Effects of long-term total fasting and insulin on ob gene expression in obese patients

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Abstract

In the present study the effect of long-term fasting (6 days) on obese (ob) gene expression was examined in nine severely obese females of 34±3 years and with a body mass index of 46.4±2.3 kg/m². Six days of fasting induced a significant weight loss (126.8±5.3 vs 120.5±5.1 kg, P<0.0001). Insulin-stimulated glucose uptake (hyperinsulinemic, euglycemic clamp, insulin infusion rate 1.5 mU/kg per min) was markedly reduced following fasting (M-value 5.96±0.74 vs 2.79±0.23 mg/kg per min, P<0.0001). ObmRNA/β-actin concentration in fat biopsies from abdominal subcutaneous adipose tissue was unchanged after 6 days of fasting (1.50±0.40 vs 1.47±0.36 arbitrary units, not significant), whereas serum leptin levels decreased significantly from 53.8±4.7 to 30.7±2.0 ng/ml (P<0.0001) during the same period. No significant correlations were found between insulin-stimulated glucose uptake and serum leptin concentration, either prior to the fast or after the fast. Serum leptin levels were unchanged by hyperinsulinemia for 3 h during the clamp prior to the fast, while hyperinsulinemia for 3 h after 6 days of fasting increased serum leptin by 25% (P<0.01). In conclusion, 6 days of fasting reduced serum leptin by about 40%. In contrast, obmRNA in abdominal subcutaneous adipose tissue was unchanged. Furthermore, after 6 days of fasting insulin was able to increase the serum level of leptin significantly, indicating that the effect of insulin on the level of leptin is dependent on the nutritional state.

European Journal of Endocrinology 137 229–233

Introduction

The adipose tissue-specific ob gene encoding the protein leptin was recently cloned in mice and humans (1). Leptin regulates food intake and energy expenditure in rodents. In ob/ob mice lack of leptin leads to obesity while administration of leptin to these obese mice leads to weight loss and increased energy expenditure (2–5). In other animal models of obesity (6, 7) and in obese humans (6, 8) leptin levels are increased, possibly indicating resistance to the central effects of leptin. A recent study suggests that a defect in the transport of leptin into cerebrospinal fluid could be involved in leptin resistance (9).

In humans, circulating leptin concentrations increase with body mass index (BMI) and body fat (6, 10). Recently, Klein et al. (11) showed that the rate of leptin production is directly related to adiposity (percent body fat). Further, it was demonstrated that the rate of leptin clearance from plasma seemed to be unrelated to adiposity. It has been established that weight loss in humans can reduce plasma leptin concentrations and obmRNA levels in adipose tissue (6, 10). In order to further explore the regulation of ob gene expression in human obesity we have examined the effect of long-term fasting (6 days) on serum leptin and obmRNA levels in adipose tissue in obese patients. In rodents, it has been demonstrated that insulin has a direct positive effect on obmRNA levels in adipose tissue (12, 13). Accordingly, we have examined the effect of insulin on serum leptin levels during a 3-h hyperinsulinemic, euglycemic clamp, before and after fasting.

Subjects and methods

Subjects

Nine obese women with BMI of 46.4±2.3 kg/m² and 34±2.9 years of age were included in the study. All the subjects were non-diabetic with fasting blood glucose levels of 4.6±0.3 mmol/l prior to inclusion in the study. The study protocol was approved by the local ethical committee and informed consent was obtained from all subjects in accordance with the Helsinki Declaration II.
Study protocol

Patients were hospitalized during the study period. During the fast, lasting 6 days, the patients were supplemented daily with a multivitamin tablet once a day. KCl tablets (750 mg) three times a day and magnesium tablets (500 mg) three times a day. Patients had free access to non-calorie-containing fluids. In all patients there was a significant fall in plasma carbonate and CO₂ during the fast and the patients started to excrete ketone bodies in the urine. Before and after the 6 days of fasting a hyperinsulinemic, euglycemic clamp was performed and a subcutaneous fat biopsy was obtained from the abdominal wall. Serum leptin was determined before and during the fast.

