Identification, and saturable nature, of signaling pathways induced by metreleptin in humans: comparative evaluation of in vivo, ex vivo and in vitro administration

Hyun-Seuk Moon¹*, Joo Young Huh¹**, Fadime Dincer¹, Benjamin E. Schneider², Per-Olof Hasselgren³, Christos S. Mantzoros¹,4

1. Division of Endocrinology, Diabetes, and Metabolism, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215, USA.
2. Division of Minimally Invasive Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215, USA.
3. Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215, USA.
4. Section of Endocrinology, Boston VA Healthcare System, Harvard Medical School, Boston, Massachusetts 02130, USA

Correspondence should be addressed to: Christos S. Mantzoros, MD, DSc, Professor of Medicine, Harvard Medical School, JP9B52A, 150 S. Huntington Ave, Boston, MA 02130, Tel: (617) 667-8630, Fax: (617) 667-8634, Email: cmantzor@bidmc.harvard.edu

*current address: Laboratory of Metabolic Engineering, Division of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, South Korea
**current address: College of Pharmacy, Chonnam National University, Gwangju 500-757, South Korea

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Abstract

Signaling pathways activated by leptin in metabolically important organs has largely been studied only in animal and/or cell culture studies. In this study we examined whether leptin has similar effects in human peripheral tissues in vivo, ex vivo, and in vitro and whether the response would be different in lean and obese humans. For in vivo leptin signaling, metreleptin was administered and muscle, adipose tissue, and peripheral blood mononuclear cells were taken for analysis of signal activation. Experiments were also done ex vivo and with primary cultured cells in vitro. The signal activation was compared between male vs. female and obese vs. lean humans. Acute in vivo, ex vivo, and/or in vitro metreleptin administration similarly activated STAT3, AMPK, ERK1/2, Akt, mTOR, NFκB and/or IKKα/β without any differences between male vs. female and obese vs. lean subjects. All signaling pathways were saturable at ~30-50 ng/ml, consistent with the clinical evidence showing no additional effect(s) in obese subjects who already have high levels of leptin. Our data provide novel information on downstream effectors of metreleptin action in humans which may have therapeutic implications.
Leptin, the prototype adipocyte-secreted hormone, primarily regulates food intake and body weight by acting in the hypothalamus (1). In addition to the role of leptin in the brain for regulation of energy balance, it has also been shown that leptin acts in peripheral tissues as a regulator of energy homeostasis, insulin action, lipid metabolism, and immune function (2; 3). Leptin receptors are found in various locations throughout the body supporting the central as well as peripheral role of leptin in metabolism (4-6). Administration of leptin has been observed to markedly decrease insulin and glucose levels prior to a decrease in food intake or changes in body weight in leptin deficient rodents (7). Also, peripheral leptin administration, but not food restriction, was able to fully correct severe insulin resistance in lipoatrophic mice (8). These results indicate that leptin may act at the level of insulin sensitive tissues independently from leptin’s central action in regulating feeding behavior (7). Therefore, it is important from the point of view of biology and human therapeutics to know which downstream signaling pathways are activated by leptin to mediate its effects in the peripheral tissues.

Recombinant human leptin (metreleptin) has been recently approved by the United States and Japan Food and Drug Administration for treatment in generalized lipodystrophy, shedding light on the therapeutic potential of leptin. Patients with lipodystrophy are deficient in leptin which in turn results in severe insulin resistance and hypertriglyceridemia (9; 10). Leptin replacement therapy has been reported to be effective in improving insulin resistance and the metabolic profile in these patients (11-13). However, whether the metreleptin treatment has a direct effect in metabolically important peripheral tissues in humans remains unknown.

