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doi:10.2337/db10-1791

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Efficacy of Metreleptin in Obese Patients With Type 2 Diabetes: Cellular and Molecular Pathways Underlying Leptin Tolerance

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OBJECTIVE—Metreleptin has been efficacious in improving metabolic control in patients with lipodystrophy, but its efficacy has not been tested in obese patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS—We studied the role of leptin in regulating the endocrine adaptation to long-term caloric deprivation and weight loss in obese diabetic subjects over 16 weeks in the context of a double-blinded, placebo-controlled, randomized trial. We then performed detailed interventional and mechanistic signaling studies in humans in vivo, ex vivo, and in vitro.

RESULTS—In obese patients with diabetes, metreleptin administration for 16 weeks did not alter body weight or circulating inflammatory markers but reduced HbA1c marginally (8.01 ± 0.93–7.96 ± 1.12, P = 0.03). Total leptin, leptin-binding protein, and antileptin antibody levels increased, limiting free leptin availability and resulting in circulating free leptin levels of ~50 ng/mL. Consistent with clinical observations, all metreleptin signaling pathways studied in human adipose tissue and peripheral blood mononuclear cells were saturable at ~50 ng/mL, with no major differences in timing or magnitude of leptin-activated STAT3 phosphorylation in tissues from male versus female or obese versus lean humans in vivo, ex vivo, or in vitro. We also observed for the first time that endoplasmic reticulum (ER) stress in human primary adipocytes inhibits leptin signaling.

CONCLUSIONS—In obese patients with diabetes, metreleptin administration did not alter body weight or circulating inflammatory markers but reduced HbA1c marginally. ER stress and the saturable nature of leptin signaling pathways play a key role in the development of leptin tolerance in obese patients with diabetes. Diabetes 60:1647–1656, 2011

Metreleptin has consistently been shown to dramatically improve insulin resistance and HbA1c in several clinical trials involving hypoleptinemic subjects with lipodystrophy, hypoleptinemia, insulin resistance, and the metabolic syndrome (1). No prior study has evaluated in detail the effect of metreleptin in obese subjects, with garden variety diabetes, obesity, and high circulating leptin levels, who are presumably resistant or tolerant to the effects of leptin (2). Furthermore, no prior study has evaluated mechanisms underlying such leptin tolerance.

In the context of a large, randomized, placebo-controlled trial, we examined for the first time the efficacy of metreleptin in regulating body weight, glycemic control, and immune function in hyperleptinemic obese subjects with type 2 diabetes. We subsequently examined whether the observed suboptimal efficacy of circulating leptin in regulating adiposity and immune function in obese diabetic individuals is attributable to specific, identifiable mechanisms at the cellular and molecular level. In this respect, we methodically explored mechanisms previously shown to underlie other hormone resistance syndromes, e.g., insulin resistance or underlying immunogenicity seen with use of other biologics. To further elucidate the role of leptin in regulating human adiposity and immune function and to study potential mechanisms underlying the development of leptin resistance or tolerance, we then performed detailed interventional and mechanistic signaling studies in humans in vivo, ex vivo, and in vitro.

More specifically, we first discovered that levels of leptin-binding protein (LBP) and antibodies against metreleptin increased in response to metreleptin treatment, limiting circulating free leptin to ~50 ng/mL despite total leptin levels of ~982.7 ng/mL in obese diabetic subjects. We then proceeded to study whether mechanisms that have been described to affect leptin signaling and thus leptin resistance in mice, i.e., endoplasmic reticulum (ER) stress (3–6), are also operative in humans. Subsequently, we investigated intracellular leptin signaling in vivo in response to metreleptin administration in lean and obese subjects by comparatively studying metreleptin signaling in human adipose tissue (hAT) and human peripheral blood mononuclear cells (hPBMCs) from both lean and obese humans in vivo. Finally, we extended these observations by studying leptin signaling in vitro and ex vivo in hAT and hPBMCs from lean and obese subjects to determine whether neuroendocrine changes induced by metreleptin in vivo or paracrine mechanisms ex vivo may differentially

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Received 28 December 2010 and accepted 16 March 2011.

DOI: 10.2337/db10-1791. Clinical trial reg. no. NCT01275053, clinicaltrials.gov.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db10-1791/-/DC1.

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affect leptin signaling in humans in vivo versus ex vivo or in vitro.

