Plasma Leptin Levels
Interaction of Obesity With a Common Variant of Insulin Receptor Substrate-1
Franz Krempler, Emanuel Hell, Carmen Winkler, David Breban, Wolfgang Patsch

Abstract—Obesity is associated with insulin resistance and other major cardiovascular risk factors. A common amino acid polymorphism at codon 972 of the insulin receptor substrate-1 (IRS-1) has been shown to interact with obesity in the expression of insulin resistance. The plasma concentration of the adipocyte-specific hormone leptin is increased in obesity and is correlated with adipose tissue mass. Because in vitro studies demonstrated inhibitory effects of leptin on insulin signaling, leptin may be involved in obesity-associated insulin resistance. To gain insight into the relationship between insulin and leptin in obesity, we studied plasma leptin levels and several cardiovascular risk factors, as well as their modification by the IRS-1 codon 972 genotype, in 156 obese individuals and 131 lean control subjects. In both groups, 10% of the subjects were heterozygous for the IRS-1 codon 972 variant. Obese individuals harboring the IRS-1 variant displayed significantly lower plasma concentrations of leptin than obese subjects without the polymorphism (means, 26.7 versus 37.8 ng/mL, P<0.0293). In a subgroup of obese patients, leptin mRNA abundance was measured in the adipose tissue and was significantly lower in carriers of the IRS-1 variant than in subjects with the wild-type variant (P<0.0291). Our data suggest that insulin signaling influences plasma leptin concentrations at the mRNA expression level and argue against leptin as a major causative factor of insulin resistance. (Arterioscler Thromb Vasc Biol. 1998;18:1686-1690.)

Key Words: obesity ■ leptin ■ insulin resistance ■ insulin receptor substrate-1

Obesity is associated with insulin resistance and hyperinsulinemia. Both conditions increase the risk for coronary heart disease.1-3 Hyperinsulinemia seems to predict ischemic heart disease independent of other risk factors.3 The mechanism(s) underlying the association between insulin resistance and obesity is not completely understood. Leptin, the product of the ob gene, is secreted specifically by adipocytes, binds to hypothalamic receptors encoded by the db gene, and conveys the size of the adipose tissue mass to centers controlling appetite.4-6 Administration of recombinant leptin to ob/ob mice resulted in decreased food intake, increased energy expenditure, and improved insulin resistance before affecting body weight.7-9 Because the reduction in glucose and insulin exhibited a more rapid time course than the changes in body weight, the inhibitory effect of leptin on neuropeptide Y gene expression and interactions with peripheral cells have been implicated in the improvement of insulin sensitivity.10 Moreover, leptin increased glucose turnover in lean wild-type mice.11 Insulin administration, on the other hand, was shown to increase leptin expression in rodents.12 In the common forms of human obesity, the relation of leptin to insulin resistance is less clear, because both leptin and insulin levels are often increased.13 Associations of plasma levels of leptin and insulin have been reported in subjects with or without insulin resistance.14 Three-hour euglycemic hyperinsulinemic clamp studies failed to increase leptin mRNA abundance in human adipose tissues,15 while infusion of insulin during a period of 8.5 hours induced an increase in plasma leptin levels.14 Moreover, hyperinsulinemia during a period of 72 hours increased serum leptin concentrations in a dose-dependent manner.16 Thus, prolonged hyperinsulinemia seems to enhance leptin expression in humans also. However, leptin itself has been suspected to be a causative factor of insulin resistance.17 Studies in cultured human and rat hepatoma cell lines have shown that leptin attenuates several insulin signals.18

A common sequence polymorphism in the insulin receptor substrate-1 (IRS-1) gene that predicts the replacement of glycine by arginine at codon 972 has been shown to be more frequent in patients with non–insulin-dependent diabetes mellitus than in control subjects and may therefore contribute to insulin resistance.19 Interestingly, this IRS-1 polymorphism
METHODS

Study Population
This study included 287 unrelated white subjects; 156 subjects were obese patients referred consecutively for weight reduction surgery, and 112 of these obese subjects fulfilled the criteria for bariatric surgery and underwent a gastric banding procedure. Adipose tissue samples were collected from comparable anatomic sites of the omentum (referred to as intraperitoneal) and the abdominal wall (referred to as extraperitoneal). One hundred thirty-one subjects were lean, healthy individuals. Among obese subjects, 6 females used oral contraceptive drugs and 27 subjects used medications, including thyroid hormones (4 subjects), calcium channel blockers or angiotensin-converting enzyme inhibitors (9 subjects), hypoglycemic drugs (1 subject), allopurinol (4 subjects), H2-receptor antagonists or antacids (3 subjects), benzodiazepines or tricyclic antidepressants (7 subjects), and theophylline and selective beta-adrenergic agonists by inhalation (2 subjects). Study subjects gave their written informed consent and the study protocol was approved by the institutional review board for human studies.

