Leptin Secretion from Adipose Tissue in Women

Relationship to Plasma Levels and Gene Expression

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Abstract

The role of expression and secretion of the ob gene product, leptin, for the regulation of plasma leptin levels has been investigated in vitro using abdominal subcutaneous adipose tissue of 20 obese, otherwise healthy, and 11 nonobese women. Body mass index (BMI, mean±SEM; kg/m^2) in the two groups was 41±2 and 23±1, respectively. Fat cell volume was 815±55 pl in the obese and 320±46 pl in the nonobese group. In the obese group, plasma leptin concentrations and adipose leptin mRNA (relative to gamma actin) were increased five and two times, respectively. Moreover, adipose tissue secretion rates per gram lipid weight or per fat cell number were also increased two and seven times, respectively, in the obese group. There were strong linear correlations ($r = 0.6$–0.8) between plasma leptin, leptin secretion, and leptin mRNA. All of these leptin measurements correlated strongly with BMI and fat cell volume ($r = 0.7$–0.9). About 60% of the variation in plasma leptin could be attributed to variations in leptin secretion rate, BMI, or fat cell volume. We conclude that elevated circulating levels of leptin in obese women above all result from accelerated secretion rates of the peptide from adipose tissue because of increased ob gene expression. However, leptin mRNA, leptin secretion, and circulating leptin levels are all more closely related to the stored amount of lipids in the fat cells of adipose tissue than they are to an arbitrary division into obese versus nonobese. (*J. Clin. Invest.* 1997. 99:2398–2404.) Key words: ob gene • obesity • fat cells • body weight • messenger ribonucleic acid

Introduction

Leptin, a product of the ob gene (1), is a peptide hormone that is produced by adipocytes and binds to specific receptors predominantly found in the brain (2, 3). The role of leptin in man is unclear so far (4). However, results from laboratory animals show that leptin may regulate body weight homeostasis, and leptin administration has major effects on body fat content in mice, as discussed in detail (5, 6).

Adipocyte mRNA levels and circulating concentrations of leptin are increased in obese subjects (7–12), although there is considerable interindividual variability in leptin expression and plasma concentrations at each body mass index level (7–13). It is not known how the circulating leptin level is regulated in man. The secretion rate from adipose tissue may be an important factor which, in turn, may be controlled by ob gene expression. Previous results have suggested that leptin secretion from cultured human fat cells may be regulated by hormones (14, 15). However, almost no data are available on leptin secretion by adipose tissue in obesity.

To investigate the mechanisms underlying increased circulating leptin concentrations in obesity, we measured the secretion rates in vitro and the mRNA levels of leptin in subcutaneous adipose tissue of obese and nonobese women and compared the results with the fasting plasma leptin concentrations.

Methods

Patients. 20 obese women, body mass index (BMI)[1] > 30 kg/m^2, who were to undergo weight-reducing surgery were included. A random control group ($n = 11$), consisting of subjects who never had been obese (BMI < 27 kg/m^2) but were undergoing elective laparoscopic cholecystectomy at Huddinge University Hospital, was also included. The two groups were matched for age and smoking habits. All subjects were Caucasians born in Sweden and, except for obesity and/or gallstone disease, they had no apparent diseases and were not on any medication. The age of the subjects ranged from 18 to 57 yr, and all but one woman in the control group were premenopausal. The study was approved by the Ethics Committee of the Karolinska Institute. All patients gave informed consent to participate in the study.

The waist-to-hip ratio and BMI were measured on the day before surgery and an overnight fast. After a rest in bed for 15 min, blood pressure was measured and venous blood samples were taken for determination of metabolic and hormone parameters. They were all analyzed by the hospital's chemistry laboratory, except insulin, which was measured with a radioimmunoassay kit (Pharmacia Fine Chemicals, Uppsala, Sweden). One aliquot (0.5 ml) of plasma was stored at −70°C for subsequent analysis of leptin.

Adipose tissue biopsies. General anesthesia was induced at 0800 h by a short-acting barbiturate and maintained by fentanyl and a mixture of oxygen and nitrous oxide. The patients had fasted from 2200 h the previous day. Intravenous saline was administered before taking the fat biopsies from the abdominal subcutaneous adipose tissue at the beginning of surgery. The adipose tissue specimens, weighing between 0.5 and 2 g, were divided into two parts. One part was immediately used for secretion studies and, if enough tissue was available, also for determination of fat cell size. The other part was used for mRNA analysis. It was immediately frozen unfixed on dry ice at the operation room and stored at −70°C until sectioned. In the event that there was not enough tissue to measure both secretion and mRNA, we gave priority to secretion measurements.