Hyperinsulinemic, euglycemic clamp

The experiments started at 0800 h after an overnight fast (before the fast). The protocol comprised a basal period (time, 0–30 min) which was followed by 3 h of hyperinsulinemia (time, 30–210 min). Soluble insulin (Actrapid; Novo-Nordisk, Copenhagen, Denmark) was infused at a rate of 1.5 μU/kg per min, while plasma glucose was maintained at about 5 mmol/l by infusion of glucose (20% w/v) depending on the plasma glucose concentration. Arterialized venous blood samples were obtained every 5 or 10 min and plasma glucose concentration was determined with a glucose oxidase method (using a glucose analyzer; Beckman Instruments, Palo Alto, CA, USA).

Adipose tissue biopsy and isolation of adipocytes

Subcutaneous adipose tissue was obtained from the abdominal region by liposuction. Briefly, the skin was anesthetized using 10 ml lidocaine (10 mg/ml). Afterwards, a small incision (0.3 cm) was made and 20 ml isotonic NaCl was injected into the adipose tissue. The liposuction cannula was inserted through the incision and vacuum was applied. Adipose tissue fragments were placed in Hanks’ balanced salt solution, supplemented with 1% BSA. A total of 10–25 g adipose tissue was removed and isolation of adipocytes was initiated within 10 min of removal. Adipocytes were isolated by collagenase digestion as previously described (14).

RNA isolation

Total RNA was extracted from isolated adipocytes using an acid–phenol single-step method (15).

Ob mRNA quantitation

The reverse transcriptase PCR assay was used for detection of ob mRNA.

The ob-primers used in the PCR spanned a PCR product of 373 bp. The sense ob-primer (5’-GATGAGCACCAAAACCTCAGTCATC) corresponds to ob cDNA 417 to 437 and the antisense ob-primer (5’-GCCACCCACCTCGTGAGTAG) corresponds to the anti-sense strand of the ob cDNA sequence 85 to 105. In addition, β-actin mRNA was amplified as a housekeeper marker in parallel tubes using β-actin primers. The reverse transcriptase and amplification were performed using the GeneAmp RNA PCR kit from Perkin Elmer Cetus (Norwalk, CT, USA). Reverse transcriptase was performed using random hexamer primers at 23 °C for 10 min, 42 °C for 60 min and terminated by increasing the temperature to 95 °C for 10 min. The cDNA product was amplified by the addition of Taq DNA polymerase supplied with the kit. Each cycle of amplification consisted of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min (β-actin: 60 °C for 1 min) and 2 min of extension (74 °C). During the final cycle the product was extended for 6 min (74 °C). Initial studies using ob-primers demonstrated that between 34 and 40 cycles the reaction was linear, therefore we chose 36 cycles when evaluating the ob mRNA. Using β-actin primers the reaction was linear between 18 and 26 cycles; therefore, 22 cycles were chosen for the β-actin.

Finally, 5 μl of the PCR product was loaded on a 2% agarose gel, stained with ethidium bromide, and photographed. The density of the bands were determined by scanning the photograph in a Shimadzu flying spot laser scanner. Each ob mRNA value was normalized by the corresponding β-actin value.

A similar protocol was used for negative controls except that the reverse transcriptase was omitted.

Serum leptin

Serum leptin was quantitated using a human leptin RIA kit (Linco Research Inc.).

Statistical analysis

Results are expressed as means±S.E.M. Statistical analyses were performed with the Statistical Package for the Social Sciences package. Comparison of data were made using Student’s paired t-test. Differences were accepted as significant at the P<0.05 level.

Results

Clinical characteristics

During the 6-day fast total bodyweight was reduced by 6.3±0.2 kg (P<0.0001). During the same period insulin resistance was augmented with a significant 50% reduction in M-value (insulin-stimulated glucose uptake rate) (Table 1). Serum insulin levels were reduced, whereas serum free fatty acid (FFA) levels were doubled (Table 1) by the end of the fasting period.
Serum insulin levels during the steady-state periods of the hyperinsulinemic, euglycemic clamp were comparable before and after fasting (212.0 ± 22.5 and 172.9 ± 13.1 μU/ml respectively).

Serum leptin

**Effect of fasting (Fig. 1)** Mean serum leptin concentrations were 53.8 ± 4.7 ng/ml prior to the fast. In comparison, Considine et al. (10) found that the mean serum leptin concentration in normal weight subjects was 7.5 ± 0.8 ng/ml (n = 136). Serum leptin levels fell gradually within the first 3 days of fasting, whereafter it reached a plateau about 40% below the prefasting level (Fig. 1). At the end of the fast, serum leptin concentrations were reduced significantly to 30.7 ± 2.0 ng/ml (P < 0.0001).

