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Leptin Impairs Metabolic Actions of Insulin in Isolated Rat Adipocytes*

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Leptin is an adipocyte hormone involved in the regulation of energy homeostasis. Generally accepted biological effects of leptin are inhibition of food intake and stimulation of metabolic rate in *ob/ob* mice that are defective in the leptin gene. In contrast to these centrally mediated effects of leptin, we are reporting here on leptin effects on isolated rat adipocytes. Leptin impairs several metabolic actions of insulin, *i.e.* stimulation of glucose transport, glycogen synthase, lipogenesis, inhibition of isoproterenol-induced lipolysis, and protein kinase A activation, as well as stimulation of protein synthesis. Insulin effects were reduced by leptin (2 nM) with a half-life of about 8 h. At low leptin concentrations (<1 nM), the insulin sensitivity was reduced leading to a shift to the right in the dose-response curve. At higher concentrations the responsiveness was diminished, resulting in nearly complete inhibition of insulin effects at >30 nM leptin. The IC_{50} value of leptin was 3.1 ± 1 nM after 15 h of preincubation of adipocytes in primary culture. The natural splice variant des-Gln^{49}-leptin exhibited a significantly lower potency. Adipocytes regained full insulin sensitivity within a few hours after leptin removal. The stimulation of glucose transport by vanadate was not affected by leptin. These data show specific and potent impairment of insulin action by leptin in the physiological concentration range of both leptin and insulin, which may be related to the pathophysiology of insulin resistance in both non-insulin-dependent diabetes mellitus and obesity.

Obesity is one of the most common pathological phenotypes of man in affluent societies. It is a metabolic disorder resulting from chronic disequilibrium between energy uptake and expenditure and is strongly associated with type II diabetes, cardiovascular disease, and hypertension.

The discovery of the *ob* gene (1) and analysis of the biology of its gene product, leptin, has given some insight into the mechanism of energy homeostasis. Leptin is exclusively synthesized in adipose tissue (1) and secreted into the circulation (2–4). Leptin gene expression is highly regulated. Except in the leptin-deficient *ob/ob* mouse, mRNA levels as well as serum protein levels are increased in all models of animal obesity, regardless of whether the obesity is caused by genetic defects, hypothalamic lesions, or brown adipose tissue deficiency (2, 5–7). Leptin protein levels in serum and mRNA levels in fat are reduced by fasting (2, 3). Similar regulatory phenomena have been seen in overweight humans (3, 8).

Recombinant leptin given to *ob/ob* mice makes them reduce their food intake, lose body weight, normalize body temperature, and can even restore fertility (4, 9–11). Thus, leptin fulfills the criteria for a circulating satiety factor, an hypothesis originally deduced from parabiosis experiments (12). It most likely defines an endocrine signaling pathway communicating the energy content of adipose tissue to the centers that regulate energy uptake.

Genetic obesity in animals defective in leptin (*ob*) or leptin receptor genes (*db*, *fa*, and *cp*) (13–15) resembles in many aspects hypothalamic obesity produced by chemical, electrolytic, or surgical lesions in the ventromedial area (16). A central action of leptin was therefore assumed by many investigators. This seems to have been proven by the findings that leptin blocks neuropeptide Y synthesis and secretion in some hypothalamic areas as well as by the fact that leptin binds to specific regions in the brain, *e.g.* the choroid plexus or some hypothalamic nuclei (17, 18). But direct action of leptin via central mechanisms has not yet been demonstrated. In leptin-treated ob/ob mice, reduction of body weight does not seem to parallel the reduced food intake, but instead weight loss is significantly higher than estimated from pair-fed animals (4, 19). This is best explained by a direct or indirect effect of leptin on the metabolic rate. Stimulation of catabolic processes in metabolically active tissues, such as muscle, liver, and white fat, would be examples of a direct leptin effect, whereas increased sympathetic activity and subsequent brown fat thermogenesis reflect a possible indirect effect.

Several isoforms of leptin receptor mRNAs derived from a common transcript by alternative splicing have been identified (13, 14, 20). In the brain, the mRNA isoform coding for a receptor with a large cytoplasmic domain, Ob-Rb, resides in some hypothalamic nuclei, and another isoform that codes for a protein with a short cytoplasmic tail, Ob-Ra, prevails in the choroid plexus and leptomeninges (21). These and other isoforms, however, are also found in diverse peripheral tissues and embryonic and hematopoietic cells (13, 20, 22). Ob-Rb mRNA, coding for the presumed functional receptor, is also existent in the testis and to a very low extent in adipose tissue where mRNAs for short isoforms and for a soluble receptor predominate (13).

To understand the full range of biological activities of leptin and to evaluate its role in the pathogenesis of obesity, we looked for direct leptin effects in peripheral tissues. Starting from the most obvious feature of leptin deficiency, the massive expansion of adipose tissue, and from some hints that *ob/ob* mice mobilize stored lipids more slowly than lean mice (23), our working hypothesis was that leptin might be directly involved in the regulation of adipocyte metabolism.

In this report, we describe a procedure to produce recombinant leptin in sufficient quantities for biological testing and the effects of this recombinant leptin on isolated rat adipocytes. While leptin does not significantly influence basal rates of

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adipocyte metabolism, it specifically inhibits insulin action in a potent and sensitive manner. This impairment of insulin actions may be of significance in the physiology and pathophysiology of both leptin and insulin actions.

EXPERIMENTAL PROCEDURES

Leptin Cloning and Plasmid Construction—Total RNA from mouse epididymal fat pads was used to synthesize leptin cDNA by reverse transcriptase (Boehringer Mannheim). For priming the cDNA synthesis, a specific oligonucleotide was used that was complementary to the coding strand in the 3′-untranslated region of the leptin nucleotide sequence (5′ GAATGCAGAATAAATAAATA; nucleotides 1061–1080 in Ref. 1). The cDNA was amplified by *Taq* polymerase (Perkin-Elmer) and specific primers in a polymerase chain reaction according to the manufacturer's instructions. The sequence of the forward primer (5′ GAAAGAAGGATCCAGTGCCTATCCAGAAAGTCCA) locates to the 5′ end of the coding region of mature leptin (nucleotides 177–198 in Ref. 1) and has a *Bam*HI sequence added. The reverse primer (5′ GGAGAGAAGCTTGAGGGAGAGAAATGAATGATGG) was complementary to the 3′-untranslated region (nucleotides 715–735 in Ref. 1) and has a *Hin*dIII sequence added. The polymerase chain reaction product was inserted into the pQE31 (Qiagen) expression plasmid via *Bam*HI and *Hin*dIII and cloned in *Escherichia coli* HB101. DNA sequences of the resulting plasmids (pQEob3-9 and pQEob3-4, differing in the presence or absence of the codon for Gln^{49}) were confirmed by dideoxy sequencing on a LI-COR 4000L sequencer (Sequitherm sequencing kit, Epicentre Technologies). The expression plasmid encoded for leptin starting with the authentic protein sequence from Val^{22} of the original proleptin sequence (1) N-terminally linked to the presequence "MRGSHHHHHHTDP." From plasmid pQEob3-9, subsequent constructions were made and DNA sequences verified as follows: 1) insertion of amino acid sequence DDDDKA in front of leptin (pQEobEK2); 2) deletion of C-terminal Cys^{167} (pQEob3S).

