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Research Article

The regulation of ob gene expression in abdominal subcutaneous adipose tissue was investigated using a reverse transcription-competitive PCR method to quantify the mRNA level of leptin. Leptin mRNA level was highly correlated with the body mass index of 26 subjects (12 lean, 7 non-insulin-dependent diabetic, and 7 obese patients). The effect of fasting on ob gene expression was investigated in 10 subjects maintained on a hypocaloric diet (1045 KJ/d) for 5 d. While their metabolic parameters significantly changed (decrease in insulinemia, glycemia, and resting metabolic rate and increase in plasma ketone bodies), the caloric restriction did not modify the leptin mRNA level in the adipose tissue. To verify whether insulin regulates ob gene expression, six lean subjects underwent a 3-h euglycemic hyperinsulinemic (846 \pm 138 pmol/liter) clamp. Leptin and Glut 4 mRNA levels were quantified in adipose tissue biopsies taken before and at the end of the clamp. Insulin infusion produced a significant threefold increase in Glut 4 mRNA while leptin mRNA was not affected. It is concluded that ob gene expression is not acutely regulated by insulin or by metabolic factors related to fasting in human abdominal subcutaneous adipose tissue.

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The Expression of *ob* Gene Is Not Acutely Regulated by Insulin and Fasting in Human Abdominal Subcutaneous Adipose Tissue

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Abstract

The regulation of *ob* gene expression in abdominal subcutaneous adipose tissue was investigated using a reverse transcription-competitive PCR method to quantify the mRNA level of leptin. Leptin mRNA level was highly correlated with the body mass index of 26 subjects (12 lean, 7 non-insulin-dependent diabetic, and 7 obese patients). The effect of fasting on *ob* gene expression was investigated in 10 subjects maintained on a hypocaloric diet (1045 KJ/d) for 5 d. While their metabolic parameters significantly changed (decrease in insulinemia, glycemia, and resting metabolic rate and increase in plasma ketone bodies), the caloric restriction did not modify the leptin mRNA level in the adipose tissue. To verify whether insulin regulates *ob* gene expression, six lean subjects underwent a 3-h euglycemic hyperinsulinemic (846 ± 138 pmol/liter) clamp. Leptin and Glut 4 mRNA levels were quantified in adipose tissue biopsies taken before and at the end of the clamp. Insulin infusion produced a significant threefold increase in Glut 4 mRNA while leptin mRNA was not affected. It is concluded that *ob* gene expression is not acutely regulated by insulin or by metabolic factors related to fasting in human abdominal subcutaneous adipose tissue. (*J. Clin. Invest.* 1996. 98:251–255.) Key words: leptin • obesity • adipose tissue • RT-competitive PCR • satiety factor

Introduction

Recently, the mouse *ob* gene (1), and subsequently its human and rat homologues (1–5), have been cloned. Leptin, the product of *ob* gene (6), is produced by the adipocytes and considered as a hormone that acts centrally to regulate body weight homeostasis through the control of appetite and energy expenditure (1, 6–10). Mutations in the *ob* gene, as seen in the *ob/ob* mice (1), or in the *ob* receptor locus, as observed in *db/db* mice

(11), result in extreme obesity in these animal models. In rodents, the *ob* gene is highly regulated and is under control of various hormonal and metabolic factors. Leptin mRNA levels are correlated with the nutritional status being markedly reduced after fasting and increased by refeeding (12–16). These changes in *ob* expression seem secondary to changes in circulating insulin levels, since *ob* gene expression is positively regulated by insulin both in vivo and in vitro (12, 13).

The relevance of leptin to human obesity is less clear. As of yet no mutation in the human *ob* gene or absence of leptin expression has been reported in obese subjects (17). In contrast several studies report a positive correlation between body mass index and leptin mRNA or plasma concentrations (17–21). This situation is reminiscent of the overexpression of leptin mRNA observed in several obese animals such as the *db/db* mice, the hypothalamus-lesioned rats and the Zucker *fa/fa* rats (4–6, 16, 22–24). This implies that obese animals and humans are unresponsive or resistant to the effects of leptin and questions the suggested role of leptin as a satiety factor, which controls food intake and/or energy expenditure.

