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.

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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 immunogenecity 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...
In vitro metreleptin signaling study

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, 33.2 ± 3.8 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 absence of a 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 titer, inflammatory marker, and HbA1c.

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 anesthetic agents, such as lidocaine or procaine hydrochloride (Novocain; 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 subject. Research Center after a 12-h fast. An intravenous cannula was placed in each subject. After baseline laboratory tests and 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 ± 1.09 kg/m²) subjects. There was no such difference in lean (lean, 63.8 ± 6.3 kg/m², obese 31.48 ± 6.1, control 25.85 ± 3.98 µg/mL, P = 0.07) or age (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 ± 12 cm, P = 0.004) and leptin levels (2.94 ± 3.16 vs. 11.3 ± 9.28 ng/mL, P = 0.04) 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, and gastric bypass surgery treated for obesity. For this study, we used a tissue bank system at the BIDMC.

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 39–50 kg/m²) men and women, respectively. The human primary adipocyte (hAD) 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 (12) from the repository and compared gene expression levels in lean and obese subjects.

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 ng/mL in PBS, pH 7.4, was placed in 96-well ELISA plate (PBI International, Spa, Milano, Italy). 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 polyvalent 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 (PNPP) (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 antileptin antibodies.

Functional activity of antileptin antibodies. The functional activity of antileptin antibodies was assessed with the human leptin-receptor (hLепR)-transfected BAF3 cell line, provided by Dr. Arieh Gertler (The Hebrew University, Rehovot, Israel). In brief, because hLепR+BAF3 cell proliferation is leptin-dependent, human hLепR+BAF3 cells were cultured in flat-bottom 96-well microtiter plates (Becton-Dickinson Falcon, Franklin Lakes, NJ) at a density of 5 × 10^3 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 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 0.1 to 10 ng/mL. Purified IgGs from sera of metreleptin-treated obese and healthy control individuals 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 β-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 IgG to block in vitro signalling 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 LепR signaling in BAF3 cells. In brief, hLепR+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 48 h, cells were washed in PBS, cell lysates were generated to perform Western blotting analyses for STAT3 phosphorylation as a readout of LепR 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 immunoradiometric 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 (InnuC sor, 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 subjects then were centrifuged for 15 min at 1,200 × g. Supernatant was used from standards, subjects, and samples in duplicate to measure free leptin by radioimmunoassay. All samples were run in one assay to eliminate interassay variability.

Cytokines and inflammatory markers were measured in serum or plasma using commercially available ELISAs: tumor necrosis factor-α (Quantikine HS; R&D Systems, Minneapolis, MN; sensitivity, 0.06 pg/mL, CV, 5.3–8.8%), soluble tumor necrosis factor receptor-1 (sTNFRI; Quantikine; R&D Systems; sensitivity, 1 µg/mL, CV, 1.6–2.5%), interleukin (IL)-10 (Amersham Biosciences, Buckinghamshire, UK; sensitivity, 0.1 pg/mL, CV, 10%), soluble intracellular adhesion molecule-1 (Quantikine; R&D Systems; sensitivity, 0.35 ng/mL, CV, 4.8–10.1%), C-reactive protein (CRP) (DSL sensitivity, 1.6 ng/mL, CV, 1.7–3.9%), and IL-6 (Quantikine; R&D Systems; sensitivity, 0.04 pg/mL, CV, 6.9–7.8%).

Researchers at the BIDMC have studied the effects of metreleptin on metabolic and immune responses in obese hyperleptinemic subjects with diabetes. In vitro studies were performed to assess the functional activity of antileptin antibodies and their effect on leptin receptors.
in metreleptin-treated patients and resulted in only a small
leptin signaling in both male and female sub-
jects. There was no difference in MAPK activation
in response to increasing leptin levels similar to those seen
in clinical trials, we performed in vivo signaling experi-
ments. In vivo metreleptin administration (0.01 mg/kg)
induced an approximately 3.2-fold increase in p-STAT3
at 30 min versus baseline in both hAT (Supplementary
Appendix 3A) and hPBMCs (Supplementary Appendix 3B),
with no significant difference in p-STAT3 levels from lean
versus obese subjects.