Leptin Expression and Purification—For large scale production of recombinant leptins, the respective *E. coli* clones were grown in a 10-liter fermenter. At the end of the fermentation (A_{546} = 20), the bacteria were harvested by centrifugation and suspended in 5 volumes of lysis buffer (6 M guanidinium HCl, 100 mM sodium phosphate, 10 mM Tris, pH 8). His_6-tagged leptin was isolated by metal affinity chromatography. For this, the lysate was applied to Ni-NTA-agarose (Qiagen) and leptin eluted by an imidazole gradient (0–0.4 M in lysis buffer). For renaturation, leptin was reduced at a concentration of 2.4 mg/ml by addition of a 10-fold molar excess of β-mercaptoethanol for 2 h, diluted 8-fold with 0.1 M Tris/HCl, pH 9, and stirred for 24 h at 16 °C. The refolded leptin was further purified by RP-HPLC[1] (PLRP-S RP-300 10 μm, Polymer Laboratories) using an acetonitrile/water system containing 0.1% trifluoroacetic acid. Leptin bound to the column was eluted by a linear gradient of acetonitrile (25–50%). The pH of the pooled fractions was adjusted to 3 by addition of Na_2HPO_4 (10 mM) and NaOH. Subsequently, the organic solvent was removed in a rotary evaporator, and the pH was increased to 7.5 with NaOH. A minor fraction of leptin precipitated at this stage and was removed by centrifugation. The supernatant was concentrated by ultrafiltration and finally purified by GPC on a Superdex 75 HiLoad 26/60 column (Pharmacia Biotech Inc.) equilibrated in PBS (10 mM sodium phosphate, 154 mM NaCl, pH 7.4). The leptin solution was sterilized by filtration and stored at −70 °C until use. His_6-tagged des-Gln^{49}-leptin was purified in the same way. Ala^0-leptin was prepared from the pQEobEK2-derived precursor that had been isolated by metal affinity chromatography, refolded, and dialyzed against 20 mM Tris/HCl, pH 8. Removal of the presequence was achieved by addition of 1 unit of enterokinase per mg of leptin (16 h, 37 °C). The resulting tag-free Ala^0-leptin was purified by RP-HPLC and GPC (see above).

Des-Cys^{167}-leptin (pQEob3S) was isolated up to the refolding step according to the protocol given above. The refolding solution was dialyzed three times against 20 mM sodium phosphate buffer, pH 8.5. As a control, normal His-tag-leptin (pQEob3-9) was prepared by the same method, except that the dialysis buffer was pH 7.4. The resulting leptin solutions were filtered sterile.

Antibodies—Polyclonal antibodies were raised in rabbits using His_6-tagged leptin as antigen and purified by leptin affinity chromatography. For this, leptin (3 mg) was coupled to 1 ml of Fractogel EMD Azlacton (Merck) according to the manufacturer's instructions. Rabbit serum (6 ml) was filtered through a 0.2-μm disposable filter and applied to the column. Bound antibodies were eluted with 0.1 M glycine, pH 2.7, and neutralized immediately by addition of 0.8 M Tris base. Subsequently, the antibodies were dialyzed four times against PBS, concentrated by ultrafiltration, and sterilized by filtration. The concentration was determined by the bicinchoninic acid method (Sigma). The last dialysis buffer was used as control solution. Polyclonal rabbit IgG was purchased from Sigma, reconstituted in PBS, and dialyzed against the same buffer.

Protein Analytical Procedures—Analytical RP-HPLC was performed on a Nucleogel RP 300 5 μ column (4.6 × 50 mm, Macherey-Nagel) using an acetonitrile/water/trifluoroacetic acid solvent system. The structural identity was confirmed by electrospray mass spectrometry, N-terminal sequencing, and amino acid analysis. The purity was evaluated by RP-HPLC and SDS-polyacrylamide gel electrophoresis. The concentration of leptin was determined by RP-HPLC using leptin as a standard that had been quantified by amino acid analysis (ninhydrin method).

Preparation of Adipocytes—Adipocytes released from epididymal fat pads of male Wistar rats (140–160 g, animal breeding station of Hoechst AG, HMR, Kastengrund) by collagenase (Biochrom) digestion (24, 25) were washed twice with KRH (25 mM HEPES free acid, 25 mM HEPES sodium salt, 80 mM NaCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM KCl, 1 mM sodium pyruvate, 0.5% bovine serum albumin (Behring, ORHD 20/21)) and once with DMEM (Life Technologies, Inc.) containing 5.5 mM glucose, 20 mM HEPES, pH 7.4, 2% fetal calf serum (Life Technologies, Inc.), 1% bovine serum albumin, 50 units of penicillin/ml, and 10 mg streptomycin/ml (Life Technologies, Inc.) by flotation (800 × *g*, 1 min) in a plastic tube and then diluted to a final volume equal to 20 ml of DMEM/g of fat tissue (final titer about 2.5×10^5 cells/ml).

Primary Culture of Adipocytes and Incubation with Leptin—Adipocytes were added to DMEM containing 25 mM glucose and 100 nM N^6-phenylisopropyl adenosine (Sigma) including the above ingredients (1 ml of cells/4 ml of DMEM, final titer about 5×10^4 cells/ml) in sterile 50-ml conical polypropylene flasks. The cells were incubated for various times at 37 °C under an atmosphere of 5% CO_2 and mild shaking in the absence or presence of leptin. Subsequently, the adipocytes were washed three times with cold (10 °C) KRH (volume equal to the culture medium) and finally suspended in 0.7 ml of glucose-free KRH or KRB (12 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.8 mM KCl, 25 mM $NaHCO_3$, 120 mM NaCl, 1.4 mM $CaCl_2$, 5 mM glucose, and 2.5% (w/v) bovine serum albumin, bubbled with 5% CO_2, 95% O_2) resulting in a titer of about 3.5×10^5 cells/ml.

To assess insulin sensitivity after primary culture, washed adipocytes were incubated with insulin (0.02–50 nM human insulin, Hoechst AG) prior to assaying lipogenesis, glucose transport, glycogen synthase activity, PKA activity, and lipolysis. Loss of cells due to incubation and washing was identical among control and leptin-treated cell groups (26).

For assaying reversibility of leptin action, the adipocytes were incubated with leptin and washed as described. After complete removal of the final washing solution, cells were suspended in 4.5 ml of DMEM (about 4×10^4 cells/ml), further incubated under the above conditions, washed twice, and assayed as described.

Assays for Metabolic Actions of Insulin—Lipogenesis was measured as incorporation of D-[3-^{3}H]glucose (5–15 Ci/mmol, Amersham-Buchler) into total toluene-extractable acylglycerols as described (25), with the following modifications. 200 μl of adipocytes in KRH were supplemented with 680 μl of KRH containing 140 μM or 3.5 mM glucose (low or high glucose concentrations, respectively) and 20 μl of insulin solution in 10-ml plastic scintillation vials for 20 min at 37 °C. Lipogenesis was started by addition of 100 μl of D-[3-^{3}H]glucose (1 or 25 μCi/ml KRH for low or high glucose condition, respectively). After 90 min, 10 ml of a toluene-based scintillation mixture were added. Dpm values for radiolabeled lipids were measured in the toluene phase and corrected for background values determined for an incubation mixture containing the same amount of [^{3}H]glucose but lacking adipocytes. At low glucose concentrations, basal and insulin-stimulated values were 300–500 and 5,000–9,500 dpm, respectively. At high glucose the corresponding values were 3,000–4,000 dpm and 6,000–9,500 dpm.

Glucose transport was assayed as uptake of the non-metabolizable and radiolabeled glucose analogue, 2-deoxy-D-[2,6-^{3}H]glucose (45 Ci/mmol, Amersham-Buchler) according to previously published procedures (26). 50 μl of adipocytes in KRH, preincubated in the absence or presence of insulin for 20 min at 37 °C, were incubated with 50 μl of KRH containing deoxy[^{3}H]glucose (0.5 μCi, 0.2 mM) for 5 min at 25 °C. Deoxy[^{3}H]glucose uptake, assessed after preincubation of the cells with 20 μM cytochalasin B, was subtracted from the total cell-associated

[1] The abbreviations used are: RP-HPLC, reversed phase high performance liquid chromatography; DMEM, Dulbecco's minimal essential medium; GPC, gel permeation chromatography; PBS, phosphate-buffered saline; PKA, protein kinase A.

radioactivity (27). Values for the basal and insulin-stimulated states were 400–700 and 6,000–10,000 dpm, respectively.