Body mass index (BMI, kilograms per meter squared) was calculated from measurements of weight and height. Sitting blood pressure was measured after a 5-minute rest. Smoking status was assessed with interviews. Subjects were classified as diabetic if they were using hypoglycemic medications or had fasting-plasma glucose concentrations >140 mg/dL.

Biochemical Measurements
After patients fasted overnight, venous blood was collected at 8 AM into tubes containing EDTA. Fasting-plasma glucose was measured using a hexokinase/glucose-6-phosphatase dehydrogenase method. Plasma insulin was measured using immunoassay (MEIA, Abbott Laboratories). Plasma cholesterol and triglyceride were measured with enzymatic procedures using a Hitachi 717 analyzer (Boehringer Mannheim Diagnostics) and the respective enzymatic kits (catalog Nos. 1489437 and 1085520, Boehringer Mannheim Diagnostics).

Statistical Analyses
Allele frequencies of the 972 polymorphism were estimated by gene counting. One-way ANOVA was used to test the distributions of the continuous variables among strata. The variation in plasma leptin levels attributable to the IRS-1 polymorphism was calculated using the R2 from the ANOVA. Kruskal-Wallis test was used, or a transformation was made on the original variable, if the equal variance and normality assumptions of the one-way ANOVA were rejected. To compare categorical variables, a contingency χ2 test was used. A factorial ANOVA was used to determine the interaction between obesity and IRS-1 polymorphism on leptin and other continuous variables adjusted for sex.

RESULTS
This study examined 156 obese patients and 131 controls. Table 1 shows sex, age, BMI, blood pressure, and prevalence of diabetes in our study subjects. About 4 times more women than men were referred for weight reduction surgery. Nonobese controls and obese patients were similar in average and median age (39 and 36 years in controls versus 36 and 35 years in obese patients, respectively). Both average and median BMI was much higher in obese patients than in controls (43.7 and 42.4 kg/m2 versus 23.3 and 23.1 kg/m2). Systolic and diastolic blood pressure readings were significantly higher in obese patients than in controls. Overt diabetes was present in 8 obese subjects compared with 1 subject in the nonobese group (P=0.0401). The proportion of cigarette smokers was similar in lean and overweight individuals (P=0.7101). The profiles of cardiovascular risk factors were markedly different between obese and nonobese individuals. Average values for glucose (91.4 versus 70.7 mg/dL), insulin (10.9 versus 6.7 μU/mL), triglyceride (342 versus 121 mg/dL), and apoB (99.5 versus 86.2 mg/dL) were significantly higher in

Northern Blot Analysis
Total RNA was isolated from 2 g of adipose tissue according to the method of Chomczynski and Sacchi.24 The integrity of RNA was ascertained by the electrophoretic pattern of rRNA in formaldehyde gels. Relative abundance of leptin mRNA was determined using Northern blot analysis as outlined by Thomas.26 After patients fasted overnight, venous blood was collected at 8 AM into tubes containing EDTA. Fasting-plasma glucose was measured using a hexokinase/glucose-6-phosphatase dehydrogenase method. Plasma insulin was measured using immunoassay (MEIA, Abbott Laboratories). Plasma cholesterol and triglyceride were measured with enzymatic procedures using a Hitachi 717 analyzer (Boehringer Mannheim Diagnostics) and the respective enzymatic kits (catalog Nos. 1489437 and 1085520, Boehringer Mannheim Diagnostics). HDL cholesterol was determined in supernatants after precipitation of plasma with phosphotungstic acid/MgCl2, and LDL cholesterol was determined in supernatants after precipitation with enzymatic procedures using a Hitachi 717 analyzer (Boehringer Mannheim Diagnostics) and the respective enzymatic kits (catalog Nos. 1489437 and 1085520, Boehringer Mannheim Diagnostics).