RESEARCH DESIGN AND METHODS

Clinical study I: Body weight, metabolic, and immune responses to metreleptin versus placebo in obese hyperleptinemic subjects with diabetes. We studied 71 obese subjects (41 male and 30 female; age, 53.3 ± 11.4 years; BMI, 33.1 ± 2.8 kg/m²) with diet-controlled type 2 diabetes who gave written informed consent to participate in the study. Inclusion criteria for participation in the study included HbA1c between 7 and 11%, BMI between 27 and 40 kg/m², and no significant evidence of stable weight—maintaining diet for at least 4 weeks before the screening evaluation. Subjects could not have taken oral hypoglycemic agents or insulin in the 12 weeks preceding the screening evaluation. Subjects were randomized in a 2:1 ratio to receive metreleptin or placebo, respectively, at a dose of 10 mg twice daily (morning and evening) by subcutaneous injection for 4 months (16 weeks), resulting in a total daily dose of 20 mg metreleptin. Blood samples were obtained at baseline (before metreleptin or placebo treatment) and after 4 and 16 weeks of treatment (with the exception of nine subjects who received metreleptin and six subjects who received placebo because of insufficient serum). Samples were stored at –70°C until assayed for the measurement of leptin, LBP, free leptin, antibody titers, inflammatory marker, and HgbA1c.

Clinical study II: In vivo metreleptin signaling in hAT and hPBMCs from lean and obese subjects. Normal volunteers were recruited from the community and screened at the Clinical Research Center at Beth Israel Deaconess Medical Center (BIDMC). Subjects were excluded if they had a history of any illness, other than obesity, that may affect insulin sensitivity, use of medications that are known to influence glucose metabolism, history of anaphylaxis or anaphylactoid-like reactions, or a known hypersensitivity to Escherichia coli derived proteins or other agents, such as lidocaine or procaine hydrochloride (Novocaine; Hospira, Inc., Lake Forest, IL). Subjects were provided with take-home meals and consumed an isocaloric diet, specifically designed for each subject, for 48 h before their main study visit to ensure stable dietary intake. On the morning of the main study visit, subjects attended the Clinical Research Center after a 12-h fast. An intravenous cannula was placed in each antecubital fossa, and samples were drawn for baseline laboratory tests. The skin of the lower abdomen was anesthetized using lidocaine, and by using an aseptic technique, a core of adipose tissue was obtained using a core biopsy instrument. The tissue sample was immediately placed in a cryotube and frozen in liquid nitrogen at the bedside. After baseline laboratory tests and baseline hAT biopsy, an intravenous bolus of metreleptin (dose 0.01 mg/kg body weight) or placebo (10 mL normal saline) was given by slow intravenous injection >1 min. The subject rested in supine position. Thirty minutes later, the adipose-tissue biopsy was repeated for metreleptin signaling experiments in hPBMCs. Serum and hAT samples were stored in liquid nitrogen in batches until later analysis. For the in vivo metreleptin signaling study, we performed experiments with hAT and hPBMCs before and after a bolus of metreleptin was administered to six lean (BMI 23.7 ± 0.66 kg/m²) and six obese (BMI 35.4 ± 2.7 kg/m²) subjects. There was no such difference in the lean (lean 41 ± 13.3, obese 43.3 ± 10.78, control 33.17 ± 14.12 years, P = 0.37) between the groups. Subjects in the lean group had lower waist circumference (87 ± 5.68 vs. 111.8 ± 25.5 cm, P = 0.004) and leptin levels (2.94 ± 3.16 vs. 11.3 ± 9.28 ng/mL, P = 0.004) compared with the obese group at baseline. All subjects provided written informed consent to participate, and the study was approved by the institutional review board at BIDMC.

Laboratory studies

Ex vivo metreleptin signaling study hAT—For the ex vivo hAT metreleptin signaling study, we used discarded hAT from subjects undergoing laparoscopic adjustable gastric band, liposuction, or abdominoplasty at the BIDMC. The age and BMI of the subjects, but no other identifiable information, were recorded for each subject. Samples were solubilized for analysis of phosphorylation of signaling proteins as described previously (7).

hPBMCs—The hPBMCs were isolated as described previously (8).

In vitro metreleptin signaling study Human primary adipocyte culture. Subcutaneous and omental hAT samples were obtained from lean (aged 35–41 years, BMI 22–25 kg/m²) and obese (aged 34–48 years, BMI 30–50 kg/m²) men and women, respectively. The human primary adipocyte (hPA) culture was performed as described previously (9).

Protein extraction and Western blotting. Protein extraction and Western blotting were performed as described previously (10).

Gene Expression Omnibus datasets analysis of published data. To compare expression of inhibitors of leptin signaling in hAT from lean and obese subjects, including SOCS3 and PTP1B, we performed a search of publicly available data on the Gene Expression Omnibus datasets website (11) using the search terms “adipose tissue” and “adipocyte.” We excluded nonhuman datasets. We searched the results for datasets comparing gene expression profiles in lean and obese humans. We found one dataset with readily available data that matched our search criteria. We extracted gene expression data for SOCS3 and PTP1B and computed the fold differences in expression between subjects.

