-b, is thought to be the signaling form in the hypothalamus. Mutations in the LEPR gene resulting in the production of a truncated receptor have been shown to cause obesity and diabetes in rodents and humans (23–26). However, in general, the leptin receptor is intact even in obese and diabetic subjects (27, 28). Several

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polymorphisms in the LEPR gene have been identified, but none has a major effect on body weight or fat mass (27–38).

We hypothesized that these polymorphisms in the LEPR gene could affect its peripheral functions. To test this hypothesis, we analyzed the associations among three different polymorphisms in the LEPR gene and serum insulin and glucose values measured in the fasted state and during an oral glucose tolerance test (OGTT) in overweight and obese women grouped by glucose tolerance state [normal glucose tolerance *vs.* impaired glucose tolerance (IGT) or type 2 diabetes].

Subjects and Methods

Subjects

Subjects were 358 Caucasian women visiting the out-patient obesity clinic at the University Hospital of Antwerp, who were overweight [25 ≤ body mass index (BMI) < 30] or obese (BMI ≥30). All patients were clinically examined by a physician and shown to be in general good health. Patients with known diabetes or with specific endocrine diseases such as Cushing's disease or hypothyroidism (TSH >5 μU/L) were excluded, as well as patients who were pregnant; using antidepressants or medication known to influence glucose tolerance, appetite, or metabolic rate; or had kidney or liver disease. Subjects of migrant origin were excluded to reduce the probability of finding LEPR variant effects because of subsample stratification caused by even slight differences in allele frequencies. Most subjects were sedentary. Of the 358 women, 255 were premenopausal, and 103 were postmenopausal. If no clinical data on menopausal state were available, subjects were defined as postmenopausal when age was above 55 yr, or when FSH was 15 U/L or more (n = 5). Subjects gave their informed consent before being enrolled in the study.

Body composition

Fat mass (in kilograms and percent total body fat) was determined by a bioimpedance measurement as described by Lukaski (39) with the formula of Deurenberg (40).

Glucose metabolism

An OGTT was performed with 75 g glucose, with blood samples taken to determine glucose and insulin levels in the fasted state and 2 h after the glucose load. In 190 subjects a complete OGTT was performed with frequent sampling for glucose and insulin (every 30 min for 3 h), and the area under the curve (AUC) was calculated for the glucose and insulin response. Plasma glucose was measured with the glucose oxidase method (on Vitros 750XRC, Johnson & Johnson), and insulin was measured by immunoradiometric assay using the Medgenix assay (Biosource Technologies, Inc., Fleurus, Belgium).

Subjects were divided into two groups according to their glucose tolerance status, based on fasting and 2 h postglucose load glucose levels, using the WHO criteria (41). Based on the OGTT, 269 patients had normal glucose tolerance, and 89 subjects were defined as type 2 diabetic of recent diagnosis (n = 25) or with IGT (n = 64).

Molecular analysis

Genomic DNA was extracted from whole blood by a method adapted from Miller *et al.* (42). Human leukocyte nuclei were precipitated, washed, and lysed in SDS and pronase (Roche Molecular Biochemicals, Indianapolis, IN). Proteins were precipitated by addition of a saturated NaCl solution, and DNA was subsequently precipitated with ethanol and dissolved in a Tris-ethylenediamine tetraacetate solution (pH 7.5).

The three restriction fragment length polymorphisms used have been described previously (27, 31). PCR was performed using 100 ng genomic DNA, 300 nmol/L of each primer, 200 μmol/L deoxy-NTPs and 0.5 U *Taq* polymerase in PCR buffer (Pharmacia Biotech, Baie d'Urfé, Canada) in a total volume of 20 μL. PCR consisted of 1 cycle of denaturation for 3 min at 95 C, annealing for 1 min at 55 C, and extension for 1 min at 72 C, followed by 40 cycles at 95 C for 30 s, annealing at 55 C for 30 s, and extension at 72 C for 30 s, and with a final extension at 72 C for 10 min (GeneAmp 9600 Thermocycler, Perkin-Elmer Corp., Foster City, CA). PCR products were digested overnight at 37 C with 5 U *Hae*III, *Msp*I, or *Bst*UI restriction enzymes (New England Biolabs, Inc., Mississauga, Canada) for the $Lys^{109}Arg$, $Gln^{223}Arg$, and $Lys^{656}Asn$ polymorphisms, respectively, and generated DNA fragments were separated on 3% agarose gel electrophoresis.