Statistical Analyses
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TABLE 1. Characteristics of Study Subjects

<table>
<thead>
<tr>
<th></th>
<th>Nonobese Subjects</th>
<th>Obese Subjects</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, male/female</td>
<td>39/92</td>
<td>31/125</td>
<td>NS</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.3 (2.9)</td>
<td>43.7 (8.6)</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>38.6 (13.9)</td>
<td>36.1 (10.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>121 (18)</td>
<td>136 (17)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>76 (9)</td>
<td>83 (11)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Diabetes, No.</td>
<td>1</td>
<td>8</td>
<td>0.0401</td>
</tr>
</tbody>
</table>

In the entire study group, 29 subjects (10.1%) were heterozygous for the glycine to arginine substitution at codon 972 of IRS-1 (Table 2), and the frequency of this polymorphism was similar in obese and nonobese individuals. Neither in the obese group nor in the control group was a subject homozygous for the IRS-1 variant identified. Among all clinical and biochemical data studied, only plasma leptin levels in obese subjects exhibited a significant difference by IRS-1 genotype. ANOVA showed that obese carriers of the IRS-1 variant had significantly lower average plasma leptin levels than obese wild-type subjects (26.7 versus 37.8 ng/mL, P<0.029) (Table 2). Adjustment for sex and BMI slightly strengthened the significance (P<0.023). Median values were also significantly different (23.5 versus 34.3 ng/mL, P<0.018) (Kruskal-Wallis). The IRS-1 codon 972 polymorphism accounted for 3.05% of the variation of serum leptin levels in our obese subjects. The average leptin values in lean subjects were 9.4 versus 8.6 ng/mL (P<0.636) and median values were 8.8 versus 7.0 ng/mL (P<0.436). Moreover, a significant interaction between the IRS-1 codon 972 variant and obesity status was observed with respect to leptin levels (P<0.035). Exclusion of diabetic subjects and subjects using oral contraceptives or any kind of medication did not substantially alter the association of IRS-1 genotype with leptin levels. Among obese subjects, the average glucose and insulin levels tended to be higher in IRS-1 variant carriers than in IRS-1 wild-type subjects (Table 2). The only nonobese patient with diabetes was a carrier of the IRS-1 variant.

In a subgroup of obese patients, relative leptin mRNA abundance in adipose tissue was determined (Table 3). Patients in the studied subgroup exhibited similar differences in plasma leptin levels by IRS-1 genotype as patients in the entire study group (40.8 versus 28.5 ng/mL, P<0.028). In carriers of the IRS-1 variant, total leptin expression was significantly lower than in wild-type patients (P<0.029).

**Discussion**

Circulating leptin levels are correlated with the mass of adipose tissue. Factors other than body weight may also play a role in the regulation of leptin. Several studies indicate that leptin expression is stimulated by insulin, whereas other studies suggest that high leptin concentrations may contribute to insulin resistance. In the present study, plasma leptin concentrations were significantly lower in obese carriers of the IRS-1 variant than in obese wild-type carriers, indicating that the insulin-signaling pathway is part of the regulatory system of leptin. Our results are therefore consistent with studies that reported stimulation of leptin expression and an increase of serum leptin by insulin.

In obese carriers of the IRS-1 variant, fasting glucose and insulin levels were higher than in wild-type subjects (Table 2), albeit these differences were not statistically significant. Similar results were reported by Clausen et al, who also found higher glucose and insulin levels in their obese IRS-1 codon 972 variant subjects. These investigators used additional tests to demonstrate a difference in insulin sensitivity between IRS-1 wild-type and variant subjects. It is therefore reasonable to assume that the higher insulin and glucose

**TABLE 2. Plasma Concentrations of Leptin, Glucose, and Insulin by IRS-1 Codon 972 Genotype**

<table>
<thead>
<tr>
<th></th>
<th>Nonobese, Mean (SD)</th>
<th>Obese, Mean (SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS-1</td>
<td>Wild-Type</td>
<td>972-Variant</td>
<td></td>
</tr>
<tr>
<td>Leptin, ng/mL</td>
<td>8.6 (6.2)</td>
<td>9.4 (5.9)</td>
<td>0.636</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>69 (20)</td>
<td>82 (54)</td>
<td>0.075</td>
</tr>
<tr>
<td>Insulin, µU/mL</td>
<td>6.7 (4.7)</td>
<td>6.6 (2.6)</td>
<td>0.912</td>
</tr>
</tbody>
</table>