Detection of antileptin antibodies and their functional/biochemical activity. Levels of serum antileptin antibodies. Levels of serum antileptin antibodies were determined with an in-house developed colorimetric sandwich enzyme–linked immunosorbent assay (ELISA). In brief, 50 µL metreleptin at a final concentration of 10 µg/mL in PBS, pH 7.4, was plated onto 96-well ELISA plate (PBI International, Inc., Sparta, MI, USA). After 16 h at 4°C, the plates were extensively washed with PBS-0.05% Tween 20, blocked with 200 µL PBS/10% FCS for 2 h, and repeatedly washed. Diluted sera in PBS-0.05% Tween 20/10% FCS (from 1/10 to 1/1,000) were added at 100 µL/well and incubated for 4 h at room temperature. After five washes, goat anti-human polyclonal immunoglobulins, such as alkaline phosphatase–conjugated antibodies (Sigma–Aldrich, St. Louis, MO), were added 100 µL/well for 1 h. The reaction was developed with Sigma-Fast p-nitrophenyl phosphate and alkaline phosphatase substrate (PNP) (Sigma–Aldrich) and read after 30 min at 405 nm in an ELISA plate-reader (Bio-Rad Laboratories, Hercules, CA). Standard curves of antileptin antibodies were developed in each assay using an antileptin monoclonal antibody (mAb) generated in our laboratory (mAb 971212). Quantification of optical density values was performed after extrapolation from standard curves of purified antileptin antibodies.

Functional activity of antileptin antibodies. The functional activity of antileptin antibodies was assessed with the human leptin-receptor (hLepR)-transfected BAF3 cell line, provided by Dr. Arieh Geltman (The Hebrew University, Rehovot, Israel). In brief, because hLepR/BAF3 cell proliferation is leptin-dependent, hLepR/BAF3 cells were cultured in flat-bottom 96-well microtiter plates (Becton-Dickinson Falcon, Franklin Lakes, NJ) at a density of 5 × 10⁴ cells/well in a total volume of 100 µL RPMI-1640 medium supplemented with 2% FCS (HyClone-Pierce; Thermo Fisher Scientific, Inc., Rockford, IL), 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (Life Technologies, Inc., Carlsbad, CA). Cells were cultured at 37°C in 100% humidity and 5% CO₂ in the presence of increasing doses of metreleptin ranging from 0.01 to 10 ng/mL. Purified IgGs from sera of metreleptin-treated and placebo-treated healthy controls, and from sera of lean and obese subjects, were added to cells in all the different conditions at a final concentration of 50 µg/mL. After 48 h, [3H]thymidine (0.5 µCi/well) (Perkin-Elmer, Milano, Italy) was added to the cultures, and cells were harvested after 12 h. Radioactivity was measured with a beta-cell plate scintillation counter (Wallac, Gaithersburg, MD). As standard of leptin neutralization, anti-human leptin mAb 971212 was used at increasing concentrations usually from 0.1 to 25 µg/mL.

Capacity of antileptin IgGs to reproduce the biochemical level. We also assessed at the biochemical level the capacity of antileptin IgGs, isolated from lean or placebo-treated subjects and healthy controls, to affect LepR signaling in BAF3 cells. In brief, hLepR/BAF3 cells were incubated for 1 h at 37°C with recombinant leptin at 2 ng/mL in the presence or absence of IgGs (50 µg/mL) purified from leptin- or placebo-treated subjects and healthy controls. After the 1-h pretreatment, cell lysates were generated to perform Western blotting analyses for STAT3 phosphorylation as a readout of LepR signaling.

Immunocytochemistry. Immunodetection was performed as described previously (12).

Blood sample measurements. Leptin and LBP were analyzed by ELISA as described previously (13). Free leptin was measured using radioimmunoassay for study (Linco Research, Inc., St. Charles, MO; sensitivity, 0.5 ng/mL; coefficient of variation [CV], 6–7%) and by immunodiagnostic assay for study (Diagnostics Systems Laboratory [DSL], Webster, TX; sensitivity, 0.1 ng/mL, CV, 3.7–6.0%). First, 100 µL of unknown serum samples were incubated at 37°C in a water bath for 2 h; 100 µL polyethylene glycol (Immucor, Inc., Norcross, GA) was then added to standards, subjects, and unknowns, vortexed for 1 min, and allowed to sit at room temperature for 10 min. Standards, samples, and controls were centrifuged to remove precipitation, cell lysates were generated to perform Western blotting analyses for STAT3 phosphorylation as a readout of LepR signaling.
Statistical analysis. For clinical study I, between-group comparison of placebo- and metreleptin-treated subjects on change in study variable measures over time were determined using repeated-measures analysis, allowing for use of all available data. P values for difference between groups at baseline and each follow-up were determined by one-way ANOVA, P values for difference within group at baseline and each follow-up were determined by one-way ANOVA followed by a least significant difference test. Pearson correlation coefficients were calculated between changes of study variables. All data were presented as means ± SE, P < 0.05 was considered statistically significant. Intention-to-treat analysis revealed similar results (data not shown). For the in vivo signaling study, baseline comparisons among the three groups were made using a nonparametric Kruskal-Wallis test. The ratios of phosphorylated protein to total protein were compared before and 30 min after metreleptin bolus, using a nonparametric, paired Wilcoxon signed rank test. To compare pSTAT3 change between lean and obese subjects, the fold change was compared between the groups using a Wilcoxon rank sum test. For the ex vivo and the in vitro signaling study, data were analyzed with one-way ANOVA followed by post hoc test for multiple comparisons. Analyses were carried out using SPSS (version 11.5, SPSS, Inc., Chicago, IL) and SAS (version 9; SAS Institute, Inc., Cary, NC).