In this study we investigated the regulation of leptin mRNA abundance in human subcutaneous abdominal adipose tissue in response to either insulin infusion or fasting. Our data confirm the correlation between leptin mRNA levels and the body mass index, previously observed by others (20, 21). Interestingly, neither insulin infusion nor a caloric restriction during a 5 day period significantly modified leptin mRNA levels. This indicates that human *ob* gene expression, unlike rodent *ob* gene expression, is not subject to an acute control by nutritional and hormonal factors, such as insulin. Our data question the role of human leptin as a tightly controlled satiety factor but rather suggest that leptin signals the size of the adipose tissue deposit.

Methods

Subjects and protocols. 26 subjects (11 men and 15 women, mean [\pm SD] age 36 ± 13 years) participated in the study. All gave their written consent after being informed of the nature, purpose, and possible risks of the study. The experimental protocol was approved by the ethics committee of Hospices Civils de Lyon and performed according to the French legislation (Hurriet law).

Seven subjects suffered from morbid obesity (body mass index (BMI)¹ = 45.1 ± 5.8 Kg/m², range 36.6–53.4) and were treated by verti-

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cal banded gastroplasty. At the time of surgery, patients were on their maximal lifetime weight and were not on restricted diet. None of them were taking any medication or had any evidence of metabolic disease other than obesity. Their fasting glycemia was 5.6 ± 0.7 mM, their fasting insulinemia was 146 ± 43 pmol/liter. Biopsies of subcutaneous adipose tissue were performed during the surgery after an overnight fast, and frozen in liquid nitrogen immediately.

We studied 12 normal lean subjects (BMI = 21.8 ± 1.8 Kg/m²), from whom six underwent a 3-h euglycemic hyperinsulinemic clamp (insulin infusion rate of 12 pmol/Kg.min) (25) in order to determine the acute effect of insulin on *ob* gene expression. The study was performed after an overnight fast. Biopsies of the abdominal subcutaneous adipose tissue were performed 2 h before and at the end of the euglycemic hyperinsulinemic clamp. After cleaning and infiltration of the skin with 2% xylocaine, adipose tissue was aspirated through a 15-gauge needle. Samples averaged 131 ± 62 mg wet weight. They were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

10 subjects participated in a five day study protocol during which they received a 1045 KJ/d diet in order to study the effect of fasting on *ob* gene expression. Seven of these subjects were overweight and had non-insulin-dependent diabetes mellitus (NIDDM) (BMI = 31.2 ± 2.7 Kg/m², ranging from 25.9 to 34.4; fasting plasma glucose concentration = 13.4 ± 3.3 mM). Three normal weight subjects served as lean controls (BMI = 21.9 ± 1.5 Kg/m², glycemia = 4.5 ± 0.2 mM). The reported body weight of the subjects was stable for at least one month. For the patients with NIDDM, usual treatment with oral antidiabetic agents was interrupted at least one week before admission. The subjects entered the hospital 3 d before the beginning of the study protocol. During these 3 d, their diet was standardized (104 KJ/kg per day). The first biopsy of abdominal subcutaneous adipose tissue was performed after an overnight fast, just before starting the period of caloric restriction. The second biopsy was taken on the morning of the sixth day of caloric restriction. Before and after the diet, the resting metabolic rate of the subjects was measured by indirect calorimetry (Datex Deltatrac Monitor, Helsinki, Finland) (26). Blood and urine samples were collected each day to measure glucose and ketone bodies concentrations.