In obese individuals than in lean individuals (all P<0.001). Average values for HDL cholesterol (34.0 versus 53.3 mg/dL) and apoA-I (117.7 versus 141.8 mg/dL) were significantly lower in obese subjects than in controls (both P<0.001). Exclusion of diabetic subjects did not alter these differences. No difference in plasma cholesterol was observed between obese patients and controls (197.6 versus 195.6 mg/dL). Average and median levels of leptin were significantly higher in cases than in controls (36.8 versus 8.6 ng/mL and 33.2 versus 7.1 ng/mL, P<0.001).
levels in our obese subjects harboring the IRS-1 codon 972 variant reflect diminished insulin sensitivity. Hence, our observations do not support a major role of leptin in promoting insulin resistance. Nevertheless, minor or transient effects of leptin-reducing insulin sensitivity may have been concealed by more dominant, leptin-independent influences of the IRS-1 codon 972 variant.

The frequency of the IRS-1 variant in our study population was comparable to that of other studies and was similar in lean and obese subjects. We can therefore conclude that, at least in our study population, the IRS-1 codon 972 polymorphism itself is not a causative factor for obesity. However, interactions of subclinical genetic defects may influence the course and expression of multifactorial diseases. Injection of MSG in neonatal mice resulted in a more severe syndrome of obesity in IRS-1–deficient animals than in controls. Moreover, heterozygosity for both insulin receptor and IRS-1 null alleles increased the incidence of overt diabetes in transgenic mice several-fold. It is thus possible that interactions of the IRS-1 codon 972 polymorphism with other predisposing factors are involved in the pathogenesis of obesity.

Arteriovenous balance studies have shown that leptin synthesis is the principal determinant of leptin plasma levels, and studies in rodents have demonstrated that insulin increases the abundance of leptin mRNA in adipose tissue. Evidence to suggest that the insulin–signaling pathway is part of the regulatory system of leptin in humans comes from leptin mRNA expression data in a subgroup of our obese patients. In carriers of the IRS-1 variant, leptin expression levels were significantly lower than in wild-type carriers (Table 3).

Experiments in myeloid progenitor cells have provided biochemical evidence that the IRS-1 codon 972 variant impairs insulin-stimulated signaling along the phosphatidyl-inositol 3-kinase pathway but also tends to reduce the association of IRS-1 with Grb2. Whether these pathways are also involved in the regulation of leptin expression is not known. IRS-1 is also used by other upstream activators such as insulin-like growth factor-1 or interleukin-4. Moreover, treatment of cultured murine adipocytes with tumor necrosis factor-α (TNF-α) converts IRS-1 into an inhibitor of the insulin receptor tyrosine kinase activity, thus attenuating insulin receptor signaling. In human obesity, the expression of TNF-α is correlated with the extent of obesity and hyperinsulinemia. An interaction of the functionally defective IRS-1 codon 972 variant with TNF-α or other mediators that are overexpressed in obesity might contribute to our finding that leptin levels in plasma are reduced only in obese carriers, but not in lean carriers, of the IRS-1 variant.

In our obese patients, the IRS-1 codon 972 polymorphism was associated with 30% lower plasma leptin levels and accounted for 3% of the variation in leptin concentration. Comuzie et al conducted a genome-wide scan and multi-point linkage analysis in pedigrees of Mexican Americans and showed that chromosomal region 2p21 accounted for 47% of the variation of serum leptin levels. Compared with this quantitative trait locus, possibly harboring several sequence substitutions in 1 or more genes, the effect of the single amino acid substitution at residue 972 of IRS-1 seems relatively small. However, in vivo studies indicate that the effect of insulin on leptin plasma concentrations is moderate. Thus, most functional mutations of the insulin-signaling components cannot be expected to have a major impact on the variation in plasma leptin levels in the population, if their frequency is as low as that of the IRS-1 polymorphism. Nevertheless, we cannot rule out the possibility that the lower leptin levels in obese carriers of the IRS-1 variant resulted from linkage disequilibrium with a functional mutation in a different gene.

Although leptin is very effective in lowering food intake and body weight in ob/ob mice, animal models with diet-induced obesity exhibit, like many obese human subjects, increased plasma leptin concentrations. Higher doses of leptin are required in these animal models in comparison with ob/ob mice to achieve weight reduction. In a recent report, low plasma concentrations of leptin predicted weight gain in Pima Indians. The identification of obese subjects with inappropriately low leptin levels, as reported here in carriers of the IRS-1 codon 972 polymorphism, may therefore identify a subset of patients who may be candidates for therapeutic regimens aimed at reducing body weight by increasing leptin levels.

**Acknowledgments**

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Insulin Receptor Substrate-1 and Leptin


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