Statistical analysis

The normality of the distribution was verified for each variable, and phenotypes that were not distributed normally were log-transformed. A general linear model procedure was used to test for differences between the genotypes at each polymorphism. Analyses were performed on data adjusted for age and fat mass. Data were analyzed separately for pre- and postmenopausal women.

As frequencies of the homozygotes for the variant alleles were low in each subgroup, analyses were performed by carrier status, *i.e.* carriers of the rare allele (heterozygotes and homozygotes together: $Lys^{109}Arg$ + $Arg^{109}Arg$, $Gln^{223}Arg$ + $Arg^{223}Arg$ and $Lys^{656}Asn$ + $Asn^{656}Asn$) were compared with the noncarriers ($Lys^{109}Lys$, $Gln^{223}Gln$, or $Lys^{656}Lys$).

All statistical analyses were performed with the SAS statistical software package (version 6.12). Subject characteristics are presented as the mean ± SD, and results of carrier analyses are the mean ± SE.

Results

Subject characteristics

The mean age of all subjects was 39 ± 10 yr (range, 18–60 yr). Anthropometric and metabolic characteristics of the two subgroups (normal *vs.* IGT) are shown in Table 1. All women were overweight or obese, with a mean BMI of 36.0 ± 6.1 (range, 25.5–59.1 kg/m^2). In brief, 25% of the premenopausal (n = 65) and 23% of the postmenopausal (n = 24) women had IGT or type 2 diabetes. Women with IGT had, on the average,

TABLE 1. Anthropometric and metabolic characteristics of the subjects

	Women with normal glucose tolerance (n = 269)	Women with impaired glucose tolerance (n = 89)	*P* value (*t* test)
Age (yr)	39 ± 11 (18–60)	40 ± 10 (18–60)	NS
Wt (kg)	93.9 ± 15.5 (64.8–150.2)	105.6 ± 20.2 (74.2–170.8)	0.0001
BMI (kg/m^2)	35.0 ± 5.4 (25.5–53.6)	39.2 ± 6.8 (26.4–59.1)	0.0001
Fat mass (kg)	46.4 ± 12.8 (20.2–91.1)	55.5 ± 16.6 (28.2–108.0)	0.0001
Fat-free mass (kg)	47.8 ± 5.4 (37.5–69.1)	50.6 ± 6.0 (38.3–74.2)	0.0001
Waist (cm)	103 ± 12 (77–142)	111 ± 14 (81–150)	0.0001
Fasting glucose (mmol/L)	4.7 ± 0.5 (3.4–6.6)	5.7 ± 2.0 (3.8–15.8)	0.0001
Glucose, 2 h (mmol/L)	5.5 ± 1.2 (2.2–7.7)	10.1 ± 2.7 (5.2–19.7)	0.0001
Glucose AUC (mmol/L)	147 ± 21 (81–195)	224 ± 56 (131–419)	0.0001
Fasting insulin (pmol/L)	105 ± 50 (14–280)	146 ± 80 (28–459)	0.0001
Insulin, 2 h (pmol/L)	471 ± 378 (50–2,848)	919 ± 493 (144–2,734)	0.0001
Insulin AUC (pmol/L)	12,334 ± 7,670 (3,666–54,322)	16,675 ± 9,198 (4,276–41,816)	0.001

Unadjusted values are presented as the mean ± SD with the range in *parentheses*.

higher weight, BMI, fat mass, and glucose and insulin levels than normoglycemic women (Table 1).

Gene frequencies

Allele frequencies for the variant alleles were 0.29 at the *Lys*^{109}*Arg* polymorphism, 0.48 at *Gln*^{223}*Arg*, and 0.18 at *Lys*^{656}*Asn*. Although there were 10% more carriers of the Arg mutation at *Lys*^{109}*Arg* in the group with IGT, allele frequencies were not significantly different between women with IGT and those with normal glucose tolerance for any of the polymorphisms.

Associations of LEPR polymorphisms with glucose metabolism

No associations were found between the LEPR polymorphisms and glucose or insulin in women with normal glucose tolerance (data not shown). In contrast, in the group of women with IGT, significant associations were found with insulin parameters in postmenopausal women, and some trends were found with glucose parameters in pre- and postmenopausal women after adjustment for age and fat mass (Tables 2 and 3).

For fasting glucose, a significant association was found with *Lys*^{656}*Asn* in premenopausal women ($P = 0.02$), and a trend was found in postmenopausal women ($P = 0.08$). For the 2 h postload glucose levels, trends were found with *Lys*^{109}*Arg* in premenopausal women and with *Lys*^{656}*Asn* in postmenopausal women. Finally, the overall glucose response during an OGTT, expressed as the AUC, showed a borderline significant association with *Lys*^{109}*Arg* and *Lys*^{656}*Asn* in premenopausal women; the AUC was 13% higher in *Arg*^{109} carriers and in *Lys*^{656} homozygotes (Table 2).