RESULTS

Clinical study I: Body weight, metabolic, and immune responses to metreleptin versus placebo treatment in obese hyperleptinemic subjects with diabetes. Circulating leptin levels increased significantly in men and women treated with metreleptin over the 4-month study period (Table 1, Supplementary Appendix 1). Non-neutralizing antileptin antibodies developed with increasing study period (Table 1, Supplementary Appendix 1). Non- and women treated with metreleptin over the 4-month interval improved in glycemic control (HbA1c in metreleptin-treated patients and resulted in only a small differential weight loss or changes in in leptin antibodies. The increase in free leptin levels to ~48.4 ng/mL was not associated with any significant differential weight loss or changes in inflammatory markers in metreleptin-treated patients and resulted in only a small but differential improvement in glycemic control (HbA1c from 8.01 ± 0.93 to 7.96 ± 1.12, P = 0.03). Changes of leptin and free leptin levels were not correlated with changes of body weight or inflammatory markers studied (Table 2). Baseline TNFR-II and monocyte chemoattractant protein-1 levels were positively correlated with circulating leptin levels (r = 0.99, P < 0.0001), circulating free leptin levels (r = 0.44, P = 0.012, SI 2) were also increased. This indicates that increasing doses of leptin can break through the resistance caused by increasing binding of leptin by leptin antibodies. The increase in free leptin levels to ~50 ng/mL was not associated with any significant differential weight loss or changes in inflammatory markers in metreleptin-treated patients and resulted in only a small but differential improvement in glycemic control. The stimulated activation of SOCS3 by ex vivo metreleptin administration in hAT and hPBMCs from lean and obese subjects

No differential activation of STAT3 signaling by ex vivo metreleptin administration in hAT and hPBMC. Dose-response curves showed that administration of up to 50 ng/mL metreleptin for 30 min significantly induced phosphorylation of STAT3 in subcutaneous and omental hAT from obese male and female subjects (Fig. 2A and B). These results were similar to what was observed in hAT and hPBMCs after in vitro metreleptin administration (Supplementary Appendix 3A and 3B). Similar to in vivo observations, there was no significant difference in p-STAT3 expression in hAT from obese versus lean subjects (Fig. 2C and D). The phosphorylated form of STAT3 was increased in metreleptin-stimulated hPBMCs (Fig. 2E and F) compared with control, showing that activation was evident as early as 5 min after ex vivo stimulation with metreleptin.

No differential activation of MAPK signaling by ex vivo metreleptin administration in hAT. Ex vivo metreleptin administration stimulated activation of MAPK by ~3.1-fold in hAT (Fig. 2F) from obese male and female subjects. There was no difference in MAPK activation from subcutaneous versus omental, male versus female, and obese versus lean subjects.

No differential expression of inhibitors of ex vivo metreleptin signaling in hAT. We observed no activation of SOCS3 by ex vivo metreleptin administration in hAT (Supplementary Appendix 4). There was no difference in SOCS3 activation from subcutaneous versus omental, male versus female, and obese versus lean subjects.
**Table 1**
Clinical study I: Study variables for obese, diabetic subjects taking placebo (n = 21) or metreleptin (n = 50) at baseline and 4 and 16 weeks of follow-up and % change in study variables from baseline to 16 weeks.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo-treated</th>
<th></th>
<th>Leptin-treated</th>
<th></th>
<th>P value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ± SE</td>
<td>%</td>
<td>n</td>
<td>Mean ± SE</td>
<td>%</td>
</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>32.8 ± 0.7</td>
<td>50</td>
<td>32.7 ± 0.5</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>19</td>
<td>32.8 ± 0.7</td>
<td>47</td>
<td>32.5 ± 0.5</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>Change after 16 wk</td>
<td>19</td>
<td>−0.5 ± 0.2</td>
<td>45</td>
<td>−0.7 ± 0.1</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>18</td>
<td>38.0 ± 6.4</td>
<td>48</td>
<td>35.2 ± 3.5</td>
<td>0.27</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LBP (ng/mL)</td>
<td>20</td>
<td>25.3 ± 1.7</td>
<td>49</td>
<td>25.0 ± 1.2</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>Free leptin (ng/mL)</td>
<td>15</td>
<td>15.8 ± 3.3</td>
<td>32</td>
<td>22.6 ± 4.7</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>Antibody titer (μg/mL)</td>
<td>7</td>
<td>0.0 ± 0.0</td>
<td>16</td>
<td>5.3 ± 4.0</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>20</td>
<td>3.2 ± 0.2</td>
<td>49</td>
<td>3.7 ± 0.4</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>CRP (μg/L)</td>
<td>20</td>
<td>9,356 ± 2,059</td>
<td>48</td>
<td>9,138 ± 1,172</td>
<td>0.95</td>
<td></td>
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<tr>
<td>stTNFR-I (pg/mL)</td>
<td>20</td>
<td>1,058 ± 63.1</td>
<td>49</td>
<td>1,129 ± 45.7</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>stTNFR-II (pg/mL)</td>
<td>20</td>
<td>2,089 ± 166.9</td>
<td>49</td>
<td>2,470 ± 111.0</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>11</td>
<td>10.4 ± 2.0</td>
<td>27</td>
<td>8.9 ± 1.0</td>
<td>0.56</td>
<td></td>
</tr>
</tbody>
</table>