Glycogen synthase was assayed by measuring the incorporation of D-[^{14}C]glucose from UDP-D-[^{14}C]glucose (300 mCi/mmol, DuPont NEN) into glycogen (28, 29). 0.5 ml of adipocytes in KRH were diluted with 0.5 ml of KRH and incubated in the absence or presence of insulin in a shaking water bath under an atmosphere of 5% CO_2 for 20 min at 37 °C. A homogenate was prepared, supplemented with 50 μl of UDP-D-[^{14}C]glucose (44 μCi/ml, 0.2 mM), and incubated for 10 min in the presence of 0.1 and 10 mM glucose-6-P (30). Glycogen was precipitated with 66% ethanol, 10 mM LiBr (−20 °C), washed, dried, and counted for radioactivity. Reaction values, generated by adding the homogenate to tubes containing the complete reaction mixture plus ice-cold ethanol, were subtracted from each experimental value. The portion of glycogen synthase active at the time of homogenization compared with total enzyme content was calculated as the ratio between the activities at 0.1 mM (I form of glycogen synthase) and 10 mM glucose-6-P (I plus D forms). Values for the basal and insulin-stimulated state were 2,000–2,500 and 5,000–7,000 dpm, respectively (I form). The values (basal and insulin-stimulated) for the I plus D form were 50,000–80,000 dpm.

Lipolysis and PKA were assayed as release of glycerol and the incorporation of ^{32}P from γ-[^{32}P]ATP into Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly, Sigma), respectively. 1 ml of adipocyte suspension in KRB was incubated with various concentrations of insulin for 10 min at 37 °C in 2-ml microcentrifuge vials under an atmosphere of 5% CO_2 and shaking (160 cycles/min). After addition of 1 μM isoproterenol hydrochloride (Calbiochem) and 1 unit of adenosine deaminase (Calbiochem), the incubation was continued for 60 min. The incubation was terminated by the addition of 200 μl of extraction medium containing Tris/HCl, pH 7.4, 6 mM 3-isobutyl-1-methylxanthine, 60 mM EDTA, and 180 mM NaF, followed by immediate vigorous mixing and homogenization by hand with 10 strokes of a Teflon pestle fitting to the incubation vials. The homogenate was centrifuged (10,000 × *g*, 15 min, 4 °C). The infranatant below the fat cake was removed with a glass Pasteur pipette, taking care not to aspirate the membrane pellet, and stored frozen in liquid N_2 until determination of PKA activity and glycerol content (31). The values for the isoproterenol-induced lipolysis were 3,000–5,000 nmol of glycerol/3 × 10^4 cells/60 min for the basal state (set at 100%) and 200–450 nmol of glycerol/3 × 10^4 cells/60 min for the insulin-stimulated state, respectively. Corresponding values for the PKA activity ratio were 25–30 and 2–8%, respectively.

Phosphorylation of Kemptide (determination of PKA activity) was initiated by addition of 50 μl of adipocyte homogenate (10,000 × *g* supernatant) to 50 μl of prewarmed assay buffer containing 1 μM Kemptide, 80 mM Tris/HCl, pH 7.2, 5 mM dithiothreitol, 25 mM $MgCl_2$, 200 μM phenylmethylsulfonyl fluoride, 1 mM 3-isobutyl-1-methylxanthine, 250 μM [γ-^{32}P]ATP (1.5 μCi) with or without 1 μM cAMP. After incubation for 10 min at 30 °C, the reaction mixture was chilled on ice, immediately supplemented with 3 ml of ice-cold 12.5 mM ATP, 100 mM NaF, and 150 mM $NaPP_i$, and spotted on phosphocellulose filters (Whatman P18). The filters were washed extensively with 150 mM $NaPP_i$ (500 ml for 50 filters, three times for 20 min each), dried, and counted for radioactivity. A blank value measured for a reaction mixture lacking homogenate was subtracted in each case. The PKA activity was expressed as the ratio between ^{32}P incorporation into Kemptide in the absence and presence of cAMP. This activity ratio is a parameter for the portion of PKA active at the time of homogenization to total cellular enzyme content. The PKA activity ratio determined for isoproterenol-stimulated adipocytes was set at 100%.

Protein synthesis was assayed as incorporation of L-[^{35}S]methionine into trichloroacetic acid-precipitable protein. 150 μl of an adipocyte suspension in DMEM depleted of methionine (Life Technologies, Inc.) was diluted with 2 volumes of the same medium and incubated in the absence or presence of insulin for 15 min in 10-ml plastic scintillation vials under gentle shaking. After addition of 6.5 μCi of L-[^{35}S]methionine (Amersham/Buchler 1000 Ci/mmol), the incubation was continued for 60 min at 35 °C. Subsequently, the cells were homogenized by addition of an equal volume of 20 mM Tris/HCl, pH 7.4, 1% SDS, 1 mM EDTA, 20 μM leupeptin, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride and vigorously mixed. After centrifugation (800 × *g*, 2 min), 600 μl of the infranatant was removed and supplemented with 400 μl of 10% trichloroacetic acid. Following incubation for 30 min on ice, the precipitated proteins were collected by centrifugation (15,000 × *g*, 15 min). The pellet was washed three times with ice-cold acetone, dried, dissolved in 1 ml of 2 M NaOH, neutralized, and measured for radioactivity by liquid scintillation counting. The amount of nonspecific radioactivity associated with the precipitate was measured by incubating insulin-stimulated adipocytes in the presence of cycloheximide (50 μg/ml, Boehringer Mannheim). Values for the basal and insulin-stimulated state were 3,500–5,000 and 7,000–9,000 dpm, respectively.

RESULTS

Cloning, Expression, and Purification of Leptins—E. coli clones carrying plasmids with murine leptin cDNA insertions of both splice versions containing and lacking Gln^{49} of proleptin amino acid sequence (pQEob3-9 and pQEob3-4, respectively) were obtained. DNA sequences coincided with the published sequence except for an A to G transition 43 nucleotides 3′ of the termination codon.

Recombinant *E. coli* clones were grown in 10-liter fermenters and expressed His_6-tagged leptin in high yield. The protein was renatured and purified by metal affinity, reversed phase, and gel permeation chromatographic procedures. This resulted in a monomeric protein with a purity greater than 97% as analyzed by HPLC and SDS-polyacrylamide gel electrophoresis. The molecular mass of the His_6-tagged leptin was determined by electrospray mass spectrometry and corresponded to the calculated value of 17,570 Da. The content of endotoxins was lower than 0.5 international units per mg (limulus amebocyte lysate assay). Biological activity was demonstrated by the induction of significant weight loss after subcutaneous application to C57BL/6J *ob/ob* mice in doses of 100 nmol/kg per day (data not shown). To remove the His_6-tag, an enterokinase cleavage site (DDDDK) was introduced with an additional alanine between the cleavage site and the leptin sequence (starting with Val^{22} according to Ref. 1). After cleavage, this resulted in a leptin that was N-terminally elongated by an alanine (Ala^0-leptin). The alanine was inserted because a precursor in which the lysine of the enterokinase recognition site had been directly joined to Val^{22} of leptin was not processed at all.

As a putative negative control, an analogue lacking the C-terminal cysteine was designed (His_6-tagged des-Cys^{167}-leptin). This variant was purified by metal affinity chromatography, refolded, and dialyzed against 20 mM sodium phosphate buffer at pH 8.5. This pH was chosen because the mutant leptin precipitated at pH values lower than 8. HPLC analysis and SDS-polyacrylamide gel electrophoresis revealed that about 80% of the leptin molecules had formed disulfide-linked dimers. In this case, the concentration was calculated as the sum of the molar concentrations of monomers and dimers.