Fasting insulin was associated with *Lys*^{109}*Arg* and *Lys*^{656}*Asn* in postmenopausal women only (both $P = 0.05$). The insulin response after an oral glucose load, both the 2 h value ($P < 0.01$) as well as the AUC ($P < 0.02$), was also significantly associated with *Lys*^{109}*Arg* and *Gln*^{223}*Arg* in postmenopausal women (Table 3). Insulin levels, adjusted for fat mass, were especially increased in *Lys*^{109}*Lys* and *Gln*^{223}*Gln* homozygotes compared with Arg carriers: fasting insulin, 2 h insulin, and insulin AUC were all about twice as high in *Lys*^{109}*Lys* and *Gln*^{223}*Gln* homozygotes compared with Arg carriers (Fig. 1). This finding persisted when analyses were carried out separately in subjects with IGT and was even more obvious in subjects with overt diabetes (data not shown).

Discussion

Leptin and its receptor are known to play a role in glucose metabolism. For instance, leptin exerts insulin- and glucose-lowering effects by enhancing peripheral insulin sensitivity and glucose uptake (14–16, 19–21). In addition, leptin receptors are present on pancreatic β-cells, which could offer one mechanism by which leptin could modulate glucose-induced insulin secretion (9–11). Therefore, we investigated whether polymorphisms located in the extracellular, binding part of the LEPR gene were associated with fasting glucose and insulin levels and with glucose and insulin responses to an oral glucose load in obese subjects with normal or impaired glucose tolerance. The glucose and insulin phenotypes were all adjusted for age and fat mass, a procedure that allowed us to investigate the effects of LEPR DNA sequence variation independently of the level of adiposity.

No significant associations were found with glucose or insulin in women with normal glucose tolerance. Only in women with IGT or (recently diagnosed, nontreated) type 2 diabetes were associations between the LEPR polymorphisms and glucose and insulin levels found. Insulin levels were associated with LEPR polymorphisms more consistently in postmenopausal women. Associations with glucose, especially with the overall response to an OGTT, were stronger in premenopausal women.

Most previous studies did not find any association of these LEPR polymorphisms with insulin metabolism phenotypes. Thompson *et al.* found no association between the *Lys*^{109}*Arg* and *Gln*^{223}*Arg* polymorphisms with the acute insulin re-

TABLE 2. Glucose metabolism in women with impaired glucose tolerance or type 2 diabetes, according to genotype

Phenotype	Genotype	Premenopausal women			Postmenopausal women		
		Mean ± SE	n	*P*	Mean ± SE	n	*P*
Fasting glucose, (mmol/L)	*Lys*^{109}*Lys*	5.4 ± 0.4	29	NS	5.6 ± 0.6	7	NS
	Lys^{109}*Arg* + *Arg*^{109}*Arg*	5.8 ± 0.3	34		6.1 ± 0.4	15	
	Gln^{223}*Gln*	5.7 ± 0.5	18	NS	5.6 ± 0.8	4	NS
	Gln^{223}*Arg* + *Arg*^{223}*Arg*	5.6 ± 0.3	45		6.1 ± 0.3	18	
	Lys^{656}*Lys*	6.0 ± 0.3	44	0.02	5.6 ± 0.4	15	0.08
	Lys^{656}*Asn* + *Asn*^{656}*Asn*	4.8 ± 0.4	19		6.9 ± 0.6	6	
Glucose, 120 min (mmol/L)	*Lys*^{109}*Lys*	9.4 ± 0.4	28	0.08	10.4 ± 1.4	7	NS
	Lys^{109}*Arg* + *Arg*^{109}*Arg*	10.4 ± 0.4	34		10.8 ± 0.9	15	
	Gln^{223}*Gln*	9.4 ± 0.6	17	NS	10.3 ± 1.9	4	NS
	Gln^{223}*Arg* + *Arg*^{223}*Arg*	10.1 ± 0.3	45		10.8 ± 0.9	18	
	Lys^{656}*Lys*	10.2 ± 0.3	43	NS	9.8 ± 0.9	15	0.07
	Lys^{656}*Asn* + *Asn*^{656}*Asn*	9.4 ± 0.5	19		13.0 ± 1.4	6	
Glucose, AUC (mmol/L)	*Lys*^{109}*Lys*	207 ± 9	27	0.03	231 ± 29	7	NS
	Lys^{109}*Arg* + *Arg*^{109}*Arg*	234 ± 9	29		234 ± 21	13	
	Gln^{223}*Gln*	205 ± 12	16	NS	229 ± 37	4	NS
	Gln^{223}*Arg* + *Arg*^{223}*Arg*	227 ± 7	40		234 ± 18	16	
	Lys^{656}*Lys*	230 ± 8	37	0.04	217 ± 18	14	NS
	Lys^{656}*Asn* + *Asn*^{656}*Asn*	203 ± 11	19		281 ± 31	5	