So far, it has been demonstrated only in animal models that leptin administration activates peripheral intracellular signaling pathways and plays a key role in the pathophysiology of insulin resistance (14-16). By contrast, whether leptin has similar effects in human peripheral tissues
(hPT) *in vivo* and whether lean and obese humans would respond differently has not yet been comparatively studied. Hence, in order to provide detailed evidence on metreleptin signaling in human peripheral metabolism, we extended previous observations (17) by studying *in vivo* metreleptin signaling in insulin sensitive tissues, i.e. human adipose tissues (hAT), human peripheral blood mononuclear cells (hPBMC), and human muscle tissues (hMT). Also, we investigated *ex vivo* and *in vitro* metreleptin signaling in hAT, hPBMC, hMT, human primary adipocytes (hPA) and human primary muscle cells (hMC). Moreover, since obesity is a state of generalized resistance or insensitivity to leptin (18; 19) it would be of value to study whether there might be an impairment of leptin signaling pathways and/or saturation in some or all signaling pathways downstream of leptin in the obese compared with the lean state. Therefore, we compared intracellular *in vivo, ex vivo and/or in vitro* metreleptin signaling pathways in hPT from male vs. female and obese vs. lean subjects.
Research Design and Methods

Subjects - Volunteers were recruited from the community and screened at the Clinical Research Center (CRC) at Beth Israel Deaconess Medical Center (BIDMC). All subjects provided written informed consent to participate and the study was approved by the institutional review board at 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 E. coli-derived proteins or anesthetic agents such as Lidocaine or Novocaine. Subjects were provided with take-home meals and consumed an isocaloric diet, specifically designed for each subject, for 48 hours prior to their main study visit to ensure stable dietary intake.

In vivo leptin administration - On the morning of the main study visit, subjects attended the CRC after a 12 hour fast. An intravenous cannula was placed in each antecubital fossa. The tissue samples (hAT from lower abdomen and hMT from thigh) were immediately placed in a cryotube and frozen in liquid nitrogen at the bedside. Blood sample were taken for metreleptin signaling experiments in hPBMC. Following baseline sampling, an intravenous bolus of metreleptin, dose 0.01 mg/kg body weight or placebo (10 cc of normal saline) was given by slow intravenous injection over one minute. The subject rested supine. 20 minutes later, the biopsy was repeated. To compare the signaling in lean and obese patients, a bolus of metreleptin was administered to 6 lean [male (n=3); BMI 25.0±1.7 kg/m$^2$; female (n=3); BMI 22.7±0.6 kg/m$^2$] and 6 obese [male (n=3); BMI 35.3±8.4 kg/m$^2$; female (n=3); BMI 37.0±6.0 kg/m$^2$].

Ex vivo leptin administration - Ex vivo leptin administration was performed as described in detail previously (17). Briefly, hAT and hMT from 3 lean females (BMI: 22.8±2.1 kg/m$^2$) and 3
obese females (BMI: 37.6±5.7 kg/m$^2$) were minced into pieces of ∼1 mm in diameter and incubated at 37°C with leptin for 30 minutes.

**The hPBMC culture** - The hPBMC was isolated from blood collected from the same subjects as *ex vivo* by density gradient sedimentation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) as described previously (17). After isolation of the hPBMC, the cells were washed twice in PBS and re-suspended in medium appropriate for cell culture (RPMI 1640 supplemented with 25 mmol/l HEPES, 2 mmol/l L-glutamine, 100 μU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B).

**The hPA culture** - The hPA culture was performed as previously described (20). Discarded subcutaneous hAT were collected from obese females (n=3, age: 47.7±3.5 years, BMI: 38.5±0.6 kg/m$^2$) undergoing laparoscopic adjustable gastric band, liposuction, or abdominoplasty at BIDMC. The hAT was then digested with PBS/collagenase solution (3 mg collagenase/g tissue and 1 ml PBS/1 mg collagenase) + 3.5% fatty acid–free BSA and then centrifuged at 1200 rpm for 10 min. The pellets were re-suspended in α-MEM supplemented with 15 mmol/l NaHCO$_3$, 15 mmol/l HEPES, 33 μmol/l biotin, 17 μmol/l pantothenate, 10 mg/ml human transferrin, 0.05 mg/ml gentamicin, and 10% FBS and then were plated overnight. To induce adipocyte differentiation, confluent cells were exposed to differentiation medium containing 66 nmol/l insulin, 100 nmol/l cortisol, 0.2 nmol/l triiiodothyronine, and 1 μg/ml ciglitazone. The medium was changed every 2 days for 28 days and then used for experiments.