In situ hybridization. This assay was performed on 14 obese and 6 nonobese subjects. As previously described in detail (7), two *ob*

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1. *Abbreviation used in this paper:* BMI, body mass index.

gene oligonucleotides complementary to nucleotides 122–169 (*ob* 1) and nucleotides 259–300 (*ob* 2) of the human *ob* sequence, and a gamma actin oligonucleotide probe complementary to nucleotides 325–372 were synthesized. All oligonucleotides were synthesized and purified by Pharmacia Fine Chemicals. They were subsequently labeled with a ^{35}S-labeled isotope at the 3′ end, using terminal deoxynucleotidyl transferase to a specific activity of 2×10^9 cpm/µg and purified in a Nensorb 20 column.

The analyses of adipose tissues were done at the same time with respect to *ob* and gamma-actin expression in subcutaneous fat. Tissues from eight subjects were analyzed simultaneously in the same detection batch (glasses, hybridization, washes, quantification, etc., as described below) to reduce the influence of between-run variations in results. We included obese and nonobese subjects in the same batch. The adipose tissue was rapidly frozen and then cut into 20-µm-thick sections in a cryostat and thawed on an electrically charged Fischer probe on + slides (Fischer Scientific Co., Springfield, NJ). The sections were incubated at 42°C for 15–18 h with a 10^6 cpm–labeled probe per 100 µl of a solution containing 50% formamide, 4× SSC, 1× Denhardt's solution, 1% sarcosyl, 0.02 M sodium phosphate (NaPO$_4$, pH 7.0) and 10% dextran sulfate mixed with 500 µg/ml sonicated salmon sperm DNA and 200 mM dithiothreitol. SSC is a solution of sodium chloride and sodium citrate. Denhardt's solution is made from Ficoll (10 g), bovine serum albumin (10 g), and polyvinylpyrrolidone (10 g) in 500 ml distilled water. The sections were rinsed in 1× SSC at 55°C for 1 h with five changes of buffer. They were then dried and exposed to Hyperfilm β-max x-ray film (Amersham International, Little Chalfont, UK) for 14 d before development and fixation. Some of the sections were subsequently dipped in NTB 2 nuclear track emulsion (Eastman Kodak Co., Rochester, NY), followed by a 5-wk exposure, developed in D19 for 3 min, fixed in 3000A (Eastman Kodak Co.) and analyzed in a Zeiss Axiophot darkfield microscope (Carl Zeiss, Inc., Thornwood, NY). Pictures of these emulsion radiographs were taken using T-max 100 film (Eastman Kodak Co.).

Quantification of in situ hybridization autoradiographs. Autoradiographic ^{14}C microscale strips (Amersham International) were used as standards by coexposure with the tissues sections. Sections of adipose tissue from the eight subjects were mounted on the same glass slides used for leptin or gamma actin. The slides were processed together in triplicate for leptin or gamma actin and were handled as a single batch through hybridization, washing, autoradiographic exposure and computerized image analysis. The signals were measured from high resolution x-ray films, where sections had been coexposed with radioactive standards to linearize the film response to radiation. This quantification was performed exactly as described by Lönnqvist et al. (7). Labeling of leptin mRNA and gamma actin mRNA, expressed in nCi/g, was calculated on each tissue section using computerized image analysis of the high resolution x-ray films. Using this method, we have shown that the expression of the control gene, gamma actin, is the same in abdominal subcutaneous adipose tissue of obese and nonobese women (7). Therefore, to exclude possible variations in mRNA yield between the samples, *ob* gene expression was presented as leptin mRNA divided by gamma actin mRNA. Typical autoradiographs depicting leptin mRNA in adipose tissue of one obese and one nonobese woman are presented in Fig. 1.