BMI, body mass index. sICAM, soluble intracellular adhesive molecule. *Data presented are on treatment. The number of subjects is variable because of occasional missing blood test results from a few subjects in a small numbers of analytes as shown. The change after 16 weeks is based on pairwise difference of subjects who had data available for both baseline and week 16 time points. Means ± SEs are presented for each variable at each follow-up visit. *P values are calculated from one-way ANOVA. **P values are calculated from repeated-measures analysis evaluating the change in study variables over time, adjusted for age and sex.
<table>
<thead>
<tr>
<th>Change in variable</th>
<th>Leptin</th>
<th>Free leptin</th>
<th>Leptin antibody</th>
<th>LBP</th>
<th>IL-6</th>
<th>CRP</th>
<th>sTNFR-I</th>
<th>sTNFR-II</th>
<th>MCP1</th>
<th>sICAM-1</th>
<th>IL-10</th>
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<tbody>
<tr>
<td>BMI</td>
<td>r</td>
<td>−0.08</td>
<td>−0.17</td>
<td>−0.11</td>
<td>0.11</td>
<td>0.20</td>
<td>0.00</td>
<td>−0.04</td>
<td>−0.02</td>
<td>0.22</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.531</td>
<td>0.372</td>
<td>0.812</td>
<td>0.395</td>
<td>0.122</td>
<td>0.991</td>
<td>0.754</td>
<td>0.866</td>
<td>0.113</td>
<td>0.432</td>
</tr>
<tr>
<td>Leptin</td>
<td>r</td>
<td>0.44</td>
<td>0.99</td>
<td>0.00</td>
<td>0.02</td>
<td>−0.17</td>
<td>0.00</td>
<td>−0.07</td>
<td>0.02</td>
<td>−0.16</td>
<td>−0.02</td>
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<tr>
<td></td>
<td>P value</td>
<td>0.012</td>
<td>&lt;0.0001</td>
<td>0.975</td>
<td>0.9</td>
<td>0.16</td>
<td>0.999</td>
<td>0.583</td>
<td>0.85</td>
<td>0.203</td>
<td>0.932</td>
</tr>
<tr>
<td>Free leptin</td>
<td>r</td>
<td>0.22</td>
<td>−0.17</td>
<td>−0.13</td>
<td>−0.22</td>
<td>−0.04</td>
<td>0.18</td>
<td>0.16</td>
<td>−0.05</td>
<td>0.29</td>
<td></td>
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<tr>
<td></td>
<td>P value</td>
<td>0.601</td>
<td>0.363</td>
<td>0.494</td>
<td>0.236</td>
<td>0.808</td>
<td>0.325</td>
<td>0.4</td>
<td>0.782</td>
<td>0.274</td>
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<tr>
<td>Leptin antibody</td>
<td>r</td>
<td>0.31</td>
<td>0.09</td>
<td>−0.25</td>
<td>0.14</td>
<td>0.15</td>
<td>0.18</td>
<td>−0.07</td>
<td>−0.13</td>
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<tr>
<td></td>
<td>P value</td>
<td>0.416</td>
<td>0.827</td>
<td>0.521</td>
<td>0.728</td>
<td>0.699</td>
<td>0.641</td>
<td>0.853</td>
<td>0.806</td>
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<tr>
<td>LBP</td>
<td>r</td>
<td>0.14</td>
<td>0.44</td>
<td>0.25</td>
<td>0.18</td>
<td>−0.08</td>
<td>0.26</td>
<td>−0.13</td>
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<tr>
<td></td>
<td>P value</td>
<td>0.241</td>
<td>0.0002</td>
<td>0.043</td>
<td>0.132</td>
<td>0.522</td>
<td>0.031</td>
<td>0.503</td>
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<td>IL-6</td>
<td>r</td>
<td>0.30</td>
<td>0.21</td>
<td>0.25</td>
<td>0.07</td>
<td>0.20</td>
<td>0.22</td>
<td></td>
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<tr>
<td></td>
<td>P value</td>
<td>0.015</td>
<td>0.081</td>
<td>0.041</td>
<td>0.619</td>
<td>0.109</td>
<td>0.27</td>
<td></td>
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</tr>
<tr>
<td>CRP</td>
<td>r</td>
<td>0.43</td>
<td>0.47</td>
<td>0.05</td>
<td>0.49</td>
<td>0.26</td>
<td></td>
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MCP, monocyte chemoattractant protein; sICAM, soluble intracellular adhesive molecule.