Effects on Nonstimulated Adipocytes—In initial experiments with isolated rat adipocytes no significant acute stimulatory or inhibitory effects of recombinant His_6-tagged leptin on the major metabolic pathways, *i.e.* lipogenesis, lipolysis, and glucose transport, have been observed in the absence of insulin stimulation. Prolonged exposure of adipocytes (up to 16 h) at high leptin concentration (100 nM) in primary culture caused an increase of the basal rates of these reactions of only 30% (data not shown). Adipocytes that were cultivated under the conditions described here maintain many features characteristic of this cell type (*e.g.* insulin stimulation of glucose transport, lipogenesis, protein synthesis, induction of lipolysis by isoproterenol, and inhibition of isoproterenol-induced lipolysis by insulin). The morphology of the cells did not change significantly. Cell viability was >80% at the end of the primary culture, as determined by release of lactate dehydrogenase and lipid into the incubation medium, and did not differ significantly between leptin-treated and control cells (data not shown). From this, we conclude that there are no major physiological changes in the traits of these cultured adipocytes.

Effects on Insulin-stimulated Glucose Transport—We then studied the effect of leptin on the glucose transport activation following stimulation by insulin. Under conditions that ensured sufficient insulin sensitivity and responsiveness of the cells for up to 19 h, primary cultured rat adipocytes were

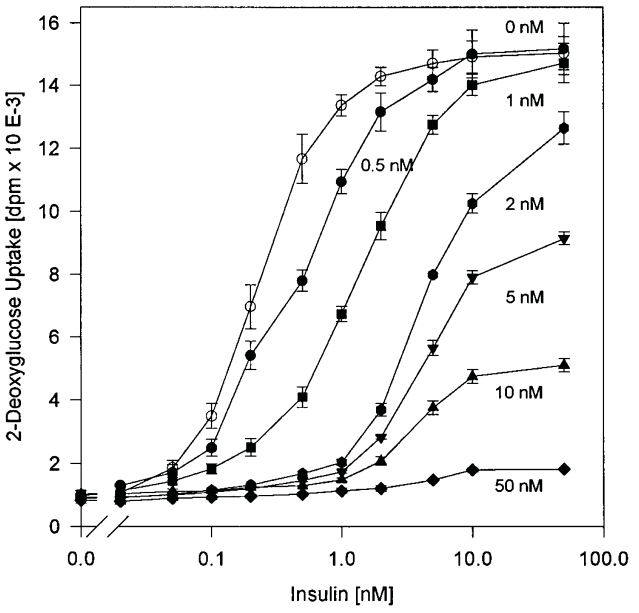

FIG. 1. **Effect of leptin on insulin stimulation of glucose transport.** Primary cultures of rat adipocytes were incubated for 16.5 h in the absence or presence of the indicated concentrations of leptin. Subsequently, the cells were washed and assayed for glucose transport activation by various concentrations of insulin. Glucose transport activity is given as 2-[^{3}H]deoxyglucose specifically associated with the cells. Each point represents the mean value; each error bar represents half-range of two independent adipocyte cultures, with glucose transport determinations performed in quadruplicate.

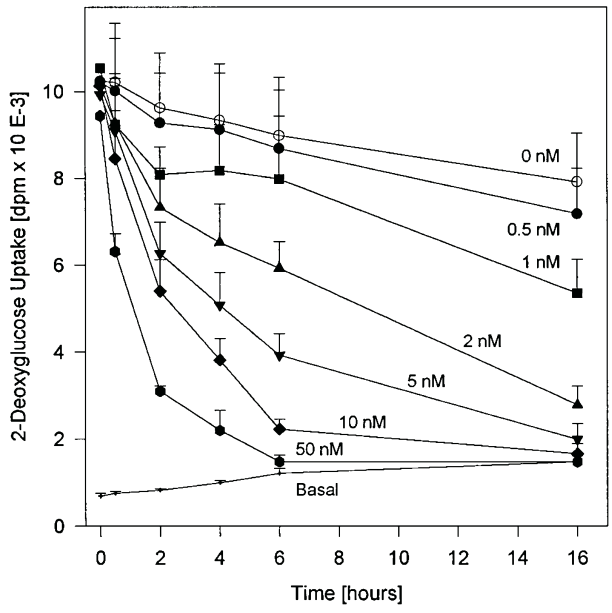

FIG. 2. **Time course of the leptin-induced desensitization of stimulation of glucose transport by insulin.** Primary cultures of rat adipocytes were incubated for 0.5–16 h in the absence or presence of various concentrations of leptin as indicated. Subsequently the cells were washed and assayed for basal and insulin (5 nM)-stimulated glucose transport. Glucose transport activity is given as 2-[^{3}H]deoxyglucose specifically associated with the cells. Each point represents the mean value; each error bar represents the half-range of two independent adipocyte cultures, with glucose transport determinations performed in quadruplicate.

incubated with increasing concentrations of leptin. This period was chosen to include putative short and long term effects of leptin. After exposure to leptin, the cells were washed to remove the culture medium and any contaminants from leaky cells that would interfere with insulin stimulation, incubated with various concentrations of insulin for 20 min, and tested for 2-deoxy[^{3}H]glucose transport (Fig. 1). In the absence of leptin, glucose transport was maximally stimulated 16.5-fold by insulin with an EC_{50} of 0.25 nM, comparable with effects of insulin in cells that were assayed immediately after isolation (18.9-fold; EC_{50} = 0.12 nM). In the presence of up to 1 nM leptin during the primary culture, the concentration response curves for insulin-stimulated glucose transport were shifted to the right with no significant reduction of the maximal insulin response. Higher concentrations of leptin (up to 50 nM) caused considerable decreases in the maximal stimulation of glucose transport by insulin (from 16.5- to 2.2-fold). The assessment of insulin sensitivity was generally performed after cells had been washed so that leptin was not present during insulin stimulation. However, identical results (data not shown) were obtained if the assay (incubation with insulin and determination of glucose uptake) was performed in the presence of leptin (same concentrations as in the primary culture). In conclusion, these results indicate that leptin induces a concentration-dependent desensitization of the glucose transport system for activation by insulin, both with regard to sensitivity and responsiveness. Remarkably, even a supraphysiological concentration of insulin (50 nM) could not overcome desensitization caused by leptin.

Kinetics of Desensitization—The time course for the desensitization of insulin-stimulated glucose transport is shown in Fig. 2. Primary cultures of rat adipocytes were incubated with various concentrations of leptin for up to 16 h prior to assay of glucose transport. The maximal insulin response was concentration dependently diminished by the addition of 1–50 nM leptin. Insulin responsiveness of the glucose transport steadily decreased during the incubation with leptin. The times required for 50% inhibition of the effects of insulin decreased dramatically with increasing leptin concentrations ranging from about 8 h at 2 nM leptin to 1 h at 50 nM leptin. At leptin concentrations in the physiological range (0.5–2 nM, see "Discussion"), there was a remarkably slow but progressive desensitization of adipocytes to insulin stimulation. Two additional experiments were performed to exclude the possibility that desensitization of adipocytes to insulin might be caused by the length of the primary culture or might depend on the sensitivity of the cells toward insulin. Adipocytes were kept in primary culture without leptin for different times (0, 2, 4, and 10 h) prior to incubation with various concentrations of leptin for 8 h. Similar dose-response curves for impairment of insulin-stimulated glucose transport were observed in each case with respect to IC_{50} values for and maximal effects of leptin (Fig. 3). The maximal glucose transport slightly decreased with increasing length of the primary culture, whereas the basal transport increased. However, this effect was also observed in the absence of leptin and reflects the aging of the adipocytes in culture. In the second experiment, adipocytes with reduced insulin sensitivity and responsiveness, induced by long term treatment with 10 nM insulin, 20 mM glucose, and 16 mM glutamine for 18 h (26, 32), showed no enhanced sensitivity toward leptin (data not shown). From this, we conclude that slow onset of effects of leptin is not due to adipocyte cultivation time and that the kinetics described in Fig. 2 actually reflect the times required for desensitization of adipocytes to insulin.