Determination of fat cell size and number. Tissue specimens, weighing ∼ 0.3 g, from 17 obese and 6 nonobese subjects were used for this purpose. Isolated fat cells were prepared by collagenase treatment according to the method of Rodbell (16). This method has been described in detail and evaluated previously (17). The cells were kept in an albumin solution, composed as described below, and the cell density of the fat cell suspension was kept constant by slow stirring. Direct microscopic determination of the fat cell diameter was calculated in 100 cells from each subject. The mean fat cell volume and weight were determined, taking into account the skewed distribution of the cell diameter. The total lipid content in each incubation was determined as described below. Assuming that lipids constitute > 95% of
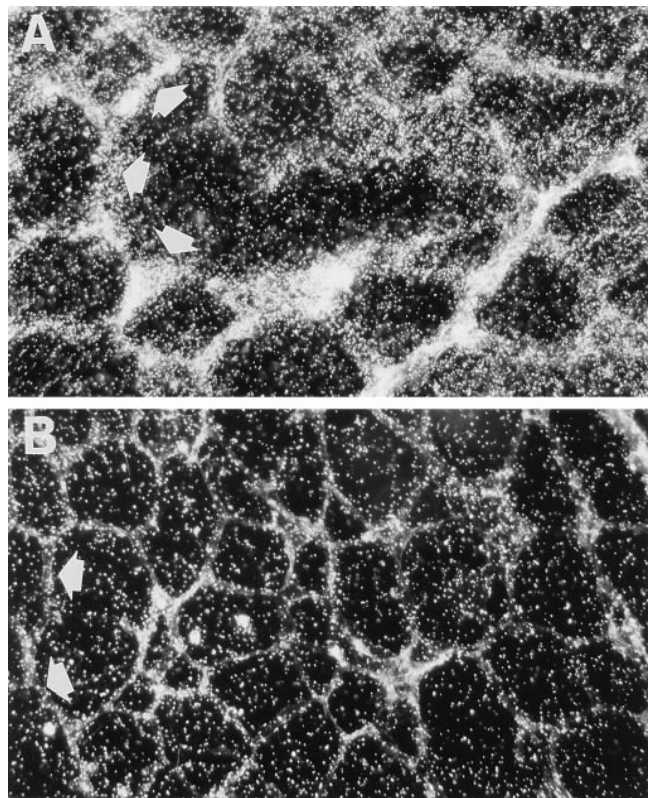

Figure 1. Dark-field emulsion autoradiograph of leptin mRNA in adipose tissue of an obese (*A*) and a nonobese (*B*) woman. The accumulation of silver grains (representing leptin mRNA) is indicated with arrows and is overlaying fat cells but not connective tissue and vascular structure. The grain accumulation is higher in the obese than in the nonobese subject.

the fat cell weight, the number of fat cells was then calculated by dividing the total lipid weight by the mean cell weight.

Leptin secretion. Leptin secretion was measured in all subjects. The adipose tissue specimens were cut into small pieces (∼ 10 mg). All visible vessels and coagulation particles were removed. The remaining tissue was rinsed in 37°C saline, and then incubated (300 mg tissue in 3 ml medium) in a Krebs-Ringer phosphate buffer (pH 7.4) supplemented with 40 g/liter of defatted bovine serum albumin and 1 g/liter of glucose. The incubation was carried out for 2 h at 37°C in a shaking water bath, with air as the gas phase. After incubation, 2 ml of the medium was removed, frozen in liquid nitrogen, and stored at −70°C. The tissue was immediately homogenized and total lipid was extracted as described (18). Leptin secretion was related to the lipid weight as well as to the number of fat cells in the incubated tissue. At the time of analysis, all the frozen medium samples were freeze-dried simultaneously and the material was redissolved in 150 µl of distilled water. One aliquot (100 µl) was used for leptin determination. We had to concentrate the leptin in the medium in this way to obtain detectable values. In methodological experiments, recombinant human leptin (Linco Research, Inc., St. Charles, MO) was added to the incubation medium that was not exposed to fat tissue. More than 95% of the recombinant leptin was recovered. In other methodological experiments, tissue pieces were incubated for 1, 2, or 3 h. The leptin release was linear with time for at least 3 h, using tissue from obese or nonobese women. In methodological experiments, isolated fat cells were prepared in parallel, as described above, in order to study leptin secretion. Packed cells (0.3 ml) were incubated in 3 ml medium, ex-

actly as described above. Leptin release from isolated fat cells and tissue fragments correlated strongly with each other ($r = 0.87$, $n = 6$).

Leptin assay. The leptin levels in plasma and medium were determined with a commercially available human leptin RIA kit (Linco Research, Inc.) using 100 µl of the sample. The assay is completely homologous, since the antibody was raised against highly purified human leptin and both the standard and tracer were prepared with human leptin. The plasma samples were run in duplicate. All samples were measured simultaneously and fell within the detection range of the kit; i.e., 1–100 ng leptin/ml. The coefficient of variance in the plasma sample was 6%. The material in this study was collected and analyzed within 9 mo. There was no evidence of leptin degradation in plasma or incubation medium during this period.