**No differential activation of AMPK signaling by ex vivo metreleptin administration in hAT and hPBMC.** Ex vivo metreleptin administration stimulated phosphorylation of AMPK in both subcutaneous and omental hAT, and hPBMCs from obese female subjects (Supplementary Appendix 5). We observed no difference in AMPK activation from subcutaneous versus omental, obese versus lean, and male versus female subjects.

**Laboratory study III: In vitro metreleptin signaling in subcutaneous and omental hPAs from lean and obese subjects**

**No differential activation of STAT3 signaling by in vitro metreleptin administration in hPA.** In vitro metreleptin administration significantly induced phosphorylation of STAT3 by ~2.1-fold at 10 min with a trend toward greater induction at 20–40 min (Fig. 3A and B). A considerable amount of p-STAT3 in metreleptin-treated cells, but only background levels of p-STAT3 in control cells, was detected in both subcutaneous and omental hPA (Fig. 3C). However, these effects were totally blocked by pretreatment with AG490, a STAT3 inhibitor (Fig. 3D), suggesting that metreleptin stimulation activates STAT3 signaling in hPA. In addition, we observed nuclear translocation of STAT3 by in vitro metreleptin administration in a dose-dependent manner (Supplementary Appendix 6). Metreleptin signaling pathways in hPA were saturable at a level of ~50 ng/mL, and these results are consistent with those observed in hAT in vivo and ex vivo. There were no significant differences in STAT3 activation from subcutaneous versus omental, male versus female, and obese versus lean subjects.

**No differential expression of inhibitors of metreleptin signaling in hPA.** We observed no early activation of SOCS3 by in vitro metreleptin administration in hPA (Fig. 3F). In addition, we observed no differences in expression of inhibitors from subcutaneous versus omental, obese versus lean, and male versus female subjects.

**Downregulation of in vitro metreleptin-stimulated STAT3 signaling by ER stress in hPA.** Stimulation of the cells with metreleptin led to a marked increase in phosphorylation of STAT3, but when challenged with ER stress (tunicamycin and dithiothreitol), the metreleptin-activated STAT3 phosphorylation was abolished totally (Supplementary Appendix 7). We observed no differences in ER stress–mediated STAT3 phosphorylation from subcutaneous versus omental, male versus female, and obese versus lean subjects.

**Laboratory study IV: Gene Expression Omnibus datasets analysis of published data on expression of inhibitors of metreleptin signaling.** To validate our in vivo, ex vivo, and in vitro metreleptin signaling data, we used publicly available datasets to examine expression of potential inhibitors of leptin signaling in hAT from lean and obese nondiabetic humans. We analyzed a dataset
comparing gene expression profiles from subcutaneous adipocytes from 20 lean and 19 obese nondiabetic Pima Indians (14), and there was no significant difference in the expression levels of PTP1B or SOCS3.

**DISCUSSION**

Several open-label trials have demonstrated that administering metreleptin to correct overt hypoleptinemia significantly improves the metabolic abnormalities and insulin resistance in patients with congenital lipodystrophy and insulin resistance (1). Likewise, randomized placebo-controlled trials in patients with human immunodeficiency virus–induced lipodystrophy and the metabolic syndrome who are also hypoleptinemic, although less so than subjects with congenital lipodystrophy, have shown significant, albeit less pronounced, effects of metreleptin (15,16). In contrast, obese hyperleptinemic subjects do not respond to exogenously administered leptin (15,16). We studied whether metreleptin administration would be effective in hyperleptinemic obese subjects with garden variety insulin resistance and diabetes who are considered leptin-resistant or -tolerant. We observed only minor improvements in the glycemic control of hyperleptinemic/diabetic subjects in this randomized trial, which, albeit statistically significant, are one order of magnitude less pronounced than those observed in hypoleptinemic, lipodystrophic subjects and apparently are not of major clinical importance (2).