**The hMC culture** - The hMC culture was performed as previously described (21). Thigh muscle (*vastus lateralis*) was collected from obese females (n=3, age 41.0±7.9 years, BMI 43.5±1.7 kg/m$^2$). Biopsied skeletal muscle tissue was minced into small pieces and incubated in dissociation media containing 0.1% BSA, 0.25% trypsin-EDTA, and 0.1% collagenase in 37°C
water bath for 1 hr. After centrifugation, the pellet was re-suspended in Skeletal Muscle Cell Growth Media (PromoCell, Heidelberg, Germany) and plated on T25 culture dish. After reaching 70-80% confluence, growth media was switched to Skeletal Muscle Cell Differentiation Media (PromoCell, Heidelberg, Germany) for the differentiation of myoblasts into myotubes. After 5 days of differentiation, the media was changed back to growth media for additional 2-4 days for a stable differentiation, according to the manufacturer's instructions.

**The protein extraction and Western Blotting** - Cells were suspended in a lysis buffer containing 20 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 5 mmol/l EDTA, 0.1 mmol/l phenylmethylsulfonyl fluoride, 0.05% aprotinin, and 0.1% Igepal and then incubated for 30 min at 4°C. The suspension was centrifuged for 25 min at 14,240 g, and the supernatant was saved as the total extract. 50 µg of tissue lysate protein per lane was resolved by SDS-PAGE (8% gel) and transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). The nitrocellulose membranes were blocked with 5% nonfat dry milk for 1 h at room temperature, incubated with primary antibody (1:500 dilution in 1% nonfat dry milk overnight at 4°C. The membranes were washed with Tris-buffered saline containing 0.05% Tween 20 (TBST) for 30 min, incubated with horseradish peroxidase secondary antibody (1:1000 dilution; Amersham Pharmacia Biotech, Arlington Heights, IL) for 2 h and washed with TBST for 30 min. The bands were visualized using the enhanced chemiluminescence system (Amersham Pharmacia Biotech) and quantified using Image J (http://rsbweb.nih.gov/ij/).

**Lipolysis** - Lipolysis assay was performed using [14C]-oleic acid method (22). Briefly, the cells were seeded on 12-well culture plates. Before pre-loading the cultures with [14C]-oleic acid, cultures were serum-starved for 24 hr. Then, 20 µl of PBS containing 6.25 nmol of [14C]-oleic acid (50 mCi/mmol) was added for 15 hr; 90% of [14C]-oleic acid was sequestered by the
cultures during this incubation. The medium was then removed and the cells were washed four times, resulting in a background radioactivity of <1000 dpm. Each well was treated with leptin in the presence or absence of isoproterenol as a positive control. After 72 hr of leptin treatment, 200 µl of medium was collected from each well and delivered to the liquid scintillation counting vial to measure $^{14}\text{C}$-oleic acid released to the medium.

**Cardiac myocyte (CM) and uterine smooth muscle (USM) cell culture** - The cells were purchased from PromoCell GmbH (Heidelberg, Germany). The cells were cultured according to the manufacture’s protocol.

**Hypertrophy** - Hypertrophy assay was performed as described in detail previously (23). Briefly, the cells grown in 48-well plates, fixed with 4% paraformaldehyde in PBS for 15 min, followed by 0.5% Triton-100 treatment for 5 min. After incubated with 0.1% rhodamine-phalloidin for 30 min, cells were washed in PBS for further interaction with DAPI. The images of cell surface area from randomly selected fields (50 for each group, at least 200 cells) were determined by Image J (http://rsbweb.nih.gov/ij/).