Drugs and chemicals. Formamide came from Baker Chemicals B.W. (Deventer, Holland). Salmon sperm DNA, sodium cacodylate, Tris base, sodium chloride, sodium citrate, defatted bovine serum albumin (fraction V), Ficoll, polyvinylpyrrolidone, sarcosyl (*N*-lauryl-sarcosine), fluorescein isothiocyanate, and clostridium difficile collagenase type 1 were from Sigma Chemical Co. (St. Louis, MO). Sodium phosphate, dextran sulphate, and dithriothreitol were from Pharmacia Fine Chemicals. Terminal deoxynucleotidyl transferase was from Amersham Corp. (Arlington Heights, IL). ^{35}S-labeled dATP NEG034H and Nensorb columns were from DuPont-NEN (Boston, MA).

Statistical analysis. Values are mean±SEM. They were compared using the Student's unpaired *t* test or linear regression analysis by the method of least squares.

Results

The clinical data are shown in Table I. The obese women showed several signs of overweight complications. Thus, plasma insulin, plasma glucose, and diastolic blood pressure were increased in obesity. In addition, fat cell volume was 2.5 times larger than in the nonobese group.

The data for leptin in the two groups are depicted in Fig. 2. Plasma levels were elevated almost fivefold and adipose tissue secretion rates were two to seven times more rapid in the obese than in the nonobese group, when expressed per lipid weight or per cell number. Leptin mRNA, relative to gamma actin mRNA, was elevated almost twofold in the obese group, which confirms data in other groups of nonobese and obese women with whom we previously investigated an identical technique (7).

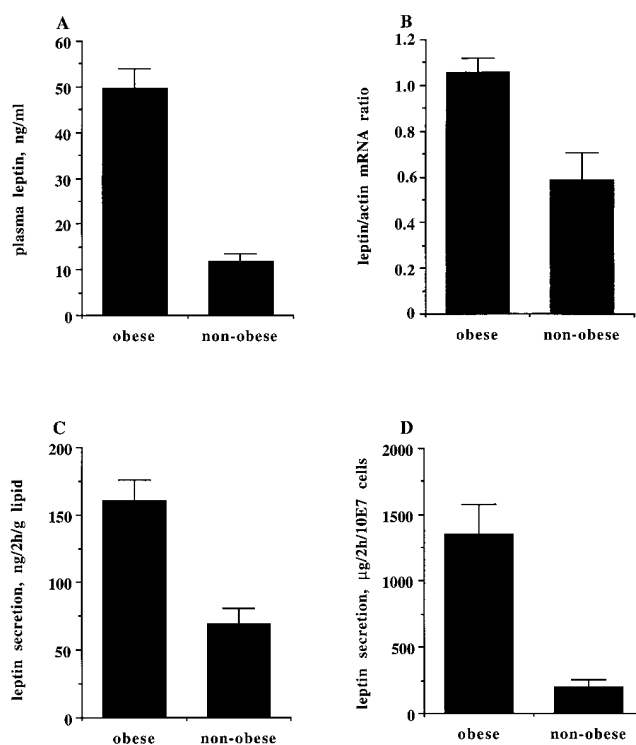The relationship between leptin mRNA, leptin secretion

Figure 2. Findings concerning leptin in obese and nonobese women. (*A*) Plasma levels ($n = 20$ obese and 11 nonobese subjects). (*B*) Adipose tissue mRNA expression of leptin in relation to gamma actin (14 obese and 6 nonobese subjects). (*C* and *D*) Secretion rates of leptin from adipose tissue per gram lipid (20 obese and 11 nonobese) and per 10^7 cells (17 obese and 6 nonobese subjects). Values are mean±SEM. They were compared using the Student's unpaired *t* test.

rate, and circulating levels of leptin was further evaluated using linear regression analysis (Table II, Figs. 3 and 4). There was a strong positive and significant correlation between all leptin parameters ($r = 0.6$ or more; $P < 0.001$). About 60% (i.e., adjusted r^2) of the variation in plasma leptin could be attributed to variations in the secretion rate (expressed per lipid weight or per cell number).