TABLE 3. Insulin levels in women with impaired glucose tolerance or type 2 diabetes according to genotype

Phenotype	Genotype	Premenopausal women			Postmenopausal women		
		Mean ± SE	n	*P*	Mean ± SE	n	*P*
Fasting insulin (pmol/L)	$Lys^{109}Lys$	131 ± 14	29	NS	226 ± 34	7	0.05
	$Lys^{109}Arg + Arg^{109}Arg$	153 ± 13	34		122 ± 22	15	
	$Gln^{223}Gln$	117 ± 18	18	NS	245 ± 44	4	NS
	$Gln^{223}Arg + Arg^{223}Arg$	154 ± 11	45		135 ± 20	18	
	$Lys^{656}Lys$	149 ± 11	44	NS	126 ± 22	15	0.05
	$Lys^{656}Asn + Asn^{656}Asn$	129 ± 18	19		225 ± 36	6	
Insulin, 120 min (pmol/L)	$Lys^{109}Lys$	838 ± 116	28	NS	1747 ± 234	6	0.004
	$Lys^{109}Arg + Arg^{109}Arg$	974 ± 105	34		616 ± 144	15	
	$Gln^{223}Gln$	727 ± 146	17	NS	2211 ± 207	4	0.0005
	$Gln^{223}Arg + Arg^{223}Arg$	982 ± 90	45		640 ± 97	17	
	$Lys^{656}Lys$	895 ± 94	43	NS	893 ± 197	15	NS
	$Lys^{656}Asn + Asn^{656}Asn$	950 ± 141	19		1171 ± 343	5	
Insulin AUC (pmol/L)	$Lys^{109}Lys$	16911 ± 1930	26	NS	24079 ± 3236	5	0.02
	$Lys^{109}Arg + Arg^{109}Arg$	17679 ± 1801	30		11408 ± 1952	13	
	$Gln^{223}Gln$	15670 ± 2526	15	NS	29661 ± 3401	3	0.006
	$Gln^{223}Arg + Arg^{223}Arg$	17930 ± 1528	41		11530 ± 1485	15	
	$Lys^{656}Lys$	17529 ± 1600	38	NS	13367 ± 2633	12	NS
	$Lys^{656}Asn + Asn^{656}Asn$	16890 ± 2325	18		19473 ± 4154	5	

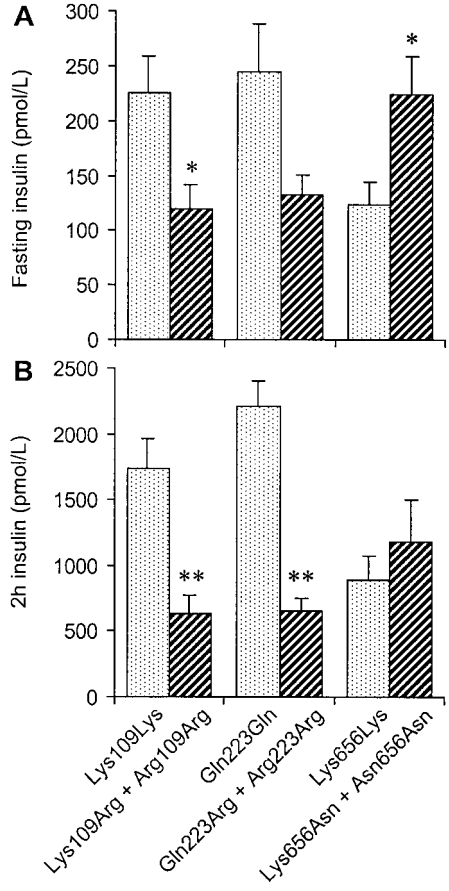

FIG. 1. Insulin levels (picomoles per L) in 25 postmenopausal women with IGT or type 2 diabetes by carrier status for 3 LEPR polymorphisms: $Lys^{109}Arg$, $Gln^{223}Arg$, and $Lys^{656}Asn$. A, Fasting insulin levels; B, insulin levels 2 h after an oral glucose load of 75 g.