**Statistical analysis** - All data were analyzed using student t-test and/or one-way ANOVA followed by post-hoc test for multiple comparisons using SPSS (version 11.5).
Results

Regulation of STAT3, ERK1/2, Akt and AMPK signaling by *in vivo* metreleptin administration in hPT

To compare the leptin-induced signaling in peripheral tissues, metreleptin was administered *in vivo* and hAT, hPBMC, and hMT were collected. Expanding from our previous study (17), *in vivo* metreleptin administration stimulated phosphorylation of STAT3 (Fig. 1A), ERK1/2 (Fig. 1B) and Akt (Fig. 1C) by ~2.5- to ~3.3-fold in hAT and hPBMC, but not hMT, when compared to control. We also observed that *in vivo* metreleptin administration stimulates phosphorylation of AMPK in all tissues (Fig. 1D). As shown in Figure 1, there was no difference in STAT3, ERK1/2, Akt and/or AMPK activation in response to *in vivo* metreleptin administration in obese vs. lean and male vs. female subjects.

Regulation of mTOR signaling by *in vivo, ex vivo* and/or *in vitro* metreleptin administration in hPT

In addition to STAT3, ERK1/2, AMPK, and Akt, we observed that *in vivo* metreleptin administration stimulates phosphorylation of mTOR (Fig. 2A) by ~2.7- to ~3.1-fold in hAT and hPBMC, but not hMT, when compared to control. We also observed that *ex vivo* metreleptin administration activates mTOR signaling in hAT (Fig. 2B) in a dose-dependent manner. Moreover, dose response experiments showed that *in vitro* administration of up to 200 ng/ml of metreleptin for 30 min significantly induces phosphorylation of mTOR in hPA (Fig. 2C) and hPBMC (Fig. 2D). Importantly, metreleptin-activated mTOR signaling was saturable at a metreleptin concentration of ~50 ng/ml. By contrast, *ex vivo* and/or *in vitro* metreleptin administration did not regulate mTOR signaling in hMT (Fig. 2E) and hMC (Fig. 2F). There was
no difference in mTOR activation in response to *in vivo* metreleptin administration in obese vs. lean and male vs. female subjects.

**Regulation of NFκB and IKKα/β signaling by *ex vivo* and/or *in vitro* metreleptin administration in hPT**

We observed that *ex vivo* and/or *in vitro* metreleptin administration stimulates phosphorylation of NFκB and IKKα/β in hAT (Fig. 3A and Fig. 4A), hPA (Fig. 3B and Fig. 4B) and hPBMC (Fig. 3C and Fig. 4C) in a dose-dependent manner. Similar to the results for mTOR signaling, metreleptin-activated NFκB and/or IKKα/β signaling was saturable at a metreleptin concentration of ~50 ng/ml. By contrast, *ex vivo* and/or *in vitro* metreleptin administration did not regulate NFκB and/or IKKα/β signaling in hMT (Fig. 3D and Fig. 4D) and hMC (Fig. 3E and Fig. 4E). There was no difference in NFκB and/or IKKα/β activation in response to *ex vivo* and/or *in vitro* metreleptin administration in obese vs. lean subjects (data not shown).

**Regulation of SHP2 signaling by *in vivo, ex vivo* and/or *in vitro* metreleptin administration in hPT**

We observed that *in vivo* (Fig. 5A) and *ex vivo* (Fig. 5B and 5C) metreleptin administration has no effects on SHP2 activation in all hPT. There was no difference in SHP2 activation in response to *in vivo* metreleptin administration in experiments comparing obese vs. lean and male vs. female subjects.

**Regulation of differentiation and lipolysis by *in vitro* metreleptin administration in hPA**
We observed that *in vitro* metreleptin administration has no effects on differentiation in hPA (Fig. 6A and 6B). By contrast, we observed that *in vitro* metreleptin administration induces lipolysis in hPA (Fig. 6C). There was no difference in differentiation and lipolysis in response to *in vitro* metreleptin administration in experiments comparing SC vs. OM.