Other linear regression analyses were also performed (Table III, Figs. 5 and 6). BMI correlated positively with all leptin

Table I. Data in 20 Obese and 11 Nonobese Women

Determination	Obese	Nonobese	*P*
Age, yr	391±3	41±4	NS
BMI, kg/m^2	41.9±1.8	22.9±0.7	NS
Waist-to-hip ratio	0.93±0.01	0.88±0.02	NS
p-Glucose,* mmol/liter	5.8±0.2	4.9±0.1	< 0.001
p-Insulin, ml	19.8±2.5	6.9±0.6	< 0.01
p-Triglycerides, mmol/liter	1.8±0.2	1.2±0.1	NS
p-Cholesterol, mmol/liter	5.2±0.2	5.3±0.4	NS
p-HDL cholesterol, mmol/liter	1.3±0.1	1.4±0.1	NS
Systolic blood pressure, mmHg	136±5	124±6	NS
Diastolic blood pressure, mmHg	81±2	71±2	< 0.01
Fat cell volume, pl	815±55	320±46	< 0.001

Values are mean±SEM. They were compared using the Student's unpaired *t* test. p, plasma.

Table II. Correlation Matrix for Interrelationships between Leptin Parameters

	Y-variable		
X-variable	Leptin/ actin mRNA	Leptin secretion/ lipid weight	Leptin secretion/ cell number
Plasma leptin	0.68	0.77	0.74
Leptin/actin mRNA	—	0.66	0.60
Leptin secretion/ lipid weight	—	—	0.97

The relationship between each parameter was through evaluation in the whole material (obese and nonobese subjects together) by linear regression analysis. Correlation coefficients are given ($P < 0.001$).
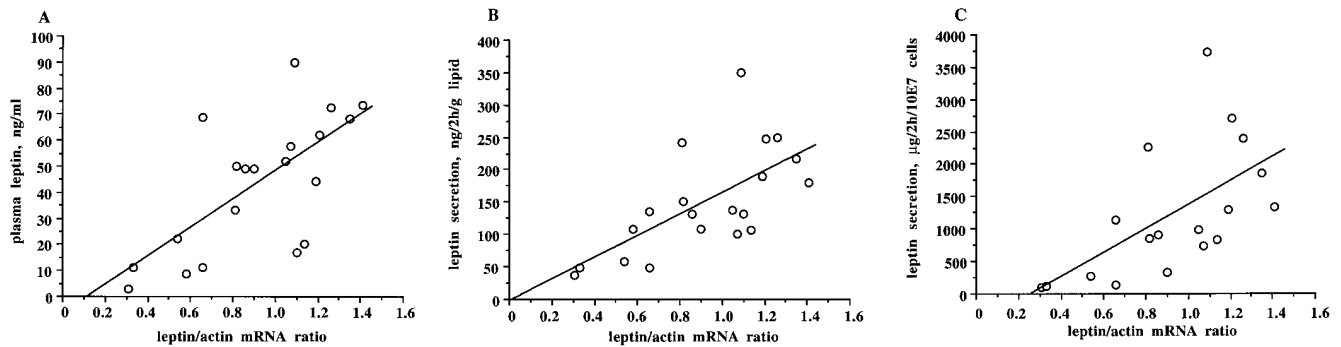
Figure 3. Relationship between leptin mRNA (in relation to gamma actin) and other leptin parameters. (*A*) Correlation with plasma leptin. (*B*) Correlation with leptin secretion by adipose tissue per gram lipid. (*C*) Correlation with adipose tissue leptin secretion per cell number. The values for the whole study group were compared using linear regression analysis. Correlation coefficients are given in Table II.

parameters ($r = 0.7$–0.8, $P < 0.001$). Of the variation in the leptin parameters, 50–70% could be attributed to variations in BMI. The fat cell volume also correlated positively with all leptin parameters ($r = 0.7$–0.9, $P < 0.001$). Fat cell volume contributed 50–80% to the variation in leptin parameters. Plasma insulin correlated weakly with the plasma leptin and leptin mRNA ($r = 0.4$) and strongly with the leptin secretion rate ($r = 0.6$–0.7, $P < 0.01$). Plasma insulin correlated positively with fat cell volume ($r = 0.70$, $P < 0.001$). Likewise, plasma insulin and BMI showed a positive correlation with each other ($r = 0.58$, $P < 0.01$). We did not assess the effects of BMI, fat cell volume, or insulin on the relationships between the various leptin parameters (using multiple regression analysis) as none of these three parameters was randomly distributed in the present material. Instead, they represented two groups of selected values due to the nature of the study groups (obese, nonobese).

