Insulin and Cortisol Promote Leptin Production in Cultured Human Fat Cells

Martin Wabitsch, Per Bo Jensen, Werner F. Blum, Claus T. Christoffersen, Piera Englaro, Eberhard Heinze, Wolfgang Rascher, Walter Teller, Hans Tornqvist, and Hans Hauner

The aim of this study was to investigate the regulation of leptin expression and production in cultured human adipocytes using the model of in vitro differentiated human adipocytes. Freshly isolated human preadipocytes did not exhibit significant leptin mRNA and protein levels as assessed by reverse transcriptase (RT)-polymerase chain reaction (PCR) and radioimmunoassay (RIA). However, during differentiation induced by a defined adipogenic serum-free medium, cellular leptin mRNA and leptin protein released into the medium increased considerably in accordance with the cellular lipid accumulation. In fully differentiated human fat cells, insulin provoked a dose-dependent rise in leptin protein. Cortisol at a near physiological concentration of 10^{-8} mol/l was found to potentiate this insulin effect by almost threefold. Removal of insulin and cortisol, respectively, was followed by a rapid decrease in leptin expression, which was reversible after readdition of the hormones. These results clearly indicate that both insulin and cortisol are potent and possibly physiological regulators of leptin expression in human adipose tissue. ***Diabetes*** **45:1435–1438, 1996**

The recent identification of the *ob* gene, which encodes a 167-amino acid protein expressed exclusively in adipose tissue (1), has drawn growing attention to the investigation of body weight control, as injection of this factor, named leptin, into obese and lean animals was found to induce a reduction of food and subsequent decrease of body fat stores (2–4). Recent studies have shown that this protein is also synthesized in human adipose tissue (1,5–8) and is apparently overexpressed in human obesity (6–8). Serum leptin concentrations are correlated not only with body fat but also with serum insulin concentrations (9). To gain a better insight into the regulation of leptin expression in humans, we investigated the effects of insulin and cortisol, two important regulators of gene expression, on leptin production in adipose tissue using the model of in vitro differentiated human adipocytes.

From the Department of Pediatrics I (M.W., E.H., W.T.), University of Ulm, Germany; the Hagedorn Research Institute (P.B.J., C.T.C., H.T.), Gentofte, Denmark; the Department of Pediatrics (W.F.B., P.E., W.R.), University of Gießen, Germany; Lilly Deutschland (W.F.B.), Bad Homburg, Germany; and the Diabetes Research Institute (H.H.), Düsseldorf, Germany.

Address correspondence and reprint requests to Dr. Martin Wabitsch, Department of Pediatrics I, University of Ulm, Prittwitzstr. 43, D-89075 Ulm, Germany.

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PCR, polymerase chain reaction; RIA, radioimmunoassay; RT, reverse transcriptase.

RESEARCH DESIGN AND METHODS

Subjects. Human adipose tissue samples (wet weight 200–500 g) were obtained from the mammary adipose tissue depot of 10 healthy women (mean age 28 ± 7 years [means ± SD], mean BMI 28.3 ± 2.3 kg/m^2) undergoing mammary reduction. All patients gave their informed consent, and the study protocol was approved by the ethical committee of the University of Ulm.

Cell culture. After removal, the adipose tissue samples were stored in phosphate-buffered saline containing 20 mg/ml bovine serum albumin and immediately transferred to the laboratory. The isolation and culture of the adipose tissue derived stromal cells was performed as described previously with minor modifications (10). Cells were cultured in serum-free DME/Ham's F-12 medium (vol/vol, 1:1) supplemented with 15 mmol/l $NaHCO_3$, 15 mmol/l HEPES, 33 µmol/l biotin, 17 µmol/l pantothenate, 10 µg/ml human transferrin, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (basal medium). To induce adipose differentiation, 100 nmol/l cortisol, 66 nmol/l insulin, and 200 pmol/l triiodothyronine (T_3) were added (adipogenic medium). The percentage of differentiated fat cells after 16 days of culture was assessed by microscopically counting the number of lipid-filled cells in relation to total cell number in 10 randomly selected areas per dish, each area representing 1 mm^2.