Total RNA preparation. Adipose tissue samples were pulverized in liquid nitrogen and total RNA was prepared from the frozen powder using the RNeasy total RNA kit (QIAGEN, Chatsworth, CA) following the instructions of the manufacturer. The ratio of absorption (260/280 nm) of all preparations was between 1.9 and 2.0 and the mean yield of total RNA was 21 ± 9 $\mu\text{g/g}$ of tissue (mean \pm SD, $n = 42$ preparations) with no significant difference between the different groups of subjects. The yield of total RNA was also not significantly different between the biopsies taken before and at the end of the euglycemic hyperinsulinemic clamp, or between the biopsies taken before and after the caloric restriction period (data not shown).

Quantification of leptin mRNA. Human leptin mRNA was quantified in adipose tissue total RNA preparations by reverse transcription reaction followed by competitive polymerase chain reaction (RT-competitive PCR) which consist in the co-amplification of leptin cDNA with known amount of a leptin competitor DNA in the same test tube (27, 28). The competitor DNA molecule was derived from the clone Ob6.1 that contained the complete coding sequence of human leptin cDNA (3). This clone was obtained by screening a human adipose tissue λ gt 11 cDNA library with a fragment of mouse *ob* cDNA clone pmob (29), and subcloned into the phagemid pBlue-Script SK (Stratagene). The leptin competitor was constructed by deleting a 80 bp-long fragment (nucleotides +324 to +404 of the coding sequence) by digestion (Eco72I/ HindIII) and ligation. The RT-PCR was performed with 23-nucleotide-long primers that hybridized to the 3' and 5' ends of the leptin coding sequence (sense primer: 5'-ATG-CATTGGGGAACCCTGTGCGG-3', located at position +1 to +23; antisense primer: 5'-TGAGGTCCAGCTGCCACAGCATG-3', located at position +465 to +487). Therefore, leptin mRNA yielded a PCR product of 487 bp while the amplification of the competitor gave

a product of 407 bp. Working solutions of the leptin competitor DNA at defined concentrations (50 – 10^{-3} pmol/liter) were prepared by serial dilution in 10 mM Tris-HCl (pH 8.3)-1 mM EDTA buffer.

The conditions of the reverse transcription reaction and of the competitive PCR were the same as we previously used to quantify insulin receptor mRNAs in rat tissues (28). Briefly, the RT reaction was performed from 0.5 μg of adipose tissue total RNA with a thermostable reverse transcriptase during 15 min at 70°C . The RT medium was then added to a PCR mix that was aliquoted in four PCR tubes, each containing a different, but known, amount of leptin competitor DNA. The tubes were subjected to 40 cycles of amplification including denaturation for 60 s at 94°C , hybridation for 60 s at 58°C and elongation for 90 s at 72°C . The PCR products were separated in 3% agarose gel and the amount of leptin mRNA in the reaction was determined at the competition equivalence point of the band densities as previously described (28).

To validate the RT-competitive PCR assay, human leptin RNA was synthesized by in vitro translation from the clone Ob6.1 (Ribo-probe system, Promega), and quantified by RT-competitive PCR over a wide range of concentrations (from 0.5 to 25 amol added in the RT reaction). The standard curve obtained was linear over the tested range ($r = 0.987$) with a slope of 1.06. The inter-assay variation of the RT-competitive assay was less than 10% with six separate determinations of the same amount of leptin RNA.

In addition to leptin mRNA, we measured Glut 4 mRNA levels in the biopsies taken before and at the end of the euglycemic hyperinsulinemic clamp. Glut 4 mRNA was also quantified by RT-competitive PCR assay, with a specific competitor DNA molecule. The construction of the competitor DNA and the validations of the RT-competitive PCR for Glut 4 are presented elsewhere (30).

Results presentation. All the results are expressed as means \pm SD. Statistical analysis were performed with a Wilcoxon nonparametric test for paired values. The threshold for significance was set at $P < 0.05$.