Final body weight and levels of inflammatory markers remained completely unaltered in metreleptin-treated hyperleptinemic obese diabetic subjects. This lack of metreleptin’s efficacy is consistent with a state of “resistance” or “tolerance” to leptin action, defined as an inability of increasing leptin levels to reduce body weight in obese individuals (17). This is the first study in obese diabetic subjects that proves the existence of clinical leptin resistance or tolerance similar to the only prior study in obese nondiabetic subjects (17). Moreover, this study demonstrated no inflammatory response to leptin, with only a minor glycemic response of no apparent clinical significance. Thus, we initiated studies to elucidate mechanisms underlying leptin resistance or tolerance.

We first explored whether mechanisms similar to those involved in other hormone resistance syndromes, such as the increase in binding protein levels, the development of antibodies, the presence of a saturable signaling system, or the presence of in vivo or ex vivo signaling inhibitors in humans, may underlie leptin resistance. A novel finding of this study was that LBP, the extracellular cleaved part of the long isoform of the leptin receptor, and the titers of antileptin antibodies increased significantly in response to metreleptin administration, but not in response to placebo administration. We previously demonstrated that short-term caloric deprivation (72 h) significantly decreases leptin levels but significantly increases LBP by >100% (18). The current study shows that LBP levels increase in response to several months of pharmacologic metreleptin versus placebo treatment. We also observed for the first time the development of non-neutralizing, leptin–binding antibodies, the titers of which increased in the circulation over time.

**FIG. 1.** Laboratory study I. Agonistic/stimulatory activity of antileptin antibodies generated during metreleptin administration. A–C: The functional activity of antileptin antibodies in hLepR+BAF3 cells was as described in detail in RESEARCH DESIGN AND METHODS. Leptin + IgG posttreatment; ◆, Leptin + IgG pretreatment. D–F: The biochemical level of the capacity of antileptin IgGs in hLepR+BAF3 cells was studied as described in detail in RESEARCH DESIGN AND METHODS. All density values for each protein band of interest are expressed as a fold increase. Data were analyzed using one-way ANOVA followed by post hoc test for multiple comparisons. Values are means (n = 6) ± SD. Means with different letters are significantly different, P < 0.05. L.N.C., lean normal control.
in the majority of subjects treated with metreleptin. The distinct possibility exists that other formulations of leptin that do not have the amino acid (methionine) substitution, which in the case of metreleptin was added to improve protein folding, but have full sequence homology with human leptin may not induce leptin antibodies; this possibility remains to be studied. In addition, because pharmacologic metreleptin doses (0.2 mg/kg in the current study) may have different effects than physiologic doses in terms of generating antibodies (C.S.M., unpublished data), future placebo-controlled randomized studies involving metreleptin administration in lower, physiologic replacement doses are needed. In any case, the apparent importance of this novel finding is that the majority of circulating leptin is bound to antileptin antibodies, whereas only a small fraction of circulating leptin is free leptin. Thus, we proceeded to explore whether this observation could also be of clinical significance.
Despite increasing LBP and antibody levels, the levels of free leptin increased and remained relatively higher (i.e., at levels ~40–50 ng/mL) in metreleptin-treated subjects. Although these levels are higher than the putative threshold for saturating the blood-brain-barrier leptin transport system (19,20), circulating free leptin levels did not correlate with weight loss, indicating clinical ineffectiveness of free leptin levels in the ~40–50 ng/mL range. Thus, we decided to study whether metreleptin administration in doses within or above the physiologic leptin range, and encompassing the ~50 ng/mL levels seen in our clinical studies described, can differentially activate signaling pathways in humans in vivo, ex vivo, and in vitro. It has been shown that leptin induces STAT3 phosphorylation in various mouse cell lines and tissues and is generally accepted that this represents a major signaling pathway through which leptin exerts its actions (7, 21–26). Despite minor differences in the timing of signaling pathway activation, we observed no major
differences in the magnitude of STAT3 activation in response to metreleptin administration in the human peripheral tissues studied in vivo, ex vivo, or in vitro. We observed no differences in leptin-activated signaling pathways when comparing obese versus lean subjects or men versus women in vivo, ex vivo, or in vitro. More important, metreleptin signaling pathways were saturable at a level of ~50 ng/mL, suggesting that above that level, i.e., the level clinically seen in obese subjects at baseline, no additional signaling effect can be observed. This explains the effectiveness of metreleptin in subjects with very low circulating leptin levels and the tolerance to metreleptin’s actions when the baseline circulating level is closer to the 40–50 ng/mL range.