Clinical study II: In vivo metreleptin signaling in hAT and hPBMCs from lean and obese subjects. To study
whether intracellular signaling pathways are differentially
activated (and thus underlie leptin resistance) in leptin-
sensitive lean subjects versus leptin-resistant obese subjects
in response to increasing leptin levels similar to those seen
in clinical trials, we performed in vivo signaling experi-
ments. In vivo metreleptin administration (0.01 mg/kg)
induced an approximately 3.2-fold increase in p-STAT3
at 30 min versus baseline in both hAT (Supplementary
Appendix 3A) and hPBMCs (Supplementary Appendix 3B),
with no significant difference in p-STAT3 levels from lean
versus obese subjects.

Laboratory study II: Comparative evaluation of ex
vivo metreleptin signaling 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 vivo metreleptin administration
(Supplementary Appendix 3A and B). 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 in-
creased 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. 2G) 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 activa-
tion 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.

RESULTS

Clinical study I: Body weight, metabolic, and immune
responses to metreleptin versus placebo treatment in
obese hyperleptinemic subjects with diabetes. Cir-
culating 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
titers, and LBP levels decreased less in metreleptin-treated
subjects compared with placebo-treated subjects. BMI
remained unchanged throughout the study in both treat-
ment groups. Free leptin levels were similar in both treat-
ment groups at baseline but increased significantly in
subjects treated with metreleptin despite the presence of
antibodies against metreleptin. Although the development
of antileptin antibodies was significantly and positively
correlated with the increase in total 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 indi-
cates 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
~48.4 ng/mL was not associated with any significant dif-
ferential 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 monocytic chemo-
attraction protein-I levels were relatively similar at
baseline in both treatment groups but increased in men
and women treated with metreleptin. By contrast, IL-6,
IL-10, CRP, sTNFR-I, and soluble intracellular adhesive
molecule-I did not change significantly in any group
(Table 1).

Laboratory study I: Agonistic/stimulatory activity of
antileptin antibodies generated during metreleptin
administration. We asked whether the antibodies pro-
duced after treatment with metreleptin in clinical study I
were agonistic (stimulate) or neutralizing leptin-related
proliferation and intracellular signaling. Thus, we used the
BAF3 cell lines after transfection with the long form of the
human leptin receptor (hLepR+BAF3). First, we developed
standard curves of antileptin antibodies using an antileptin
mAb generated in the laboratory (Supplementary Appendix
2A). Antibodies isolated from metreleptin-treated obese
subjects were able to stimulate proliferation up to ~50-fold
more than leptin alone but only at lower leptin concen-
trations (Fig. 1A). On the contrary, no additional stimulatory
effect was observed in the same subjects before treatment,
in the placebo-treated subjects, or when using IgGs from
normal subjects (Fig. 1B and C). These functional effects
were also analyzed at the signaling level in terms of STAT3
phosphorylation. IgGs from metreleptin-treated obese sub-
jects significantly increased p-STAT3 levels (Fig. 1D, Sup-
plementary Appendix 2B), whereas no effect was observed
after addition of either placebo-treated IgGs or IgGs isolated
from normal controls (Fig. 1E and F, Supplementary
Appendix 2B). These results suggest that metreleptin
treatment results in the production of antileptin anti-
bodies that are not neutralizing but may demonstrate minor
agonistic activities on hLepR measured both as increased
proliferation of a leptin-dependent cell line and as increased
hLepR-mediated STAT3 signaling. The stimulatory effect
of these antibodies is small, however, and easily overridden by
relatively higher leptin doses.