Total RNA extraction and reverse transcriptase (RT)-polymerase chain reaction (PCR). Total RNA of cultured cells was prepared according to the method of Chomczynski and Sacchi (11). The detailed description of reverse transcriptase (RT)-polymerase chain reaction (PCR) is given elsewhere (12). Briefly, total RNA was diluted to 0.2 µg/µl in Tris/EDTA buffer, and first-strand cDNA was prepared using 5 µl RNA, Superscript RT kit, and random hexamers (both Gibco/BRL) according to the instructions of the manufacturer. cDNA was diluted 1:2 with H_2O and PCR carried out using 3 µl diluted cDNA and a PCR primer mix containing 2.5 U Taq polymerase in its buffer (Promega), 40 µmol/l of dCTP, dGTP, dTTP, 20 µmol/l dATP, 2.5 µCi of 1,000–3,000 Ci/mmol (α-^{33}P)-dATP (Amersham), and 10 pmol of each primer in a 50 µl volume overlaid with 50 µl mineral oil. PCR conditions were a denaturation step at 95°C for 1 min followed by 23 cycles of 94°C, 30 s; 55°C, 1 min; 72°C, 1 min. PCR products were analyzed on a 6% polyacrylamide/7 M urea gel exposed to a Phospho-Imager screen suitable for quantification.

In this semiquantitative RT-PCR method, two primer sets were used simultaneously in the same tube whereby variation in amount of cDNA and between tubes was taken into account. One primer set was specific for leptin cDNA and the other specific for the transcription factor Sp1, which is ubiquitously expressed and is necessary for the transcription of many housekeeping genes as well as for other genes and, thus, can be used as internal standard (12). When using only 23 cycles of PCR amplification, we made sure that the amplification of both leptin and Sp1 cDNA was within the exponential range, verified by running different numbers of PCR cycles (20–28 cycles). Human leptin gene specific primers: 5′ primer 5′-gacttcattcctgggctccacc-3′, 3′-primer 5′-cctgaagcttccaggacacc-3′. Human Sp1 specific primers: 5′ primer 5′-gagagtggctcacagcctgtc-3′, 3′ primer 5′-gttcagagcatcagacccctg-3′ (13).

Since it is essential to demonstrate that only mRNA is measured by RT-PCR and not DNA contamination, we performed the PCR also in the absence of reverse transcriptase. In this case, no PCR products could be detected.

Measurement of leptin concentration in the culture media. Leptin was measured in the culture media at the time intervals indicated using a specific radioimmunoassay (RIA), which has been described in detail elsewhere (W.F. Blum, unpublished observation). In brief, recombinant human leptin (gift from Eli Lilly Research Laboratories, Indianapolis, IN) was used for the production of antiserum in rabbits and for the preparation of tracer by the chloramine T method (14) and of standards. The assay buffer was composed of 0.05 mol/l sodium phosphate, pH 7.4, 0.1 mol/l Na Cl, 0.05% (wt:vol) NaN_3, 0.1%

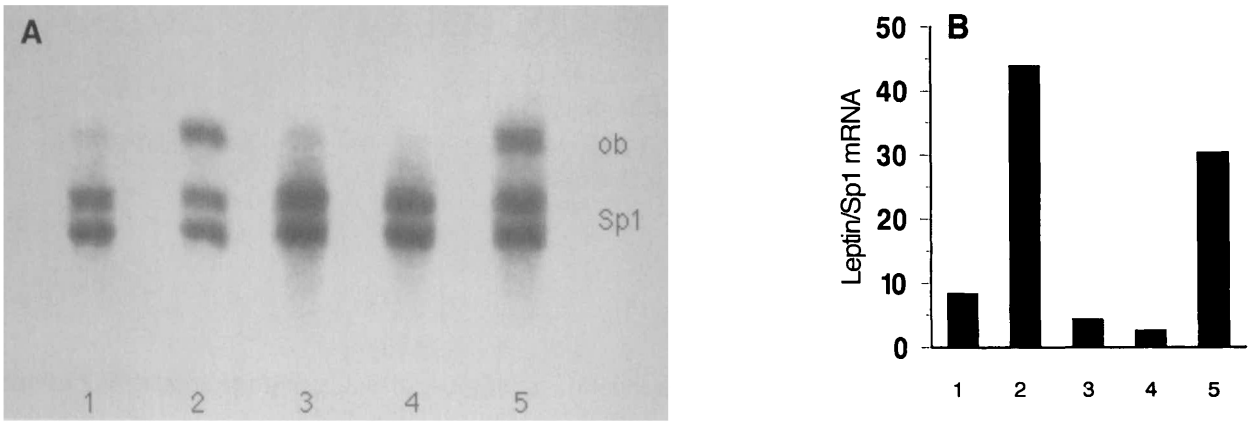

FIG. 1. *A*: **leptin mRNA expression measured by RT-PCR in human adipose tissue samples (*lane 1*), in isolated adipocytes (*lane 2*), in stromal cells isolated from adipose tissue (*lane 3*), in stromal cells cultured in basal medium for 16 days (*lane 4*), and in newly differentiated human fat cells after 16 days of culture in adipogenic medium (*lane 5*). *B*: the same data as in Fig. 1*A* presented as leptin/Sp1 mRNA ratio.**

(vol:vol) gelatine from teleost fish (Sigma, München, Germany), 0.1% (vol:vol) triton X 100 and 0.2% normal horse serum, which showed no cross-reaction in the assay. The assay volume was 0.3 ml. After incubation at room temperature overnight, bound and unbound tracer were separated by a second antibody technique. Maximal tracer binding was 37–45% and half-maximal binding occured at 0.9 µg/l unlabeled leptin. Sensitivity was 0.03 µg/l. The intra- and interassay coefficients of variation were 0.8% and 8.5%, respectively.