**Regulation of cell growth signaling and hypertrophy by *in vitro* metreleptin administration in cardiac myocytes (CM) and uterine smooth muscle cells (USM)**

*In vitro* metreleptin administration increased STAT3 activation but this effect was abolished by co-administration of STAT3 inhibitor in both CM and USM (Fig. 7A). Also, we observed that *in vitro* metreleptin administration increases ERK1/2, JNK and/or p38 phosphorylation in CM (Fig. 7B). *In vitro* metreleptin administration also increased ERK1/2 and/or p38 phosphorylation in USM (Fig. 7B). By contrast, JNK was not regulated by *in vitro* metreleptin administration in USM (Fig. 7B). Hypertrophy was increased by *in vitro* metreleptin administration in both CM and USM (Fig. 7C).
Discussion

With the recent approval of metreleptin for the treatment of lipodystrophy-related metabolic complications, understanding leptin biology in humans has become of major importance. To determine the direct effect of leptin on peripheral tissues in humans, we studied the \textit{in vivo} metreleptin signaling in insulin sensitive tissues such as hAT and hPBMC, and hMT, and found that leptin activates peripheral STAT3, AMPK, ERK1/2, Akt, mTOR, NFκB and/or IKKα/β signaling. Subsequently, we found no difference in \textit{in vivo}, \textit{ex vivo}, and/or \textit{in vitro} metreleptin signaling between male vs. female and obese vs. lean subjects.

JAK2/STAT3 pathway is one of the first identified signaling downstream of leptin receptor activation (24; 25). STAT3 is a transcription factor which mediates the expression of a variety of genes in many cellular processes such as cell growth and apoptosis (26). We observed herein that metreleptin activates STAT3 signaling in hAT and hPBMC, but not in hMT. Interestingly, unlike our results presented herein, \textit{in vivo} metreleptin administration has been shown to increase STAT3 activation in hMT from young healthy males (27). The different response could have derived from the differences in the duration of leptin infusion. Whereas this prior paper has shown STAT3 activation after 1 hr infusion, the \textit{in vivo} metreleptin infusion studied herein was 1 min and the signaling was observed 20 min after infusion. Thus, the activation of STAT3 by \textit{in vivo} metreleptin in hMT needs to be further elucidated in future studies.

We have previously shown that leptin-induced ERK/mTOR/Akt signaling is important for cell survival and/or proliferation in hAT and hPBMC \textit{ex vivo} (20), and mouse hypothalamic, liver and muscle cell lines \textit{in vitro} (14). Others have also reported that leptin stimulates glucose uptake in mouse muscle cells by activation of ERK2 (28). Similarly, we observed herein that \textit{in}
In vivo metreleptin administration activates ERK1/2, mTOR and Akt signaling in hAT and hPBMC, through the activation of which metreleptin may have a beneficial effect on cell proliferation and survival in humans.

Leptin has been reported to directly stimulate fatty acid oxidation in skeletal muscle (29; 30), and in the obese state, leptin-induced fatty acid oxidation seems to be impaired partly through inactivation of AMPK signaling by suppressor of cytokine signaling 3 (SOCS3) (31; 32). Contrary to these findings, we observed that in vivo metreleptin administration activates AMPK signaling in all hPT without any difference between lean and obese subjects showing that leptin could still stimulate AMPK signaling in obese humans which could play a pivotal role in peripheral glucose/lipid metabolism. Therefore, metreleptin-stimulated AMPK signaling in hPT may be an attractive therapeutic target for reducing lipotoxicity in obesity and type 2 DM. There is still a possibility that although the signaling pathways appear normal in obese patients, downstream functional effects such as fatty acid oxidation may be impaired, which needs to further examined.

SHP2 has been shown to promote ERK1/2 signaling in response to insulin and epidermal growth factor (EGF) binding to their receptors (33; 34). In the brain, SHP2 activates leptin signaling and regulates the hypothalamus in controlling energy balance and metabolism (35). In contrast, we did not find any association between SHP2 and ERK1/2. In fact, we did not observe any SHP2 activation in in vivo, ex vivo and/or in vitro metreleptin administration in all hPT. Therefore, the involvement of the SHP2/MAPK pathway in metreleptin action may be restricted to its central but not peripheral effect in humans (36).