Results

To analyze whether leptin mRNA levels correlate with adipose tissue mass, leptin mRNA was quantified in the subcutaneous abdominal adipose tissue of 26 subjects after an overnight fast. The absolute leptin mRNA level was about eight times lower ($P < 0.001$) in the normal weight subjects (12.2 ± 7.1 amol/ μg total RNA, $n = 12$) than in the subjects with morbid obesity (94 ± 34 amol/ μg total RNA, $n = 7$). Leptin mRNA content of the adipose tissue was strongly correlated with the BMI ($r = 0.897$, $P < 0.001$; Spearman correlation test) (Fig. 1) and with the body weight of the subjects ($r = 0.810$, $P < 0.001$). There was no correlation between leptin mRNA levels and the amount of tissue sampled or the yield in total RNA.

Next, we investigated the effects of a caloric restriction on leptin mRNA levels. Seven overweight NIDDM patients with elevated basal level of leptin mRNA (68 ± 14 amol/ μg of total RNA) and three control subjects (22.2 ± 1.6 amol/ μg of total RNA) were maintained on a restricted diet of 1045 KJ/d for 5 d. The diet induced a mean weight loss of 3 ± 2 Kg (range 1–6 Kg) in all the subjects, which corresponded to a reduction of their original body weight by $3.7 \pm 2.1\%$. In the NIDDM patients, the plasma β -hydroxybutyrate concentrations increased from 0.6 ± 1 to 1.9 ± 1.4 mM ($P < 0.001$) while fasting glycemia decreased from 13.4 ± 3.3 to 8.6 ± 2.2 mM ($P < 0.01$) and fasting insulin concentrations from 78 ± 27 to 53 ± 20 pmol/liter ($P < 0.01$). Increase in plasma ketone bodies concentration (from 0.17 ± 0.12 to 2.14 ± 1.13 mM) and decreases in glycemia (from 4.5 ± 0.2 to 3.5 ± 0.5 mM) and insulinemia (from 50 ± 10 to 28 ± 6

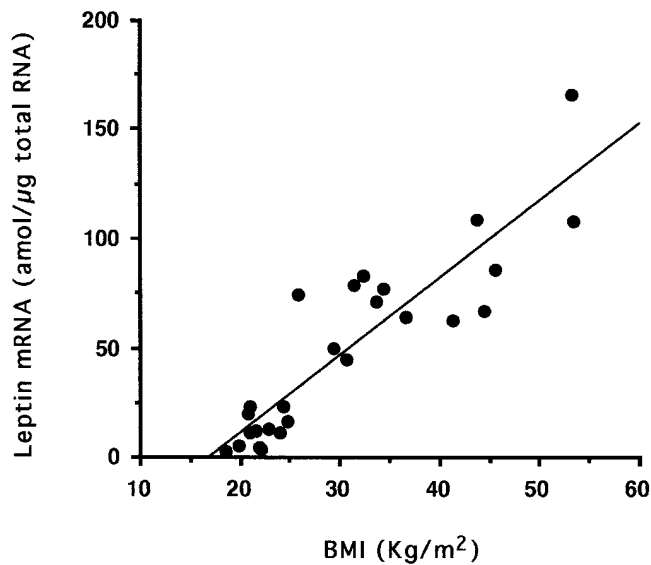


Figure 1. Correlation of leptin mRNA levels with body mass index. The absolute amounts of leptin mRNA were determined by RT-competitive PCR in abdominal subcutaneous adipose tissue biopsies as described in Methods. The equation of the regression was: $y = -59.8 + 3.5 X$, $r = 0.897$ with $P < 0.001$.

pmol/liter) were also observed in the control subjects. During the caloric restriction period, all the subjects were hungry and their resting metabolic rate, measured by indirect calorimetry, decreased by $8.5 \pm 5.5\%$ ($P < 0.01$) from 6358 ± 1087 to 5785 ± 848 KJ/d. Fig. 2 shows that the abundance of leptin mRNA in the adipose tissue did not change significantly during the caloric restriction. The average modification induced by the diet was $+17\%$, varying from a 46% diminution to a 69% increase. The large variability in basal leptin mRNA levels in this group was related to the difference in BMI of the subjects (Fig. 1). In addition, we found that there was no relationship between