RESULTS

Between 70 and 80% of the stromal cells isolated from human adipose tissue acquired adipocyte morphology within 16 days of culture upon stimulation by insulin, glucocorticoids, and T_3. Semiquantitative measurement of leptin mRNA by RT-PCR revealed that compared with freshly isolated adipocyte precursor cells substantially higher amounts of specific mRNA were measured in newly differentiated adipocytes that were comparable to the amount found in freshly isolated human adipocytes (Fig. 1). Even after 8 days of culture, a significant increase in leptin mRNA was evident, whereas cells cultured without adipogenic medium were devoid of any significant leptin mRNA expression. In the culture medium of stimulated cells, leptin production was measurable not before 8 days of culture but then increased markedly in accordance with the morphological maturation of the cells (Fig. 2). This observation suggests that the newly synthesized hormone is rapidly secreted by the fat cells and accumulates in the culture medium. On day 16, when adipocytes were fully developed, leptin production reached maximum levels and remained relatively stable at 2 ng/10^5 adipocytes/48 h. When cells were cultured in basal medium without adipogenic factors, adipose differentiation of the cells did not occur and leptin was undetectable in the medium (Fig. 2).

Replacement of the adipogenic medium by a medium without insulin led to a significant and rapid decrease of leptin mRNA by up to 70% and of leptin protein by up to 40% after 24 h. This decrease was more pronounced when cortisol was also removed (data not shown). Readdition of insulin at rising concentrations for another 24 h increased leptin production in a dose-dependent manner being significant at concentrations not lower than 10^{-9} mol/l. If cortisol was present at a concentration of 10^{-8} mol/l, the insulin effect on leptin expression was clearly potentiated. A more than threefold increase in leptin concentration from 0.35 to 1.40 ng/10^5 adipocytes/24 h was observed, when a combination of 10^{-7} mol/l insulin and 10^{-8} mol/l cortisol was supplemented for 24 h (Fig. 3).

The effect of cortisol alone on leptin expression was also pronounced. Removal of cortisol at a persistent insulin concentration of 10^{-8} mol/l for 24 h resulted in a 80% decrease in both leptin mRNA and leptin protein content in the medium. When cortisol was supplemented again to the medium at a concentration of 10^{-8} mol/l, leptin mRNA and protein returned to the initial values within 24 h (Fig. 4).

DISCUSSION

The results of this study clearly show that leptin gene expression and production in cultured human preadipocytes and adipocytes is dependent on the stage of differentiation. In fully developed human adipocytes, similar amounts of leptin mRNA were measured as in freshly isolated human fat cells. Both insulin and cortisol were found to stimulate leptin production, the former in a dose-dependent manner. It was also apparent from these experiments that changes of leptin mRNA levels are paralleled by corresponding changes of leptin in the culture medium.

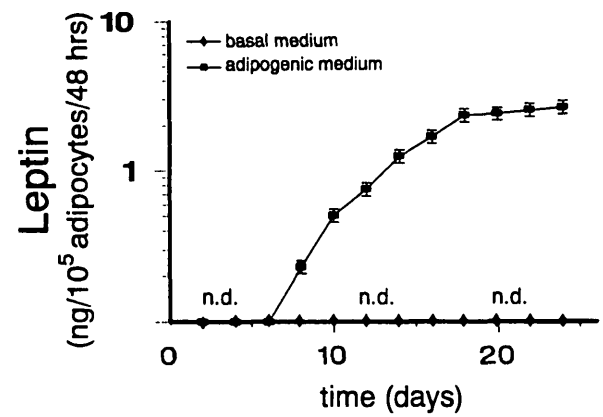

FIG. 2. Leptin production by cultured human preadipocytes during adipose differentiation. Preadipocytes were cultured in adipogenic medium and leptin concentrations were measured every 48 h from day 2 until day 24 in the aspirated medium, which was renewed thereafter. Control cultures were kept in basal medium without adipogenic factors. Means ± SE of three experiments in duplicate.