We and others have recently shown that metreleptin can reverse the suppression of the immune system and maintain the balance between pro- and anti-inflammatory cells (37; 38).
Moreover, it has been demonstrated that leptin, as a cytokine, stimulates immune cells, specifically CD4+ T lymphocytes, to synthesize and release inflammatory proteins such as interleukin-2 (IL-2) and interferon-gamma (IFN-γ) (39). Specifically, metreleptin is closely linked to the activation of inflammatory pathways NFκB/IKK (40) and increased monocyte chemo-attractant protein (MCP)-1, which can attract the immune cells to the site of inflammation (41). In addition to its direct effects on immune system physiology, metreleptin may indirectly affect immune system function by improving insulin resistance, blood vessel inflammation, and cardiovascular disease associated with hyperleptinemic states such as obesity. Here, we observed that in vivo and/or ex vivo metreleptin administration activates NFκB/IKK cascade signaling in hPBMC, but it is still unclear whether this can lead to regulation of the immune system in obese/diabetic subjects, similar to what we have seen in women with hypothalamic amenorrhea (39).

As mentioned above, the molecules observed herein are well known for their general role in cell growth/survival/metabolic/immune regulation. Therefore, the observed in vivo signaling after metreleptin infusion in humans could be a result of an indirect effect via activation of other cytokines such as TNFa and interleukins. However, our in vitro study rules out this possibility by clearly showing that leptin can directly activate these pathways in PBMCs, adipocytes, and muscle cells.

Although it is well documented that one of the mechanisms for extreme leptin resistance is the impairment of leptin receptor signaling due to leptin receptor mutations (1), the underpinnings of leptin resistance in the vast majority of leptin resistant states remains unknown. Interestingly, we did not observe any major differences in metreleptin signaling activation in tissues from obese vs. lean subjects. Also, the effect of leptin on adipocyte function, such as
differentiation and lipolysis, was unaltered in adipocytes from obese compared to lean subjects, indicating that the ability to respond to leptin remains unchanged in obese state. However, all metreleptin signaling pathways studied in hPT were saturable at ~50 ng/ml, suggesting that above that level, i.e., the level clinically seen in obese subjects at baseline (17), no additional signaling effects could be observed. In fact, we have previously reported in pharmacokinetic studies that acute leptin infusion increases circulating leptin levels by several orders of magnitude, over 100 ng/mL, within 20-30 min (42-44). Leptin at a dose of 10 mg twice daily by subcutaneous injection increased circulating total leptin by 10-fold after 4 weeks, and by 30-fold after 16 weeks, whereas free leptin levels peaked at 50-60 ng/mL even after 16 weeks (17). The fact that no difference exists in hPT of lean vs. obese subjects implies that administration of leptin may still have a beneficial effect on peripheral leptin signaling and downstream metabolism, but hyperleptinemic states observed in obese individuals make it difficult to enhance the peripheral signaling pathway in vivo. It has been demonstrated that central administration of metreleptin affects insulin sensitivity and metabolism in peripheral tissues (45), and therefore metreleptin-activated signaling pathways may prove to be much more complex and possibly involve both the CNS and the periphery. It is also possible that the observed responses and/or the lack of differences between lean and obese subjects in response to metreleptin treatment in hPT could possibly be totally different from signaling responses in hypothalamus and/or other CNS areas.