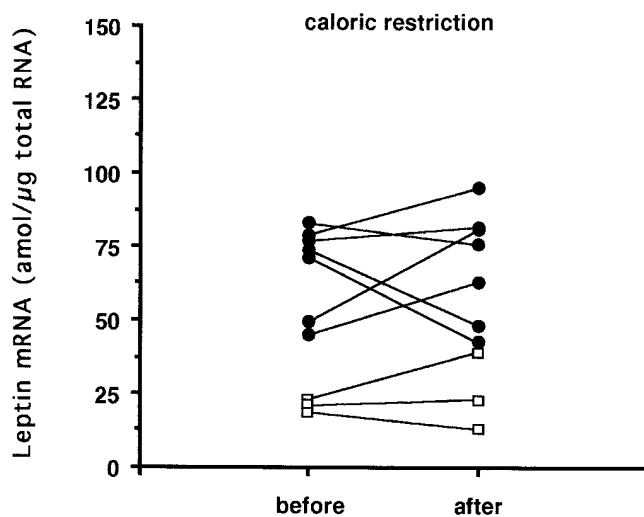


Figure 2. Effect of 5 days of caloric restriction on leptin mRNA levels. The absolute amounts of leptin mRNA were determined in the adipose tissue of 7 NIDDM patients (circle) and 3 control subjects (square) before and after 5 d of hypocaloric diet (1045 KJ/d).

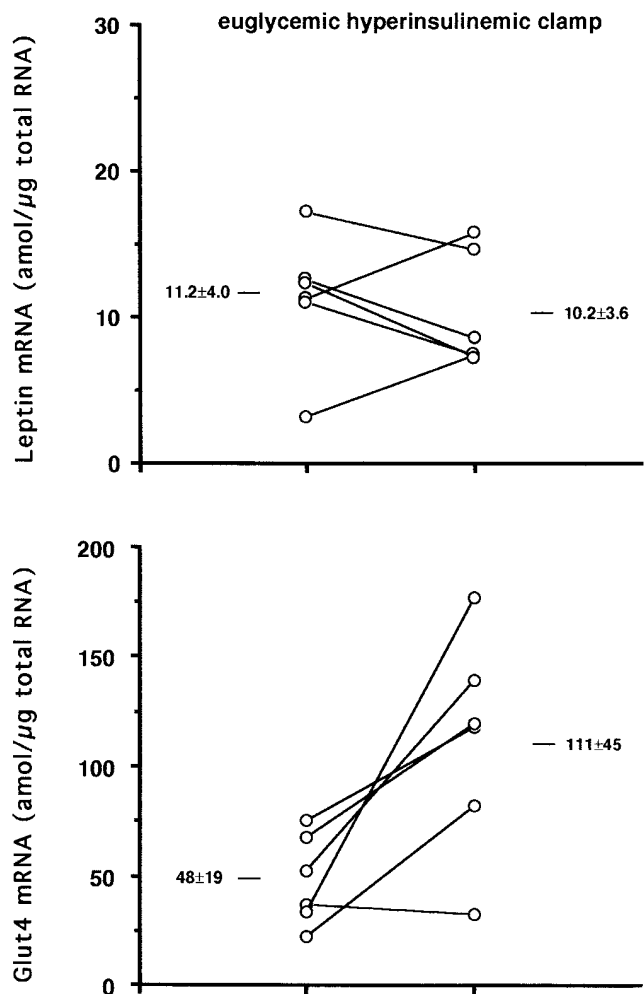


Figure 3. Effect of insulin infusion on leptin and Glut 4 mRNA levels. The absolute amounts of leptin (upper panel) and Glut4 (lower panel) mRNA were measured in the adipose tissue of six control subjects before and at the end of 3 h of euglycemic hyperinsulinemic clamp as described in Methods.

changes in the levels of leptin mRNA and changes in the resting metabolic rate of the subjects measured by indirect calorimetry before and after the diet (not shown).