sponse in Pima Indians (29). Gotoda *et al.* examined the same three polymorphisms for associations with plasma glucose and insulin (fasting and 2 h OGTT), but with negative results (33). Similarly, Silver *et al.* found no association between the same phenotypes and the $Gln^{223}Arg$ and $Lys^{656}Asn$ polymorphisms (32). One report found a lower 2 h insulin response in Gln homozygotes compared with Arg carriers, comparable to what we found in premenopausal women (although not significant), but in contrast to our results in postmenopausal women (43). However, all of these studies were performed in nondiabetic subjects and usually in mixed populations, with males and females, obese and lean subjects taken together, whereas we selected only overweight and obese women. Classifying these women further into groups based on glucose tolerance and menopausal state, we found associations with these polymorphisms only in women with IGT, which were even more obvious in women with overt diabetes. A limitation of this design is the small number of subjects remaining in each subgroup, which could have affected the results. However, we believed that it was necessary to take glucose tolerance status into account, because an association between these polymorphisms and glucose metabolism could be missed if this was not done. In this group of overweight and obese women, one of four premenopausal and one of five postmenopausal women showed impaired glucose metabolism. This group had significantly higher body weight and body fat plus higher glucose and insulin levels compared with the normoglycemic women.

The fact that associations between LEPR polymorphism and insulin levels were only found in women with IGT, and not in normoglycemic women, could indicate that the LEPR gene is interacting with factors associated with an IGT state, such as hyperinsulinemia or insulin resistance, or perhaps other genes, such that the effects of the LEPR polymorphisms on insulin levels become only apparent in this condition of a (pre-)diabetic state. For instance, glucose toxicity could affect the modulation of insulin secretion by leptin at the level of the pancreatic β-cells. Leptin normally inhibits insulin secretion through its receptor on the β-cells (10–12). If the presence of a given polymorphism in the LEPR gene decreases this action of leptin, the stimulating effect of glucose on insulin secretion might be increased. This effect could become stronger in the case of IGT, when higher glucose

levels could increase insulin secretion without an efficient inhibitory system by leptin.

It is important to note that the effects of the LEPR mutations persisted when insulin levels were adjusted for these higher glucose levels. Indeed, we obtained similar results as when insulin data were adjusted only for age and fat mass. Moreover, the associations persisted when the ratio of insulin AUC to glucose AUC was used as a phenotype instead of insulin AUC alone (data not shown). As the ratio of insulin/glucose gives an idea of insulin sensitivity, we can assume that a potential effect of these mutations would be an effect on insulin sensitivity rather than on insulin secretion itself.

Another polymorphism in the LEPR gene, a 3′-untranslated region insertion/deletion polymorphism, was shown to be associated with fasting insulin (36) and the insulin response during an OGTT (30) in obese nondiabetic subjects. The latter was significant at 30 min after the glucose load, but not at 60 min. This suggests that DNA sequence variation in LEPR may influence primarily the acute insulin response to glucose, which could explain why no associations are found with glucose levels after 2 h, but only with the AUC, in which more early measurements are incorporated. In our study measurements of insulin levels at 15, 30, and 60 min after an oral glucose load showed similar associations with the LEPR polymorphisms as the 2 h insulin response (data not shown in results). The only additional result from these analyses was a very significant ($P = 0.005$) association of $Lys^{656}Asn$ with glucose levels 30 min after an oral glucose load in premenopausal women, whereas there was no association with the 2 h response in this group.

An important question is whether polymorphisms in the LEPR gene could be involved in the development of insulin resistance. We found no differences in the frequencies of the polymorphisms considered in the present study between the group of women with normal glucose tolerance and the group with IGT. Similarly, the insulin phenotype results remained unchanged when glucose levels were taken into account. Thus, we believe that there is no compelling evidence in the present data for an effect of LEPR gene polymorphisms on insulin resistance *per se*.

Finally, associations found in population studies do not necessary imply the existence of a causal relationship between a mutation and a phenotype. An association can also be the result of a linkage disequilibrium between the marker and the sequence variant truly affecting the phenotype. Associations can also arise as a consequence of population stratification for a variety of causes or by chance. Therefore, as for any other such studies, replications are clearly desirable in other populations.

Conclusion

In summary, our results show an association between polymorphisms in the LEPR gene with glucose and insulin metabolism in overweight and obese women with IGT. This suggests that these genetic polymorphisms could affect the peripheral function of the LEPR in the regulation of insulin secretion and especially on insulin action.

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