This study is the very first attempt to map the intracellular signaling pathways downstream of metreleptin in hPT in vivo. Our data from in vivo, ex vivo and in vitro provide novel insights into the metreleptin signaling pathways which would mediate energy homeostasis, insulin resistance/metabolism and immune function in humans and thus adds value on the
biology of leptin, i.e. a molecule that was recently approved for use in humans. The limitation of this study is that we have used only 3 subjects in each study. Nevertheless, the similar pattern throughout *in vivo*, *ex vivo*, and *in vitro* among female and male subjects in all signaling pathways observed provide evidence that there is no difference in the signaling between lean vs. obese or men vs. women. It is possible that differences in methodology and procedures, including timing of blood sampling, could have resulted in differences between this and prior studies by others, and *in vitro* manipulation may have induced desensitization of the tissues. Also, there is a possibility that the study is underpowered given the small number of subjects herein but these data are consistent with our prior independent study which had twice as many subjects. Larger studies need to be conducted to extend these observations and confirm our findings. This study, followed by future *in vivo* metreleptin studies involving additional signaling pathways in other various hPT, will eventually allow the full mapping and characterization of signaling pathways downstream of leptin in human tissues. We believe that our data enhance the current knowledge on the mechanisms underlying metreleptin actions in humans and provide crucial information for development of new therapies for the treatment of insulin resistance syndromes such as type 2 DM, inflammation, and obesity.
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Author Contribution

Hyun-Seuk Moon, Joo Young Huh and Christos S. Mantzoros wrote the manuscript. Hyun-Seuk Moon, Fadime Dincer, Joo Young Huh, Benjamin E. Schneider and Christos S. Mantzoros participated in the study design, performance and coordination. Christos S. Mantzoros conceived the study. All authors read and approved the final manuscript.

Disclosure statement: The authors have nothing to disclose.
References


Figure legends

Figure 1. Regulation of STAT3, ERK1/2, Akt and AMPK signaling by *in vivo* metreleptin administration in human peripheral tissues - (A-D) Blood draw and fat (lower abdomen) and muscle (thigh) biopsy was performed at baseline. Metreleptin, dose 0.01 mg/kg body weight or placebo (10 cc of normal saline), was given by slow intravenous injection over one minute, and after 30 min, blood draw and fat and muscle biopsy was performed. Total and phosphorylated STAT3, ERK1/2, Akt, and AMPK were examined by Western Blotting. All data were analyzed using student t-test. Values are means (n=3) ± SD.

Figure 2. Regulation of mTOR signaling by *in vivo*, *ex vivo* and *in vitro* metreleptin administration in human peripheral tissues - (A) *In vivo* signaling in subcutaneous fat, muscle, and PBMCs before and 30 minutes after metreleptin administration in female vs. male and lean vs. obese subjects. (B) Signaling in subcutaneous (SC) and omental (OM) fat from female obese subjects before and 30 min after *ex vivo* metreleptin administration. (C) Time- and dose-dependent signaling by *in vitro* metreleptin administration in primary human adipocytes from female obese subjects. (D) Time- and dose-dependent signaling in human peripheral blood mononuclear cells from 3 female obese subjects by *ex vivo* metreleptin administration. (E) Signaling in muscle from 3 female obese subjects before and 30 min after *ex vivo* metreleptin administration. (F) Time- and dose-dependent signaling in primary muscle cells from 3 female obese subjects by *in vitro* metreleptin administration. Total and phosphorylated mTOR were examined by Western Blotting. All data were analyzed using student t-test and/or 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, whereas means with similar letters are not different from each other. SC: subcutaneous fat, OM: omental fat.
Figure 3. Regulation of NFκB signaling by *ex vivo* and *in vitro* metreleptin administration in human peripheral tissues - (A) Signaling in subcutaneous (SC) and omental (OM) fat from 3 female obese subjects before and 30 min after *ex vivo* metreleptin administration. (B) Time- and dose-dependent signaling by *in vitro* metreleptin administration in primary human adipocytes from 3 female obese subjects. (C) Time- and dose-dependent signaling in human peripheral blood mononuclear cells from 3 female obese subjects by *ex vivo* metreleptin administration. (D) Signaling in muscle from 3 female obese subjects before and 30 min after *ex vivo* metreleptin administration. (E) Time- and dose-dependent signaling in primary muscle cells from 3 female obese subjects by *in vitro* metreleptin administration. Total and phosphorylated NFκB were examined by Western Blotting. All data were analyzed using student t-test and/or 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, whereas means with similar letters are not different from each other. SC: subcutaneous fat, OM: omental fat.