**Table 1** Clinical characteristics of the study participants before and after 6 days of fasting. Values are means ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>After fasting</th>
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<tbody>
<tr>
<td>Body weight (kg)</td>
<td>126.8 ± 5.3</td>
<td>120.5 ± 5.1*</td>
</tr>
<tr>
<td>M-value (mg/kg per min)</td>
<td>5.96 ± 0.74</td>
<td>2.79 ± 0.23*</td>
</tr>
<tr>
<td>Serum FFA (μmol/l)</td>
<td>0.71 ± 0.07</td>
<td>1.53 ± 0.10*</td>
</tr>
<tr>
<td>Serum insulin (μU/ml)</td>
<td>25.4 ± 4.1</td>
<td>15.1 ± 3.9*</td>
</tr>
<tr>
<td>Serum carbonate plus CO₂ (mmol/l)</td>
<td>27.8 ± 0.6</td>
<td>19.8 ± 1.2*</td>
</tr>
</tbody>
</table>

Normal range for serum carbonate plus CO₂ is 22.0–30.0 mmol/l. *P < 0.05 or less.

Serum insulin levels during the steady-state periods of the hyperinsulinemic, euglycemic clamp were comparable before and after fasting (212.0 ± 22.5 and 172.9 ± 13.1 μU/ml respectively).

No significant correlation was found between serum leptin and BMI and no significant correlations were found between insulin-stimulated glucose uptake (M-value) and serum leptin concentration, either prior to the fast or after the fast in these very obese women. Further, no significant correlations between serum insulin levels and serum leptin were found either before or after the fast. Finally, no correlations were found between changes in serum insulin, FFA and M-value in relation to changes in serum leptin.

**Effect of insulin (Fig. 2)** Prior to the fast, hyperinsulinemia for 3 h had no effect on serum leptin levels (52.1 ± 6.4 vs 54.9 ± 6.1 ng/ml, not significant). After 6 days of fasting, hyperinsulinemia for 3 h increased serum leptin in all patients (26.5 ± 2.1 vs 33.3 ± 2.9 ng/ml, P < 0.01).

**Ob mRNA (Fig. 3)**

Ob mRNA/β-actin levels in abdominal subcutaneous adipose tissue were unchanged when the levels measured before the fast were compared with the levels obtained after 6 days of fasting (1.50 ± 0.40 vs 1.47 ± 0.36 arbitrary units, not significant).

Figure 1 Serum leptin concentrations in obese women (n = 9) during 6 days of fasting (hatched box) expressed as percent of basal value (pre-fast value). Data are expressed as means ± S.E.M. *P < 0.05 or less.

Figure 2 Serum leptin concentrations in the study participants before and after 3 h of hyperinsulinemia. (A) Results before fasting and (B) after 6 days of fasting. *P < 0.01.
Discussion

It has recently been shown that the rate of production of leptin is correlated positively with percent body fat, whilst rate of clearance and plasma leptin half-life seems to be unrelated to adiposity (11). In the present study serum leptin concentration was reduced by approximately 40% after 6 days of fasting. Total body weight was reduced about 5% in the same period. A great part of the weight loss during the first week of a fast is due to loss in total body water (16). Consequently, only a fraction of the loss is due to reduction of fat mass. Therefore, factors other than adiposity and total fat mass seem to be of importance for leptin production turnover. Presumably the circulating level of leptin is also reflecting the nutritional state. Leptin transcription rate and \(ob\) mRNA stability seem to be unchanged by long-term fasting, since we were not able to demonstrate any changes in \(ob\) mRNA levels in fat tissue after 6 days of fasting. The decrease in leptin levels after long-term fasting could therefore be due to post-transcriptional events such as decreased translation, increased clearance or decreased half-life. However, we cannot exclude the possibility that changes in \(ob\) mRNA may have occurred initially during the 6-day fast. Thus, time-course studies will have to be performed in order to characterize the regulation of \(ob\) gene expression during fasting in greater detail.