Discussion

In this study, we investigated the effect of leptin secretion on the circulating levels of peptide. The fasting plasma leptin level was markedly elevated in the obese women and there was a strong positive correlation ($r = 0.8$) between the circulating leptin level and BMI in the entire material. This confirms the data from a number of previous studies (7–13). In two recent

studies, a close relationship was found between fat mass and circulating leptin in women (19, 20). This agrees with the present results. BMI accounted for 60% (i.e., adjusted r^2) of the variation in plasma leptin in the whole material.

The adipose secretion rate of leptin was twice as rapid when expressed per gram lipid and as much as seven times higher when expressed per fat cell number in obese than in nonobese women. Compared with lean women, the obese women had much larger adipocytes and therefore fewer cells per gram lipid. Accordingly, it is to be expected that leptin secretion becomes very high in large cells when secretion rates are expressed per cell.

There was a strong correlation between secreted and circulating leptin. The secretion rate of leptin from adipose tissue accounted for ~ 60% of the variations in plasma leptin, no matter whether secretion data were expressed per gram lipid or per cell number. These data strongly suggest that an increased secretion rate of leptin from adipose tissue caused the elevated plasma leptin levels in obese women. Furthermore, BMI and fat cell volume correlated strongly with the leptin secretion rate per gram lipid or per cell number. BMI accounted for 50–60% of the variations in the leptin secretion rate. Fat cell volume accounted for as much as 70–80% of the variations in the leptin secretion rate, either expressed per lipid weight or per cell number. It is therefore likely that the leptin secretion rates are much more closely related to fat mass than to any ar-
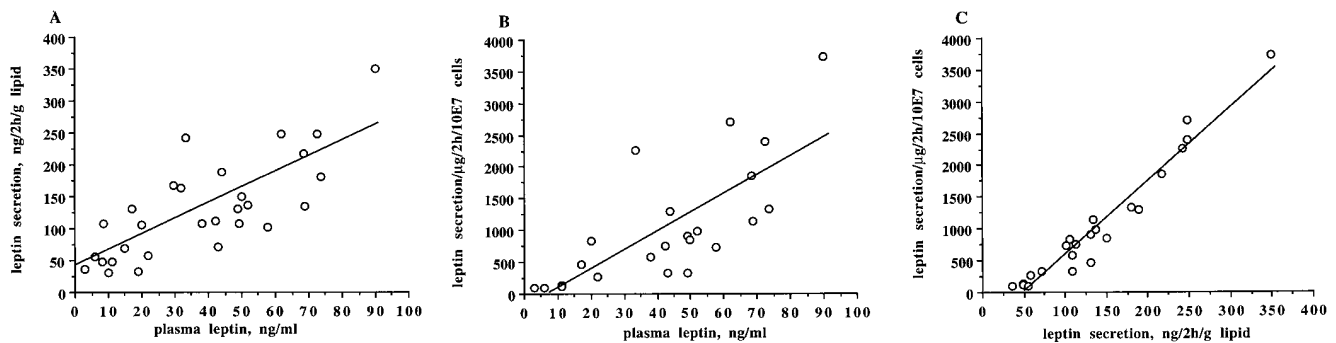

Figure 4. Relationships between different leptin parameters. (*A*) Plasma leptin vs. adipose tissue secretion rate of leptin per gram lipid. (*B*) Plasma leptin vs. adipose tissue secretion rate per cell number. (*C*) Leptin secretion rate per gram lipid vs. per cell number. See Fig. 3 for further details.

Table III. Correlations between Body Mass Index, Fat Cell Volume, and Plasma Insulin on the one Hand, and Leptin Parameters on the Other

| | Y-variable | | | |
X-variable	Plasma leptin	Leptin/ actin mRNA	Leptin secretion/ lipid weight	Leptin secretion/ cell number
BMI	0.84	0.80	0.76	0.71
Fat cell volume	0.78	0.71	0.85	0.89
Plasma insulin	0.40*	0.39‡	0.60§	0.67

The parameters were compared by linear regression analysis. Correlation coefficients are given. *$P < 0.05$, ‡not significant, §$P < 0.01$. $P < 0.001$ for all other values.

bitrary division between obese and nonobese subjects. When data with BMI, adipocyte volume, circulating leptin, and leptin secretion are considered together, it appears that leptin secretion and circulating levels in a continuous way reflect the amount of lipids stored in the fat cells of adipose tissue. However, this assumption is based on measurements performed when the energy intake and output are in balance. As discussed in detail (6), leptin may not be a static index of the amount of lipids stored in adipose tissue during non-steady state energy balance situations. Furthermore, it should be noted in this respect that leptin clearance and protein binding may also influence the circulating leptin levels in obesity (21–23).