Figure 4. Regulation of IKKα/β signaling by *ex vivo* and *in vitro* metreleptin administration in human peripheral tissues - (A) Signaling in subcutaneous (SC) and omental (OM) fat from 3 female obese subjects before and 30 min after *ex vivo* metreleptin administration. (B) Time- and dose-dependent signaling by *in vitro* metreleptin administration in primary human adipocytes from 3 female obese subjects. (C) Time- and dose-dependent signaling in human peripheral blood mononuclear cells from 3 female obese subjects by *ex vivo* metreleptin administration. (D) Signaling in muscle from 3 female obese subjects before and 30 min after *ex vivo* metreleptin administration. (E) Time- and dose-dependent signaling in primary muscle cells from 3 female obese subjects by *in vitro* metreleptin administration.
obese subjects by *in vitro* metreleptin administration. Total and phosphorylated IKKα/β were examined by Western Blotting. All data were analyzed using student t-test and/or 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, whereas means with similar letters are not different from each other. SC: subcutaneous fat, OM: omental fat.

**Figure 5. Regulation of SHP2 signaling by *in vivo* and *ex vivo* metreleptin administration in human peripheral tissues** - (A) Signaling in female/male, lean/obese subjects before and 30 min after *in vivo* metreleptin administration. (B) Signaling in subcutaneous (SC) and omental (OM) fat from 3 female obese subjects before and 30 min after *ex vivo* metreleptin administration. (C) Time- and dose-dependent signaling in human peripheral blood mononuclear cells from 3 female obese subjects by *ex vivo* metreleptin administration. Total and phosphorylated SHP2 were examined by Western Blotting. All data were analyzed using student t-test and/or 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, whereas means with similar letters are not different from each other. SC: subcutaneous fat, OM: omental fat.

**Figure 6. Regulation of differentiation and lipolysis by *in vitro* metreleptin administration in human primary adipocytes** - (A) Primary cultured subcutaneous (SC) and omental (OM) adipocytes were treated with metreleptin (50 ng/ml) *in vitro* for 28 days and Oil Red O staining was then performed. (B) Cell differentiation rate was quantified by extraction of Oil Red O with isopropanol. (C) SC and OM adipocytes were treated with metreleptin (50 ng/ml) *in vitro* for 36
hr. Lipolysis was measured using \(^{14}\)C-oleic acid method. Isoproterenol (ISO, 10 µM for 30 min) was used as a positive control. All 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, whereas means with similar letters are not different from each other. SC: subcutaneous fat, OM: omental fat, L: leptin, C: control, D: day.

Figure 7. Regulation of cell growth signaling and hypertrophy by \textit{in vitro} metreleptin administration in cardiac myocytes (CM) and uterine smooth muscle cells (USM) - (A) The cells were treated with metreleptin (50 ng/ml) \textit{in vitro} for 30 min with/without AG490 (STAT3 inhibitor, 1uM for 1hr). (B) The cells were treated with metreleptin (50 ng/ml) \textit{in vitro} for 30 min. Total and phosphorylated STAT3, ERK, JNK, p38 were examined by Western Blotting. (C) The cells were treated with metreleptin (50 ng/ml) \textit{in vitro} for 36 hr and hypertrophy assay was then performed using 0.1% rhodamine-phalloidin. All data were analyzed using student t-test and/or 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, whereas means with similar letters are not different from each other. L: leptin, C: control, D: day, CM: cardiac myocytes, USM: uterine smooth muscle cells.
Figure 2

A

B

C

D

E

F
Figure 4

A

B

C

D

E

Diabetes
Figure 5

Diabetes

A

FAT

PBMC

Muscle

B

C

Figure 5
Figure 6

Day 0 vs Day 28:

- Control:
  - SC
  - OM

- Leptin (ng/ml):
  - 50 ng/ml
  - 100 ng/ml

Cell Differentiation (Fold Increase):

- SC
- OM

Lipolysis (Fold Increase):

- SC
- OM

36 hr vs 36 hr:

- C
- L
- ISO