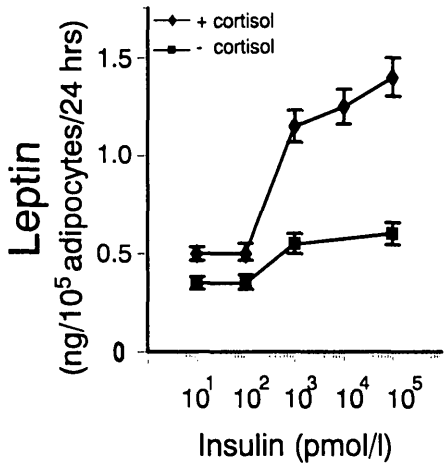

FIG. 3. Effect of rising concentrations of insulin on leptin content in the culture medium of newly differentiated human adipocytes. After differentiation, insulin was removed for 24 h and then readded for another 24 h in the absence or presence of 10^{-8} mol/l cortisol. Leptin was was measured in the medium by RIA. Data are expressed as means ± SE of three experiments in duplicate.

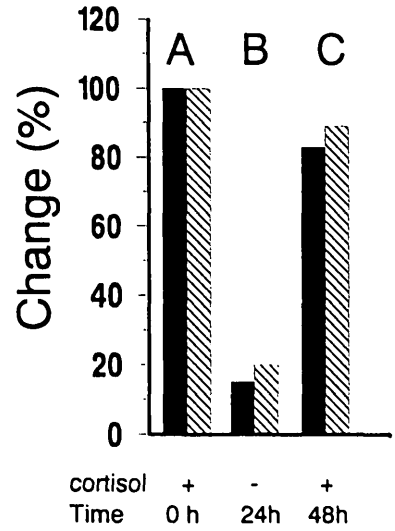

FIG. 4. Effect of cortisol on leptin mRNA and protein in newly differentiated human fat cells. After having developed the adipocyte phenotype (*A*), cortisol was removed from the culture medium for 24 h (*B*) and then readded for another 24 h at a concentration of 10^{-8} mol/l (*C*). Leptin mRNA was determined by RT-PCR and expressed as leptin/Sp1 ratio (■), leptin concentration in the culture medium was measured by RIA (▨). The results of one representative experiment is shown. Leptin/Sp1 mRNA (■); Leptin (▨)

Recent studies in animal models of obesity as well as in established adipocyte cell lines have indicated that insulin may play an important role as a regulator of leptin gene expression (15–19). This has been shown mainly at the mRNA levels. In one of these studies, it has been clearly worked out that the insulin effect takes place at the transcriptional level and is rapidly reversible (18). Our findings extend this observation by demonstrating that *1*) insulin also induces an increased leptin production in adipocytes and *2*) insulin is an important regulator of leptin gene expression in humans.

The assumption that insulin may be involved in the regulation of leptin expression is only partly supported by clinical studies. While Considine et al. (9) have demonstrated a clear correlation between serum leptin and insulin concentrations, recent studies using the euglycemic-hyperinsulinemic clamp technique failed to show an increase of serum leptin concentrations at least under short-term hyperinsulinemia (20,21). In addition, 24-h profiles of circulating leptin were not correlated with insulin levels (22). However, this does not exclude the possibility that the chronic hyperinsulinemia, which characterizes obesity, may cause and maintain an elevated leptin expression. On the other hand, fasting and weight loss not only are associated with low insulin levels, but also were found to lead to a substantial decline in leptin mRNA and protein levels (9).

It is also known from animal experiments and in vitro studies that glucocorticoids may be involved in the control of leptin expression. Treatment of normal rats with different types of glucocorticoids at catabolic doses rapidly induced leptin expression in adipose tissue followed by a concordant decrease in body weight gain and food intake (23). Exposure of isolated rat adipocytes to dexamethasone was reported to increase leptin mRNA within hours by four- to eightfold (24) as well as leptin secretion (25). In contrast, in 3T3 L1 adipocytes dexamethasone was found to exert a slightly inhibitory effect on leptin mRNA levels (19), indicating substantial differences in the regulation of leptin expression among fat cell models. Our in vitro experiments in human adipose cells clearly suggest that cortisol is a potent stimulator of leptin gene expression and may potentiate the effect of insulin. This action is obviously independent of the differentiation-promoting effect of cortisol, since leptin gene expression was also triggered in fully differentiated fat cells and was rapidly reversible after removal of cortisol. The physiological importance of this finding remains to be demonstrated, as the reported 24-h profile of circulating serum leptin (22) does not correspond with the circadian pattern of serum cortisol levels.

In conclusion, the results of this study suggest that both insulin and cortisol potently induce leptin production in cultured human fat cells. Although these data may indicate that both hormones are contributing to the elevated leptin concentrations found in obesity, it is likely that other hormonal and/or metabolic factors are also involved in the regulation of leptin production in vivo.

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