Which factors regulate the secretion rate of leptin from fat cells? The present data point to an important role of the steady state mRNA level, which presumably controls the production rate of leptin in the adipocytes. Confirming earlier data (7), we observed an approximately twofold elevation of adipocyte leptin mRNA concentration in obese women. A new observation in the present study was the strong correlation ($r = 0.6$–0.7) between mRNA levels on the one hand, and plasma levels or secretion rates of leptin (per gram lipid or cell number) on the other. Leptin mRNA accounted for ∼ 40% of the variations in circulating and secreted leptin. Using these data, one might speculate that, in obesity, increased gene expression enhances leptin production in fat cells, so that the leptin secretion rates from adipose tissue increase, thus raising the circulating leptin levels. As with leptin secretion and plasma leptin, leptin mRNA reflects the lipid content in fat cells of adipose tissue rather than a division between nonobese and obese subjects. On the other hand, variations in gene expression may also cause differences in plasma leptin between subjects with almost identical fat masses. The mechanisms underlying elevated leptin mRNA levels in obesity are not known. Chronic hyperinsulinemia could be one factor, since plasma insulin correlated strongly ($r = 0.6$–0.7) with the leptin secretion rate. It has been shown experimentally in man that a marked elevation of the circulating insulin level for several hours increased plasma leptin levels and stimulated adipose *ob* gene expression (24, 25). However, leptin mRNA (and plasma leptin) correlated only weakly ($r = 0.4$) with plasma insulin in the present study, which may suggest only a minor role of hyperinsulinemia in regulating leptin production.

In the present study, we used subcutaneous fat. Although the subcutaneous region is the major fat deposit (∼ 80% of the total fat mass), regional variations in adipose tissue function are well known as reviewed (26). Regional variations in leptin expression cannot be excluded (27), although they may not necessarily be present in human adipose tissue (7). However,
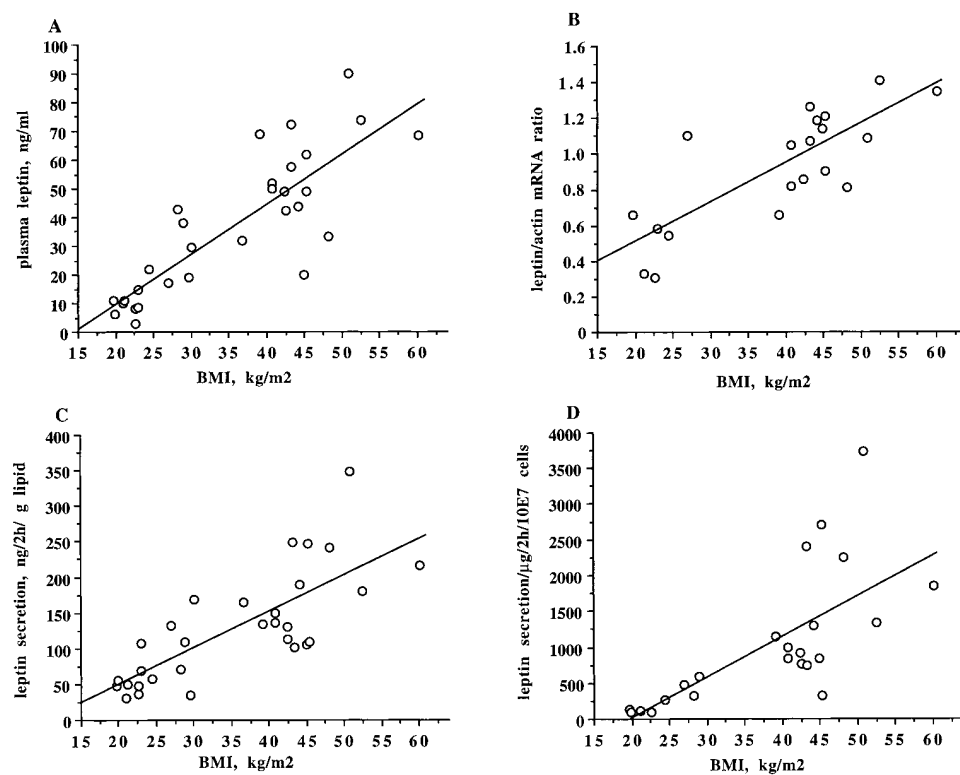