Finally, we analyzed whether changes in circulating insulin concentration affect leptin mRNA levels in human adipose tissue, as reported in rodent models (12). To this aim, six normal subjects underwent a 3-h euglycemic hyperinsulinemic clamp. During the clamp, insulin concentrations rose from 49 ± 23 to 846 ± 138 pmol/liter whereas glycemia was maintained constant at 4.1 ± 0.2 mM (glucose infusion rate = 52.8 ± 7.7 mmol/Kg.min). Plasma non-esterified fatty acid concentrations decreased from 0.4 ± 0.2 mM to 0.04 ± 0.01 mM ($P < 0.001$) during the clamp. Fig. 3 (upper panel) clearly shows that leptin mRNA level did not change during insulin infusion. To verify whether 3 h of insulin infusion is able to modify gene expression in human adipose tissue, we measured in parallel the abundance of Glut 4 mRNA in the same biopsies. In contrast to leptin mRNA levels, Glut 4 mRNA increased significantly ($P = 0.002$) during the euglycemic hyperinsulinemic clamp in all subjects but one (Fig. 3, lower panel).

Discussion

In this study, we investigated the *in vivo* regulation of the human *ob* gene expression estimated by the quantification of leptin mRNA in abdominal subcutaneous adipose tissue. Leptin, the product of *ob* gene, modulates body weight and energy balance in mouse (6–10) and *ob* gene expression is closely related to the nutritional status and the levels of circulating insulin in rodents (12–16). Therefore, leptin is considered as a satiety factor that could control food intake and/or energy expenditure in these models. In human, plasma leptin concentrations and adipose tissue mRNA abundance correlate with the body mass index and the percentage of body fat (17, 20, 21), but the *in vivo* regulation of *ob* gene expression has not been yet studied.

To investigate the regulation of *ob* gene expression in human, we developed a RT-competitive PCR based assay (27, 28) which allows to determine the absolute concentration of leptin mRNA. It is well demonstrated that standard RT-PCR should not be used as a quantitative assay to measure a given mRNA, mainly because of the uncontrolled efficiency of the PCR (27, 31). The addition of a competitor DNA molecule which is amplified in the same test tube and with the same primers as the target mRNA overcomes this problem allowing real quantitation (27, 28, 30, 31). Using known amounts of *in vitro* synthesized leptin RNA, we confirmed that the RT-competitive PCR assay developed in this work is quantitative, reproducible and very sensitive. It allows the accurate determination of leptin mRNA down to 0.5×10^{-18} moles in the assay which corresponds to $\sim 300,000$ molecules of leptin mRNA. In view of the low yields in total RNA in human adipose tissue and in view of the low levels of leptin mRNA in normal weight subjects, this RT-competitive PCR assay, allowing measurement of minute amounts of mRNA, is a powerful method to study variations in leptin mRNA and thus to investigate the regulation of *ob* gene expression *in vivo*.

Absolute levels of leptin mRNA in the abdominal subcutaneous adipose tissue showed large differences between the individuals. Leptin mRNA was about eightfold higher in patients with morbid obesity relative to lean normal subjects and strongly correlated with the BMI of the subjects. This observation confirmed recent findings (17, 20, 21) and thus served as an additional validation of our RT-competitive assay. Interestingly, the leptin mRNA levels of patients with NIDDM also correlated positively with their BMI, suggesting that the expression of *ob* gene is mainly related to the fat mass of the subjects rather than to the metabolic and hormonal changes associated with NIDDM.