Laboratory study II: Comparative evaluation of ex
vivo metreleptin signaling 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 vivo metreleptin administration
(Supplementary Appendix 3A and B). Similar to in vivo
observations, there was no significant difference in p-STAT3
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compared with control, showing that activation was
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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 activa-
tion 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 metformin (n = 50) at baseline 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>Leptin-treated</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>
</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>32.8 ± 0.7</td>
<td>50</td>
<td>32.7 ± 0.5</td>
</tr>
<tr>
<td>BMI</td>
<td>15</td>
<td>15.8 ± 3.3</td>
<td>20</td>
<td>15.8 ± 3.3</td>
</tr>
<tr>
<td>LBP (ng/mL)</td>
<td>18</td>
<td>38.0 ± 6.4</td>
<td>18</td>
<td>38.0 ± 6.4</td>
</tr>
<tr>
<td>Free leptin (ng/mL)</td>
<td>15</td>
<td>5.3 ± 0.4</td>
<td>16</td>
<td>5.3 ± 0.4</td>
</tr>
<tr>
<td>Antibody titer (µg/mL)</td>
<td>7</td>
<td>0.0 ± 0.0</td>
<td>16</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>20</td>
<td>3.7 ± 0.4</td>
<td>49</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>CRP (µg/L)</td>
<td>20</td>
<td>9,138 ± 1,172</td>
<td>19</td>
<td>9,702 ± 1,215</td>
</tr>
<tr>
<td>sTNFR-I (pg/mL)</td>
<td>20</td>
<td>1,129 ± 45.7</td>
<td>19</td>
<td>1,147 ± 51.6</td>
</tr>
<tr>
<td>sTNFR-II (pg/mL)</td>
<td>20</td>
<td>2,470 ± 1,110</td>
<td>20</td>
<td>2,373 ± 23.8</td>
</tr>
<tr>
<td>sICAM-1 (ng/mL)</td>
<td>20</td>
<td>313.2 ± 14.1</td>
<td>20</td>
<td>305.6 ± 14.1</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>11</td>
<td>8.9 ± 1.0</td>
<td>9</td>
<td>8.5 ± 1.1</td>
</tr>
</tbody>
</table>

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. <sup>b</sup>P values are calculated from one-way ANOVA. <sup>c</sup>P values are calculated from repeated-measures analysis evaluating the change in study variables over time, adjusted for age and sex.
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 activation of AMPK signaling by in vitro metreleptin administration in hPA. In vitro metreleptin administration increased AMPK phosphorylation by ~2.5-fold in both subcutaneous and omental hPA from male and female subjects (Fig. 3E). We observed no differences in AMPK phosphorylation 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 lepitin signaling in hAT from lean and obese nondiabetic humans. We analyzed a dataset

<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>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<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>
<td>−0.18</td>
</tr>
<tr>
<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>
<td>0.424</td>
</tr>
<tr>
<td><strong>Leptin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>r</td>
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MCP, monocyte chemoattractant protein; sICAM, soluble intracellular adhesive molecule.

TABLE 2
Clinical study I: Pearson correlation coefficients of the changes in study variables before and after 16 weeks of treatment

H.-S. MOON AND ASSOCIATES

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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. 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 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.
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.

FIG. 2. Laboratory study II. Comparative evaluation of ex vivo metreleptin signaling in hAT and hPBMCs from lean and obese subjects. Ex vivo metreleptin administration in hAT and hPBMCs was performed as described in detail in RESEARCH DESIGN AND METHODS. hAT (A–D) and hPBMCs (E) were incubated and stimulated with or without ex vivo metreleptin at the indicated concentrations for 30 min. F: PBMCs were incubated and stimulated with or without ex vivo metreleptin at the indicated times. G: hAT was incubated and stimulated with or without ex vivo metreleptin at the indicated concentrations for 30 min. All lysates were examined by Western blotting 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 = 3) ± SD. Means with different letters are significantly different, P < 0.05. OM, omental; SC, subcutaneous.
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 40–50 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

FIG. 3. Laboratory study III. In vitro metreleptin signaling in subcutaneous (SC) and omental (OM) hPA from lean and obese subjects. In vitro metreleptin administration in hPA was performed as described in detail in RESEARCH DESIGN AND METHODS. A: Cells were treated with metreleptin at the indicated concentrations for 30 min. B: Cells were treated with metreleptin at the indicated times. C: Cells were treated with metreleptin (50 ng/mL) for 30 min. Immunodetection was carried out as described in detail in RESEARCH DESIGN AND METHODS. All pictures were 30 magnification. D: Cells were pretreated with the STAT3 inhibitor AG490 (AG, 1 μmol/L) for 1 h, followed by treatment with 50 ng/mL metreleptin for 30 min. E and F: Cells were treated with metreleptin at the indicated concentrations for 30 min. All lysates were examined by Western blotting 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 = 3) ± SD. Means with different letters are significantly different, P < 0.05. (A high-quality digital representation of this figure is available in the online issue.)
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

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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.

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