Effects on Other Metabolic Actions of Insulin—We next studied whether or not other metabolic effects of insulin were affected as well. Adipocytes were incubated with leptin and then tested for maximal stimulation of lipogenesis by insulin at low and high glucose concentrations as well as of glycogen synthase and protein synthesis. In addition, maximal insulin inhibition of isoproterenol-induced lipolysis and activation of PKA was investigated (Fig. 4).

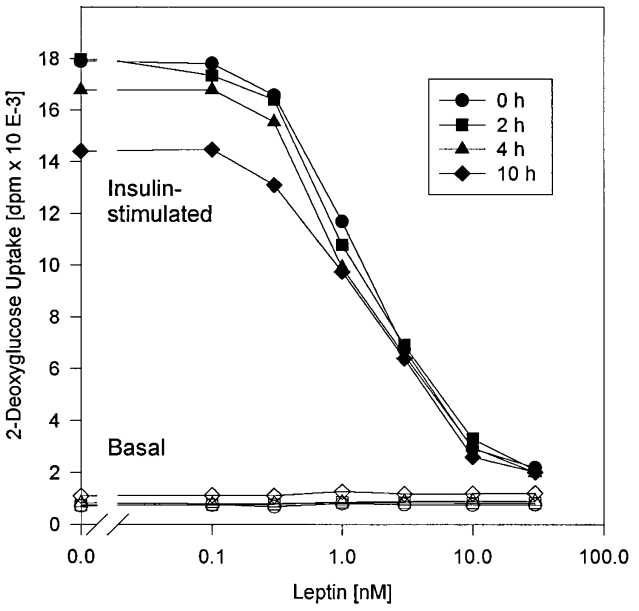

FIG. 3. **Effect of primary culture on the sensitivity to leptin.** Primary cultures of rat adipocytes were incubated for the times indicated. After addition of leptin to final concentrations as indicated, the incubations were continued for 8 h. Subsequently the cells were washed and assayed for basal (*open symbols*) and insulin (10 nM)-stimulated (*closed symbols*) glucose transport. Each point represents the mean of two independent adipocyte cultures, with glucose transport determinations performed in triplicate.

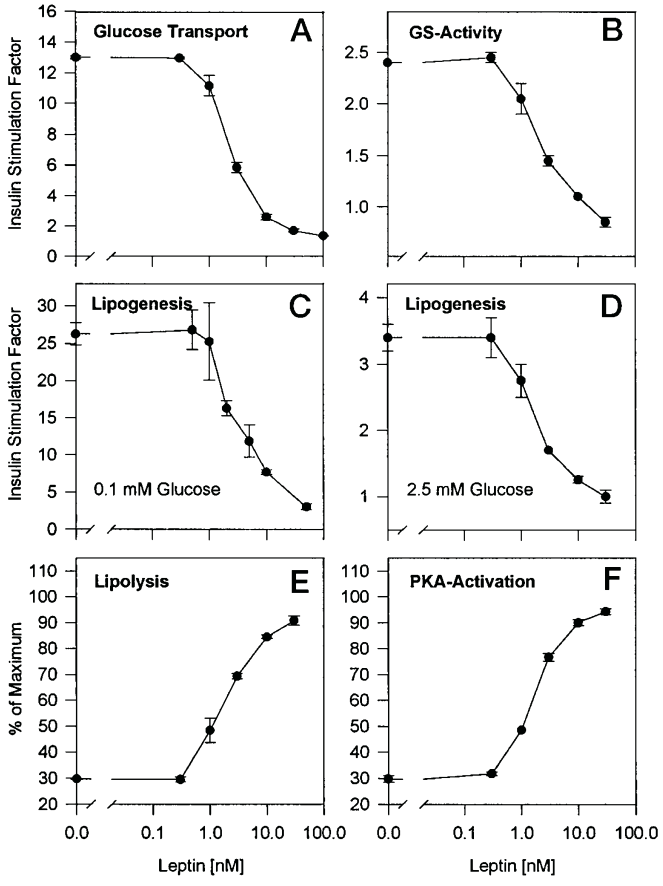

FIG. 4. **Leptin-induced desensitization of various metabolic actions of insulin.** Primary cultures of rat adipocytes were incubated for 15 h in the absence or presence of various concentrations of leptin. Subsequently the cells were washed and assayed for basal and insulin (10 nM)-stimulated glucose transport (*A*), glycogen synthase (*B*), lipogenesis at 0.1 mM glucose (*C*), or 2.5 mM glucose (*D*) as well as for basal and insulin (2 nM)-inhibited isoproterenol (1 μM)-induced lipolysis (*E*) and activation of PKA (*F*). Insulin-inhibited lipolysis (*E*) and PKA activation (*F*) at each leptin concentration was calculated as percentage of the basal isoproterenol-induced value. Each point represents the mean value; each error bar represents half-range of two independent adipocyte cultures, with activity determinations performed in triplicate.

Leptin almost completely inhibited the insulin-stimulated lipogenesis at 2.5 mM glucose (Fig. 4*D*) in a concentration-dependent manner. At this glucose concentration, the esterification reactions (in particular glycerol-3-phosphate acyltransferase) that are activated 2–3-fold by insulin (33) determine the rate of lipid synthesis. At low glucose concentration (0.1 mM), the glucose transport step is rate-limiting for the insulin stimulation of lipogenesis (34, 35). Thus, the leptin concentration response curve for inhibition of insulin-stimulated lipogenesis (Fig. 4*C*) confirms that of 2-deoxyglucose transport (Fig. 4*A*). Isolated rat adipocytes exhibit glycogen synthesis albeit to a moderate degree (36). The rate-limiting enzyme is glycogen synthase. The activation of this enzyme by insulin was inhibited by leptin in a concentration-dependent manner (Fig. 4*B*).

One of the most sensitive insulin actions in isolated rat adipocytes is the inhibition of lipolysis induced by β-adrenergic agonists (37). In our experiments, insulin reduced isoproterenol-induced lipolysis and the underlying regulatory mechanism, activation of PKA, by about 70% of the maximal value in each case. Leptin antagonized insulin inhibition of both isoproterenol-induced lipolysis (Fig. 4*E*) and PKA activation (Fig. 4*F*). The initial isoproterenol-induced rate of lipolysis and PKA activation was nearly fully restored. The IC_{50} values for desensitization by leptin of the insulin stimulation of glucose transport, lipogenesis at low and high glucose and glycogen synthase as well as of the insulin inhibition of isoproterenol-induced lipolysis, and PKA activation were in the same range (2–4 nM leptin). In all cases, leptin at concentrations around 30 nM decreased the insulin effects on the investigated metabolic effects by 85–95%. Furthermore, protein synthesis that is stimulated 2-fold by insulin (10 nM) was also decreased by leptin in a concentration-dependent manner with an IC_{50} value of about 3 nM (Fig. 5). In conclusion, the insulin desensitizing activity of leptin in isolated rat adipocytes is not restricted to glucose transport but covers other major metabolic stimulatory as well as inhibitory effects of insulin.

Activity of Leptin Variants—We compared the inhibitory activity of four leptin variants (Fig. 6). His_6-tagged leptin exhibited an activity comparable with tag-free leptin that was N-terminally elongated by an alanine. This demonstrates that the His_6-tag does not affect the described effects on adipocytes and more importantly rules out the possibility that the effects are caused by the His_6-tag *per se*.