Weight loss has previously been shown to be associated with reduced levels of plasma leptin in humans (6, 10). In both studies, changes in \(ob\) mRNA were also investigated and it was demonstrated that weight loss was associated with a fall in \(ob\) mRNA levels. However, in the study by Considine and coworkers (10) \(ob\) mRNA levels returned to preweight loss levels after 4 weeks of weight stability. In the present study we were not able to demonstrate any changes in \(ob\) mRNA levels after 6 days of fasting with a significant weight reduction. However, it is difficult to compare our data with those from the studies mentioned above because of different designs. In our study the patients underwent a 6-day fast and they were catabolic, while in the other studies weight loss was induced with a diet over several months and at the time of the second biopsy patients had been weight-stable for several weeks.

In accordance with our data, Vidal et al. (17) did not find any change in \(ob\) mRNA in adipose tissue from patients maintained on a hypocaloric diet for 5 days leading to weight reduction and a rise in plasma \(\beta\)-hydroxybutyrate.

In a recent study it was demonstrated that insulin resistance is associated with elevated plasma leptin levels (18). In the present study 6 days of fasting induced insulin resistance and a fall in serum leptin concentration. However, we were not able to demonstrate any association between serum leptin levels and insulin sensitivity (M-value) either in the basal state or after 6 days of fasting. On the basis of our data it may be suggested that insulin sensitivity per se does not seem to influence leptin production in severely obese patients associated with extreme insulin resistance.

Recently it was shown that serum insulin levels correlate positively with plasma leptin levels and that insulin infusion for 8 h in supraphysiological concentrations increases plasma leptin (19). In the present study we could not demonstrate any significant correlations between serum insulin levels and serum leptin. Neither could we find any correlation between the changes in serum insulin and serum leptin. However, in the correlation studies the small number of patients and the fact that the patients are extremely obese should be taken into account. Furthermore, prolonged fasting is a ‘non-physiological’ condition inducing many changes in substrate and hormone concentrations, making interpretation of the effect of fasting per se on leptin regulation difficult.

In other studies a positive correlation has been demonstrated between serum leptin levels and BMI. The lack of correlation between serum leptin and BMI in the present study is probably due to the high BMI of all the patients. At very high BMI levels the usual positive correlations between BMI and serum leptin more or less disappear (authors’ unpublished observations).

The lack of effect of hyperinsulinemia for 3 h on serum leptin levels prior to the fast is in accordance with previous studies (8, 17, 20). In these studies insulin did not affect serum leptin levels in subjects that were lean, obese or with non-insulin-dependent diabetes mellitus (NIDDM). However, in a study by Malmström et al. (19), hyperinsulinemia for 6–8.5 h increased serum leptin by 38% in lean healthy subjects and lean patients with NIDDM. In the present study we were able to
demonstrate an increase in serum leptin after 3 h of hyperinsulinemia, when the subjects had experienced 6 days of total fasting. These data are highly interesting, especially when we take into account the fact that 6 days of total fasting induces severe insulin resistance in muscle and adipose tissue. For the moment we do not have any good explanation for this paradox of hyper-responsiveness of leptin production to insulin, when the patients in general are severely insulin resistant. However, since ob mRNA was unchanged after fasting, the effect of insulin on ob mRNA translation and/or ob mRNA transcription may be increased by fasting.

It is concluded that, during 6 days of fasting, serum leptin concentration is gradually reduced to a level about 40% below the prefast level within the first 3 days of the fast, whereas it is stable for the rest of the fasting period. Reduced fat mass alone cannot explain the decreased serum leptin levels. The current experimental design does not allow us to define one factor that is responsible for the changes in serum leptin. However, in contrast to serum leptin levels, ob mRNA levels in abdominal subcutaneous adipose tissue are found to be unchanged after 6 days of fasting. The decrease in leptin levels after long-term fasting could be due to post-transcriptional events such as decreased translation, increased clearance or decreased half-life. Furthermore, it is suggested that insulin sensitivity per se does not seem to influence leptin production in these very obese women. Finally, 6 days of fasting seems to disclose an insulin-stimulating effect on the circulating level of leptin.

Acknowledgements
We thank Lene Trudso, Anette Mengel and Dorte Philip for excellent technical assistance. The study was supported by the Danish Diabetes Association and the Danish Medical Research Council.

References