We then focused on ER stress, which has recently been shown to play a role in the development of leptin resistance in the hypothalamus of rodents (2). It has been suggested that ER capacity is directly related to leptin sensitivity (3,4,27), and thus, it has been proposed that ER stress reversal could be used as a strategy to sensitize obese mice and, by extension, humans to leptin. These previous studies have shown that the reduction in ER function creates ER stress, blocks leptin action, and generates leptin resistance in mice, suggesting that ER stress provides a potential mechanism for the development of leptin resistance in which increased ER stress antagonizes and inhibits leptin-mediated STAT3 signaling at a step upstream of STAT3 phosphorylation (27). Because ER stress cannot yet be studied in humans in vivo, we performed in vitro metreleptin signaling studies in hPAs to explore whether ER stress could underlie the development of leptin resistance in human primary cells. We report for the first time that ER stress limits leptin signaling in hPAs in vitro, indicating that ER stress may induce leptin resistance in humans similar to induction of leptin resistance in mice in vivo (3,4) and suggesting that improving ER stress could be used as a strategy to sensitize not only obese mice (3,27) but also humans to metreleptin. Because in vivo leptin actions may differ in comparison with in vitro, studies of in vivo leptin signaling in humans are needed to prove or disprove this hypothesis, but it is currently impossible to perform human in vivo ER stress studies.

The blinded and simultaneous administration of metreleptin or placebo in the current study provides a clear and contemporaneous assessment of the role of metreleptin in regulating body weight, metabolic, and immune function in obese diabetic individuals. It could be argued that metreleptin may have different actions in the pharmacologic range (as used in the current study) than in the physiologic range, in terms of generating antileptin antibodies or possibly by resulting in differential downregulation of leptin receptors. It is unlikely that the latter is the case in the current study because we observed an increase in LBP, which reflects an increased number of cell surface receptors (18). The possible differences between the effects of pharmacologic and physiologic doses of metreleptin, however, need to be studied by future well-designed clinical trials, in which significant weight loss will be induced and replacement dose of metreleptin will be administered and studied in relation to weight maintenance. Finally, although it is difficult to perform in vivo time course signaling experiments in humans, this is an area that also needs to be addressed in future studies. Future in vivo leptin signaling studies involving additional signaling pathways and other peripheral human tissues are also needed. However, leptin signaling in central nervous system tissues (i.e., hypothalami of humans) cannot be studied in vivo or ex vivo, and thus we and others have initiated indirect studies of leptin’s actions in the brain using functional magnetic resonance imaging techniques. It is also possible that inducers of leptin resistance other than those in the current study may exist in humans and corresponding leptin sensitizers, if any, remain to be identified.

We believe that beginning to elucidate the mechanisms underlying the physiologic and pharmacologic actions of metreleptin, using studies with direct relevance to humans as those presented in this article, offers distinct advantages over the studies in rodents that have been published to date. These initial translational studies in humans are of direct potential clinical and therapeutic significance.

In summary, in obese patients with diabetes, metreleptin administration for 16 weeks did not alter body weight or circulating inflammatory markers but reduced HbA1c marginally. Furthermore, total leptin, LBP, and antileptin antibody levels increased, limiting free leptin availability and resulting in circulating free leptin levels of ~50 ng/mL. These data identify several steps mediating leptin “tolerance” in humans. Most important, we demonstrate for the first time the saturable nature of leptin signaling pathways in humans and the inhibition of leptin signaling by environmental factors inducing ER stress that contribute significantly to the development of leptin tolerance in obese humans. The mechanisms reported lend themselves to future studies with an ultimate goal of identifying and overcoming leptin resistance in the path toward developing novel therapies for the treatment of excess adiposity and associated abnormalities in humans.

ACKNOWLEDGMENTS
The current study was supported by grant F32-DK64550-01A1 (G.K.S.); the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases grants DK081913, DK79929, DK58785, and AG032030 (C.S.M.); and a Veterans Affairs Merit Review grant (C.S.M.). The Mantzoros Laboratory is also supported by a discretionary grant from the Beth Israel Deaconess Medical Center (BIDMC). G.M. is supported by grants from the EU Ideas Programme, ERC-Starting Independent Grant “LeptinMS” 202579, and Telethon-Juvenile Diabetes Research Foundation Grant GJT08004. The studies were also supported by Amgen and grants UL1-RR-025758 and M01-RR-01032, Harvard Clinical and Translational Science Center, from the National Center for Research Resources.

A.M.D. was an employee of Amgen. Amgen and Amylin Pharmaceuticals supplied metreleptin for this study and approved the clinical studies presented but had no role in interpretation of the data or the preparation, review, or approval of the article. No other potential conflicts of interest relevant to this article were reported.

H.S.M. and C.S.M. wrote the article; H.S.M., G.M., A.M.B., J.P.C., X.L., G.H.M., C.J.W., L.A., F.C., B.E.S., A.M.D., C.G.F., and C.S.M. participated in the performance and coordination of the study; A.M.D. conceived an initial version of clinical study I; and C.S.M. conceived and designed the studies as presented. All authors read and approved the final article.

The authors thank Dr. Young-Bum Kim, Division of Endocrinology, Diabetes, and Metabolism, BIDMC, Harvard Medical School, for technical assistance in the ex vivo

ACKNOWLEDGMENTS
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