From eight glucose transport assays of four different batches of His_6-tagged leptin an IC_{50} value of 3.1 $\pm$ 1 nM was calculated. The natural splice variant des-Gln^{49}-leptin, which was also His_6-tagged, showed a lower potency with a 5–6-fold higher IC_{50} value of 18 $\pm$ 2.2 nM (two batches, four assays). To exclude the possibility that an undefined agent present in the *E. coli*-derived leptin preparations induced the observed effects, we tested a His_6-tagged variant lacking the C-terminal cysteine, since the single disulfide bridge present in natural leptin was expected to be essential for native conformation. The inhibitory activity of des-Cys^{167}-leptin on insulin effects was dramatically reduced (IC_{50} = 370 nM). This decrease was not due to the modified purification scheme (no HPLC and GPC chromatography), since normal leptin prepared in parallel by the same method as a positive control exhibited full potency (IC_{50} = 3 nM, data not shown). In conclusion, these findings demonstrate that the structurally modified leptin exhibits a

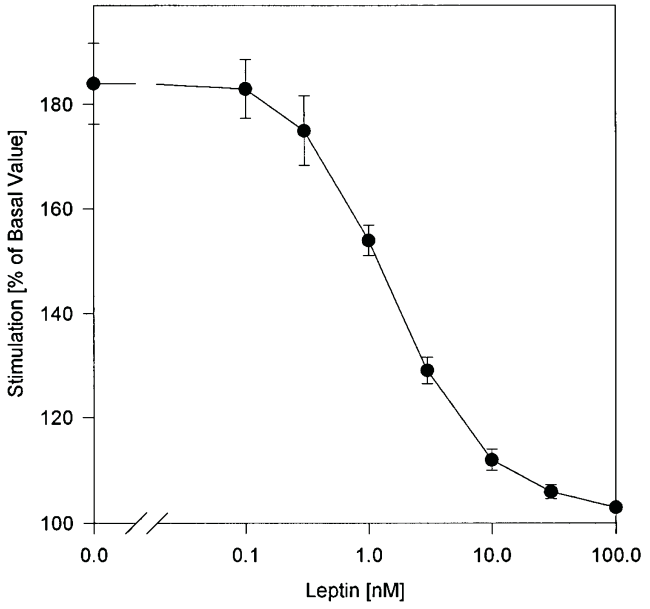

FIG. 5. **Leptin-induced desensitization of insulin-stimulated protein synthesis.** Isolated rat adipocytes were treated with leptin as described in Fig. 2. Subsequently the cells were washed and assayed for the stimulation of protein synthesis by insulin (10 nM). The values were normalized by setting the basal rate of synthesis at 100%. Each point represents the mean value ± standard deviation from four adipocyte cultures, with protein synthesis determinations performed in triplicate.

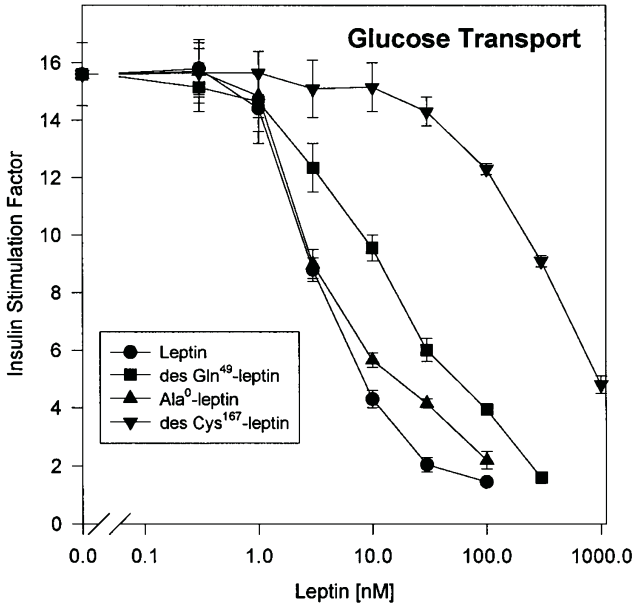

FIG. 6. **Desensitization of the insulin stimulation of glucose transport by different leptin variants.** Primary cultures of rat adipocytes were incubated for 19 h in the absence or presence of various concentrations of different leptin variants. Subsequently the cells were washed and assayed for basal and insulin (10 nM)-stimulated glucose transport. The insulin stimulation factor at each leptin concentration was calculated as ratio between insulin-stimulated and basal glucose transport. Each point represents the mean value, each error bar represents half-range of two independent adipocyte cultures, with glucose transport determinations performed in triplicate.

100-fold lower potency when compared with normal leptin. This further excludes that contaminations from *E. coli* as well as from the preparation are responsible for the desensitization of adipocytes for metabolic insulin actions induced by recombinant leptin. The effects observed at high concentrations of des-Cys^{167}-leptin may be due to the residual activity of this mutant leptin.

Specificity of the Observed Effects—To assess the specificity of the observed effects further, two additional experiments were performed (Fig. 7). Leptin (10 nM) was incubated with increasing amounts of an affinity purified anti-leptin antibody for 1 h prior to addition to the cell suspension. An equimolar amount of affinity purified anti-leptin antibody did not influence the desensitization of insulin-stimulated glucose transport by leptin. However, a 5- and 15-fold molar excess of this antibody (50 and 150 nM, respectively) reduced the inhibitory action of leptin in a concentration-dependent way. Stimulation of glucose transport by insulin increased from 3.5-fold in the presence of leptin alone to 10-fold in the presence of leptin plus antibodies (Fig. 7*A*). Stimulation of glucose transport by insulin (5 nM) without leptin was 14-fold. Polyclonal rabbit IgG, as well as buffer (PBS), which was used for dialysis, had no effect on leptin action. Neither control rabbit IgG nor anti-leptin antibody alone affected the basal or insulin-induced glucose transport. Therefore, the observed decrease of leptin activity is considered a consequence of the partial neutralization of leptin by the anti-leptin antibody.

In a second control experiment, the leptin solution was heat-denatured under different conditions and assayed for the inhibitory effect on insulin-stimulated glucose transport (Fig. 7*B*). Leptin (30 nM) decreased the fold stimulation by insulin from 13 to 2. Incubating the leptin stock solution at 95 °C for 15 min reduced the inhibitory activity of leptin restoring an insulin stimulation factor of 7. Combination of heat denaturation and centrifugation yielded a supernatant that was nearly inactive. Reduction of leptin with dithiothreitol (5 mM) and heat inactivation led to a decrease in activity that was slightly more pronounced than after heat treatment alone. The heat lability and precipitation upon boiling provides strong evidence of the proteinaceous nature of the described activity. The residual activity of the total sample observed after heat denaturation without centrifugation can best be explained by a partial renaturation of leptin in the cell culture medium during incubation with adipocytes.

Effects on Metabolic Vanadate Activity—Vanadate compounds have been extensively described as eliciting insulin-like effects in adipocytes, presumably via inhibition of tyrosine-specific protein phosphatases (38, 39). Incubation of isolated rat adipocytes with $NaVO_3$ (1 mM) for 20 min stimulated 2-deoxyglucose transport and lipogenesis (measured at 0.1 mM glucose) to up to 90% of the maximal insulin effect (data not shown). We studied whether leptin interferes with these insulin-like effects of vanadate. Adipocytes were incubated with increasing concentrations of leptin for 15 h prior to assaying stimulation of glucose transport and lipogenesis at three concentrations of vanadate. Leptin up to a concentration of 100 nM did not significantly reduce maximal (1 mM $NaVO_3$) or partial (0.2–0.5 mM $NaVO_3$) activation of glucose transport and lipogenesis. Apparently, these insulin-like activities of vanadate are not impaired by leptin indicating that vanadate bypasses the block in the insulin signaling system that is set by leptin.