If leptin is a satiety factor in humans, it could be expected that its expression correlates with changes in the nutritional status and/or with modifications in the circulating insulin concentrations, as it has been demonstrated in rodents models (12–16). To test this hypothesis, we investigated the regulation of *ob* gene expression in two defined situations. In a first experiment, calory intake was restricted for 5 d in a group of overweight subjects to verify the effects of fasting on *ob* gene expression. Caloric restriction is associated with complex metabolic adaptations involving numerous hormonal changes which include a decreased insulin/glucagon ratio (32). As a consequence, lipolysis and β -oxidation of fatty acids are enhanced resulting in increased levels of circulating ketone bodies such as β -hydroxybutyrate (32). Therefore, the observed

changes in glucose, insulin and β -hydroxybutyrate concentrations in this study are indicative of an efficient caloric restriction in the subjects maintained on the hypocaloric diet. Moreover, their resting metabolic rate decreased significantly during the period of caloric restriction. Interestingly, and in contrast to data obtained in rodents, the caloric restriction was not associated with a significant change in leptin mRNA levels. This suggests therefore that *ob* gene expression is not controlled by metabolic factors related to caloric restriction and fasting in humans. Our data seem, however, to be in apparent conflict with the data generated by Maffei et al. (20) and by Considine et al. (21) who observed a decrease in leptin mRNA and protein levels in very obese patients after long-term caloric restriction. However, the diet resulted in a substantial decrease in body weight and BMI in their patients (20, 21) and therefore, the observed decrease in leptin mRNA level might be associated with the loss in adipose tissue mass. In contrast, short-term caloric restriction with a tiny decrease in body weight and BMI as observed in our study did not affect leptin mRNA levels. Taken together, these observations are in line with the results of Hamilton et al. (19), who demonstrated that leptin expression is positively correlated with adipocyte hypertrophy and hyperplasia.

In a second experiment, plasma insulin concentration was maintained at supraphysiological levels during 3 h in a group of control subjects to investigate the acute effect of insulin on *ob* gene expression. Leptin mRNA levels in the adipose tissue did not change during the hyperinsulinemic clamp. The lack of variations of leptin mRNA during insulin infusion contrasted with the observation of significant increase in glucose transporter (Glut 4) mRNA levels in the same tissue biopsies. Increased mRNA levels of Glut 4 during euglycemic hyperinsulinemic clamp has already been reported in normal human skeletal muscle (30, 33). However, the regulation of Glut 4 expression by insulin is incompletely understood. Diabetes and fasting decrease Glut 4 mRNA level, whereas feeding restores it in animal models, suggesting a positive role of insulin in the control of Glut 4 gene expression (34). On the other hand, chronic insulin administration reduces the transcription of Glut 4 gene in 3T3-L1 adipocytes (34). In the present study we demonstrate that in human adipose tissue, like in skeletal muscle (30, 33), insulin infusion at supraphysiological concentration increases Glut 4 mRNA levels suggesting that insulin exerts a positive control on Glut 4 gene expression in human tissues. The insulin-induced increase in Glut 4 mRNA levels reinforces the observation of an absence of effect on leptin mRNA and strongly suggests that *ob* gene expression is not acutely regulated by insulin *in vivo* in humans.

The lack of regulation of *ob* gene expression by caloric restriction and by insulin is in marked contrast to the observed changes in leptin expression induced by similar protocols in rodents (12–16). In these latter models, it is clear that leptin is a satiety factor that controls food intake and energy expenditure (6–10). It is suggested that these effects are mediated via interaction of leptin with its specific receptor in the hypothalamus (11) resulting in changes in the levels of neuropeptide Y (35), a major inducer of appetite. Our results suggest that care should be taken to extrapolate this model, based on studies in rodents, to the *in vivo* situation in man.

In summary, our data show that a precise quantification of leptin mRNA level can be performed in small biopsies of adipose tissue in human. Leptin mRNA levels are correlated with

the body mass index and are eight times higher in obese than in lean subjects. However, *ob* gene expression is not affected by insulin infusion nor by 5 d of caloric restriction, suggesting that leptin is not a tightly controlled satiety factor but rather a consequence or a concomitant of the size of the fat deposit in human.

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