Figure 5. Relationships between body mass index and leptin parameters. Correlation coefficients are given in Table III. See Fig. 3 for further details.
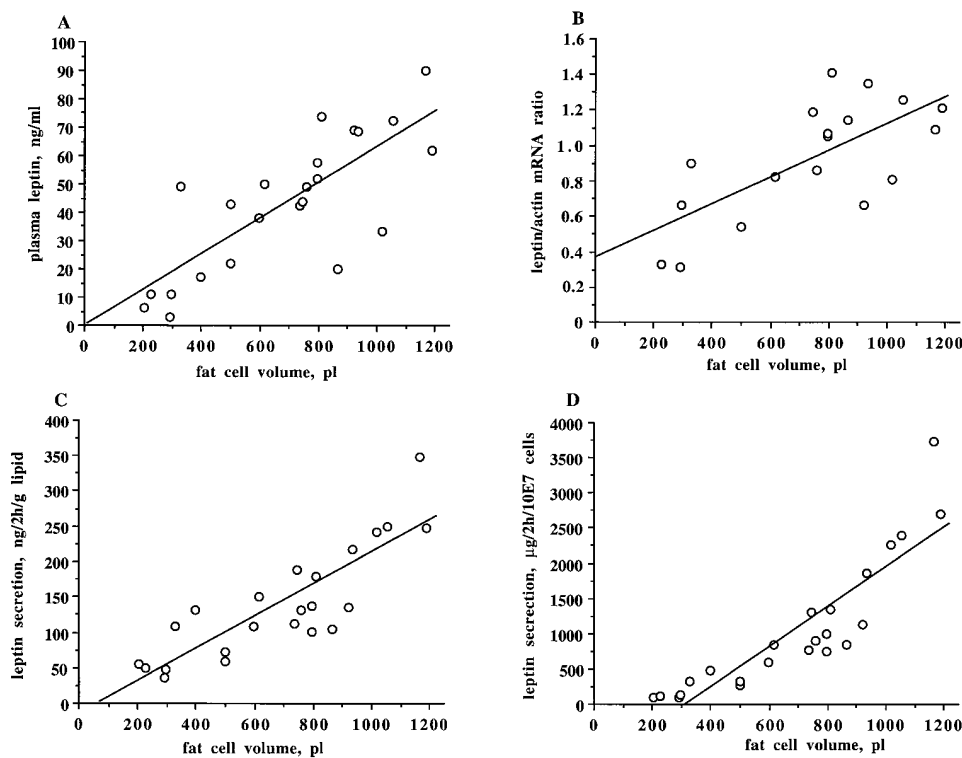
Figure 6. Relationships between fat cell volume and leptin parameters. See Figs. 3 and 5 for further details.

circulating leptin concentrations appear to be associated with all adipose deposits and not disproportionately with regional body fat distribution (28).

Since the present study was performed in women, we know of no gender differences in leptin secretion from adipose tissue. However, Klein et al. determined abdominal adipose leptin production in vivo by arteriovenous balance in 12 essentially normal-weight male subjects (BMI < 30 kg/m^2) and two obese men (BMI > 40 kg/m^2) (29). The net rate of abdominal adipose tissue leptin production correlated directly with the percentage of body fat ($r = 0.59$, $P = 0.016$). On the other hand, divergent results were obtained in the two obese subjects: one had a normal value and the other had an increased value (28). No attempts were made in the present study to compare in vitro and in vivo techniques for measuring leptin secretion. The arteriovenous balance technique is the only technique available for measuring in vivo release of large molecules such as proteins from human subcutaneous adipose tissue (30). Unfortunately, the method is difficult to perform and it has a low success rate ($\sim 70\%$), even in skillful hands. Only a few laboratories have used it on a regular basis.

In summary, this study suggests that an accelerated secretion rate of leptin from adipose tissue, due to increased adipocyte *ob* gene expression, is a cellular mechanism responsible for elevated circulating leptin levels in obese women. Leptin mRNA, leptin secretion, and circulating leptin levels relate much more to the lipid content of fat cells in adipose tissue than to any arbitrary separation into nonobese and obese subjects.

Acknowledgments

The excellent technical assistance of Maria Nilsson, Britt-Marie Leijonhufvud, Catharina Sjöberg, Eva Sjölin and Kerstin Wåhlin is greatly appreciated.

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