Reversibility of Desensitization—Finally, we investigated whether the leptin-induced impairment of metabolic actions of insulin was reversible. Immediately after preparation, adipocyte primary cultures were kept with or without leptin (10 nM) for 6 h. They were then washed extensively and incubated for increasing periods without leptin prior to assaying the cells for basal and maximally insulin-stimulated glucose transport (Fig. 8*A*). This was done to measure the kinetics of reactivation of the insulin signaling cascade in partly desensitized adipocytes after leptin withdrawal. In adipocytes preincubated with leptin, continued cultivation after leptin withdrawal resulted in increasing rates of insulin-stimulated glucose uptake, which

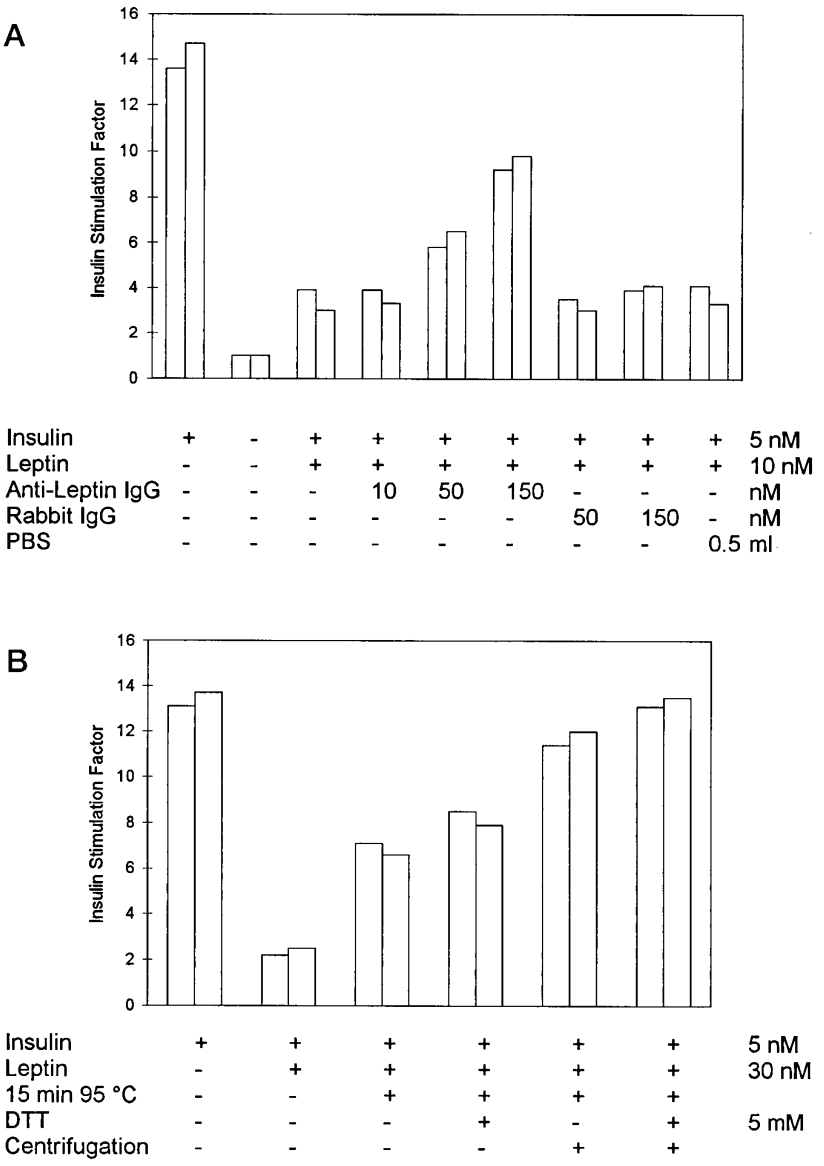

FIG. 7. **Specificity of the leptin-induced desensitization of glucose transport activation by insulin.** *A*, primary cultures of rat adipocytes were incubated for 19.5 h in the absence or presence of 10 nM leptin that had been pretreated with various concentrations of affinity purified polyclonal anti-leptin antibodies or rabbit IgG in PBS or an equivalent volume of PBS for 1 h at 4 °C or left untreated. *B*, primary cultures of rat adipocytes were incubated for 17 h in the absence or presence of 30 nM leptin that had been pretreated at 95 °C for 15 min in the absence or presence of 5 mM dithiothreitol or left untreated. Some leptin samples were centrifuged (14,000 × *g*, 15 min) as indicated, and the supernatants were added to the adipocytes. The cells were washed and then assayed for basal and insulin (5 nM)-stimulated glucose transport. Each bar represents an independent adipocyte culture with glucose transport determinations performed in triplicate.

successively approached the rates obtained with adipocytes never exposed to recombinant leptin. Thus, in these cells, insulin responsiveness increased from 3.9- to 5.9-fold within the first hour after withdrawal of leptin and, after 4 h, was almost similar to that of control adipocyte cultures. This demonstrates that effects of leptin on insulin responsiveness and sensitivity are almost completely reversible. Recovery of desensitized adipocytes occurred with a half-time of about 1 h (Fig. 8*B*). As can be seen from unchanged EC_{50} values for insulin stimulation of glucose transport, there was no significant influence of experimental manipulations or culture time on insulin sensitivity of the cells. Although maximal insulin stimulation decreased from 10.7- to 3.4-fold, the insulin effect was still sufficient for the assessment of the recovery from leptin-induced desensitization.

From this and from the experiment with vanadate, we conclude that leptin does not produce unspecific effects on the viability and metabolic activity of the cells. Furthermore, these data provide first hints of the underlying mechanisms of leptin action on isolated adipocytes.

DISCUSSION

Leptin is a signal linking the energetic status of adipose tissue to the brain control of energy homeostasis. Cloning and expression of recombinant leptin has permitted studies of the physiological role of circulating leptin. These experiments have already shown two major effects of leptin that are related to whole-body energy balance, *i.e.* (i) reduction of food intake (4, 9, 10) as well as (ii) increases in thermogenesis (40) and metabolic rate (19). Both effects seem to be mediated by hypothalamic areas that are well known for regulation of appetite, and the increases in metabolic rate can easily be explained by brown fat thermogenesis activated by the sympathetic nervous system. Until recently, direct action of leptin outside the brain was not anticipated. However, consistent with the existence of leptin receptors outside the brain (13, 20, 22), there is some indication of additional effects of leptin in peripheral tissues (41, 42). Suppression of acetyl-CoA carboxylase, lipid synthesis, and glycerol phosphate dehydrogenase in a preadipocyte cell line transfected with a leptin gene was reported recently (41).

Here, we present comprehensive biochemical data characterizing a third leptin effect that is unrelated to those contributed by the brain. Leptin impairs insulin action in isolated rat adipocytes. In these cells that are highly sensitive to insulin, leptin affects several important metabolic effects of insulin including stimulation of glucose transport, glycogen synthase, lipogenesis, and protein synthesis as well as inhibition of iso-

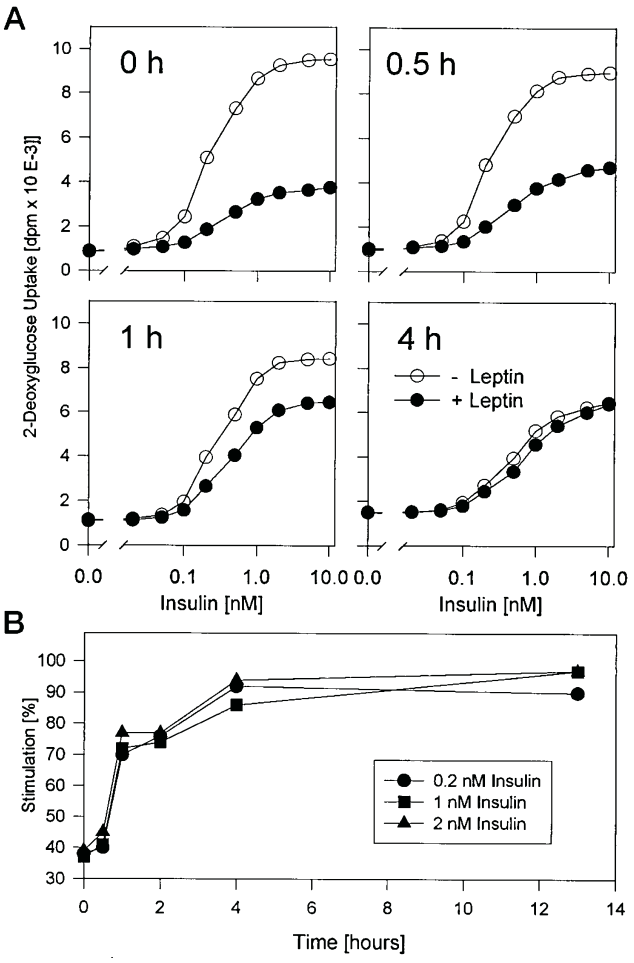

FIG. 8. **Reversibility of the leptin-induced desensitization of glucose transport stimulation by insulin.** Primary cultures of rat adipocytes were incubated for 6 h in the absence or presence of 10 nM leptin. The cells were washed three times, and the incubations were continued in the absence of leptin for the periods indicated. The cells were washed again and assayed for activation of glucose transport by various concentrations of insulin. Each point represents the mean of six determinations. *A*, glucose uptake at four selected time points for cells preincubated with (*closed circles*) and without (*open circles*) leptin (10 nM). Glucose transport activity is given as 2-[^{3}H]deoxyglucose specifically associated with the cells. *B*, recovery of adipocytes at three different insulin concentrations after withdrawal of leptin. Maximal insulin stimulation in the absence of leptin was set at 100% at each time point, respectively.

proterenol-induced lipolysis and PKA activation. Significant effects on insulin sensitivity were seen with leptin in the low nanomolar range, increasing the EC_{50} values for insulin. Higher concentrations of leptin (>1 nM) decreased the maximal response to insulin with nearly complete inhibition at >30 nM leptin. In contrast to these dramatic inhibitory actions of leptin on insulin-stimulated effects, leptin, in the absence of insulin, did not reduce glucose uptake and lipogenesis but slightly increased these effects (1.3-fold at 100 nM). Therefore, we conclude that, at least in freshly isolated rat adipocytes, leptin impairs a broad spectrum of insulin actions in a specific manner.

The pleiotropic character of the leptin effects suggests early interference with the insulin signaling cascade, close to the insulin receptor. This is compatible with the preliminary findings that leptin inhibits insulin receptor kinase activity and phosphorylation of IRS-1 following insulin stimulation in Rat-1 fibroblasts overexpressing the human insulin receptor (43). In our experiments, interaction of leptin with insulin binding to the insulin receptor is highly unlikely since leptin had been removed prior to assaying the cells for effects of insulin. Vanadate-stimulated glucose transport and lipogenesis were not affected by leptin. Although vanadate primarily inhibits phosphotyrosine phosphatases (33, 39), those phosphatases mediating the effects of vanadate on elements of the insulin signaling cascade remain unidentified. From the insensitivity of the effects of vanadate toward leptin, it is concluded that leptin acts at a site upstream of those elements. Another early interference with the insulin signaling has been found. The desensitization of 3T3-L1 adipocytes for insulin actions after long term (typically 4 d) treatment with tumor necrosis factor α was explained at least partly by increased serine phosphorylation of the insulin receptor β-subunit and/or insulin receptor substrate-1 (44, 45).

From the experiments presented here, the IC_{50} values for leptin inhibition of metabolic actions of insulin are around 3 nM. At insulin concentrations (0.1–0.5 nM) and leptin concentrations (0.5–3 nM (8, 46)) that are both in the physiological range of serum levels, a significant reduction in metabolic actions of insulin is observed *in vitro*, suggesting that leptin may also reduce insulin sensitivity under physiological conditions *in vivo*. Raised leptin serum levels in obese subjects and animals (8, 46) implicate an etiological link between leptin expression and insulin resistance, a state that is usually associated with obesity. It remains open, however, whether leptin is causally involved in this etiology or whether hyperinsulinemia or a putative leptin resistance might prevent the increased action of leptin on adipocytes, at least in subjects with manifest obesity.

Kinetic experiments demonstrate that the desensitization of adipocytes by leptin is a slow process and requires several hours. Leptin incubation of isolated adipocytes was restricted to 15–19 h due to the successive loss of insulin responsiveness at longer incubation times. This effect, however, can be clearly distinguished from leptin-induced desensitization. (i) Typically, increased culture time reduces insulin responsiveness by around 50% without any impairment of insulin sensitivity, whereas incubation with leptin produces a dose- and time-dependent decrease in both insulin responsiveness (up to 90%) and sensitivity (see Fig. 1). (ii) Culture time causes a small reduction (by 20–30%) in maximal values and a more pronounced increase in basal values (by 50–100%, Fig. 8*A*), whereas leptin selectively affects maximal values (dose- and time-dependently up to around 90%).

It is reasonable to assume that prolonged or even chronic exposure to leptin will further decrease the insulin sensitivity of adipocytes. This lends additional support to the significance of the insulin-desensitizing effect of leptin under physiological conditions.

The slow kinetics may be a first hint of a gene expression effect but does not exclude the possibility of direct interference at the level of signaling factors. Cells regain full responsiveness to insulin within a few hours after withdrawal of leptin. Thus, the molecular mechanism of desensitization consists of components being subject to rapid turnover and/or inactivation.

Leptin synthesis by adipose tissue depends on multiple factors, including both number and size of fat cells (2, 5, 47). It is remarkable that white adipose tissue itself is a sensitive target for the action of its product, leptin. This would mean an autocrine, paracrine, or endocrine action of leptin. In an auto- or paracrine loop, leptin would be synthesized by well nourished adipocytes and would protect the same or adjacent cells from further insulin-driven glucose uptake and lipid storage. In this respect, free fatty acids released by adipose tissue due to diminished antilipolytic activity of insulin are certainly energetically more important than impaired glucose utilization. As an

endocrine signal, in addition to acting on the brain, leptin could communicate the fuel state of adipocytes from one fat depot to other metabolically active tissues at distant sites, *e.g.* muscle or other adipose tissues. The minor splice variant of leptin, des-Gln^{49}-leptin, has a decreased potency as an insulin-desensitizing factor and is thus less active in this respect.

From our experiments it might be concluded that, in the interplay with insulin and catecholamines, leptin causes a catabolic net effect *in vivo*. However, we hypothesize that only the major isoform of leptin, Gln^{49}-leptin, has its primary role in redirecting fuel from adipose to other tissues, *e.g.* liver or muscle. In contrast to the well known effects on the brain, the leptin effects described in the present study are not related to whole body-energy uptake or expenditure. It still remains to be tested whether the minor form of leptin is less active in general or whether its activity is more confined to the two central effects and, in the latter case, would be more attached to the regulation of energy homeostasis.

In addition to the putative effect of Gln^{49}-leptin on the bulk flow of glucose and free fatty acids, there might be a direct stimulatory effect of leptin on the utilization of these substrates in liver and/or muscle. First hints pointing in this direction come from the observation that, in contrast to our findings on adipocytes, leptin seems to exert an insulin-like effect on glucose transport and glycogen synthesis in mouse C_2C_{12} myotubes (48) and isolated rat diaphragms.[2]

An important item for further investigations will be whether leptin impairs insulin action *in vivo*. If so, there might be a link between the high levels of leptin and the insulin resistance commonly observed in obese subjects. For the early pathogenesis of type II diabetes, in addition, elevated free fatty acid levels due to leptin action on adipose tissue would cause deterioration of β-cell function, as recently suggested (49).

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Note Added in Proof—During the reviewing process of this paper, Cohen *et al.* (Cohen, S. L., *et al.* (1996) *Science* **274,** 1185–1188) reported modulation of insulin activities by leptin in cultured liver cells. They found that leptin affects insulin signaling and antagonizes insulin-induced repression of phosphoenolpyruvate carboxykinase mRNA. It remains to be elucidated whether the observed mechanisms underlying the short term effects on insulin signal transduction observed by Cohen *et al.* and the long term effects of leptin described in our paper are related.

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