Identification and Saturable Nature of Signaling Pathways Induced by Metreleptin in Humans: Comparative Evaluation of In Vivo, Ex Vivo, and In Vitro Administration

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Signaling pathways activated by leptin in metabolically important organs have 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 versus female and obese versus lean humans. Acute in vivo, ex vivo, and/or in vitro metreleptin administration similarly activated STAT3, AMPK, ERK1/2, Akt, mTOR, NF-κB, and/or IKKα/β without any differences between male versus female and obese versus 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 that may have therapeutic implications.
Recombinant human leptin (metreleptin) has been recently approved by the U.S. and Japanese 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 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 (hPTs) 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 (hATs), human peripheral blood mononuclear cells (hPBMCs), and human muscle tissues (hMTs). Also, we investigated ex vivo and in vitro metreleptin signaling in hAT, hPBMCs, hMT, human primary adipocytes (hPAs), and human primary muscle cells (hMCs). 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 versus female and obese versus lean subjects.

**RESEARCH DESIGN AND METHODS**

**Subjects**

Volunteers were recruited from the community and screened at the Clinical Research Center 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, and changes in weight or fasting insulin levels during the 3 months before study entry. Subjects were screened for smoking, alcohol intake, use of medications that might affect glucose or lipid metabolism, and history of drug or alcohol dependence. Exclusion criteria included pregnancy, cancer, any systemic illness except hypertension, history of major surgery or liposuction, and use of medications affecting glucose metabolism. Subjects were screened for smoking, alcohol intake, use of medications that might affect glucose or lipid metabolism, and history of drug or alcohol dependence. Exclusion criteria included pregnancy, cancer, any systemic illness except hypertension, history of major surgery or liposuction, and use of medications affecting glucose metabolism.

Subjects were provided with take-home meals and consumed an isocaloric diet, specifically designed for each subject, for 48 h 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 Clinical Research Center after a 12-h 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 samples were taken for metreleptin signaling experiments in hPBMCs. 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 1 min. The subject rested supine. Twenty minutes later, the biopsy was repeated. To compare the signaling in lean and obese patients, a bolus of metreleptin was administered to six lean (male [n = 3], BMI 25.0 ± 1.7 kg/m²; female [n = 3], BMI 22.7 ± 0.6 kg/m²) and six obese patients (male [n = 3], BMI 35.3 ± 8.4 kg/m²; female [n = 3], BMI 37.0 ± 6.0 kg/m²).

**Ex Vivo Leptin Administration**

Ex vivo leptin administration was performed as described in detail previously (17). In brief, hAT and hMT from three lean females (BMI 22.8 ± 2.1 kg/m²) and three obese females (BMI 37.6 ± 5.7 kg/m²) were minced into pieces of ~1 mm in diameter and incubated at 37°C with leptin for 30 min.

**hPBMC Culture**

The hPBMCs were 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 hPBMCs, the cells were washed twice in PBS and resuspended 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).

**hPA Culture**

The hPA culture was performed as described previously (20). Discarded subcutaneous hATs were collected from obese females (n = 3, age 47.7 ± 3.5 years, BMI 38.5 ± 0.6 kg/m²) 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) 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 mmol/L triiodothyronine, and 1 μg/mL cigitazone. The medium was changed every 2 days for 28 days and then used for experiments.

**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 ± 6.0 kg/m²).
1.7 kg/m²). 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 h. After centrifugation, the pellet was resuspended 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 an additional 2–4 days for a stable differentiation, according to the manufacturer’s instructions.

**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 14,240g, and the supernatant was saved as the total extract. Fifty micrograms 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 and 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 (1:1,000 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 ImageJ (http://rsweb.nih.gov/ij/).

**Lipolysis**

Lipolysis assay was performed using [14C]oleic acid method (22). In brief, the cells were seeded on 12-well culture plates. Before preloading the cultures with [14C]oleic acid, cultures were serum starved for 24 h. Then, 20 µL PBS containing 6.25 nmol [14C]oleic acid (50 mCi/mmol) was added for 15 h; 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 <1,000 dpm. Each well was treated with leptin in the presence or absence of isoprotenerol as a positive control. After 72 h of leptin treatment, 200 µL of medium was collected from each well and delivered to the liquid scintillation counting vial to measure [14C]oleic acid released to the medium.

**Cardiac Myocyte and Uterine Smooth Muscle Cell Culture**

The cells were purchased from PromoCell GmbH (Heidelberg, Germany). The cells were cultured according to the manufacturer’s protocol.

**Hypertrophy**

Hypertrophy assay was performed as described in detail previously (23). In brief, the cells were grown in 48-well plates and fixed with 4% paraformaldehyde in PBS for 15 min, followed by 0.5% Triton-100 treatment for 5 min. After incubation 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 ImageJ (http://rsweb.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, hPBMCs, 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 hPBMCs, but not hMT, when compared with control. We also observed that in vivo metreleptin administration stimulates phosphorylation of AMPK in all tissues (Fig. 1D). As shown in Fig. 1, there was no difference in STAT3, ERK1/2, Akt, and/or AMPK activation in response to in vivo metreleptin administration in obese versus lean and male versus 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 hPBMCs, but not hMT, when compared with 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 hPAs (Fig. 2C) and hPBMCs (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 hMCs (Fig. 2F). There was no difference in mTOR activation in response to in vivo metreleptin administration in obese versus lean and male versus female subjects.

**Regulation of NF-κB 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 NF-κB.
Figure 1—Regulation of STAT3, ERK1/2, Akt, and AMPK signaling by in vivo metreleptin administration in hPTs. A–D: Blood draw and fat (lower abdomen) and muscle (thigh) biopsy were 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 1 min, and after 30 min, blood draw and fat and muscle biopsy were 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.
and IKKα/β in hAT (Fig. 3A and Fig. 4A), hPAs (Fig. 3B and Fig. 4B), and hPBMCs (Fig. 3C and Fig. 4C) in a dozedependent manner. Similar to the results for mTOR signaling, metreleptin-activated NF-κB 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 NF-κB and/or IKKα/β signaling in hMT (Fig. 3D and Fig. 4D) and hMCs (Fig. 3E and Fig. 4E). There was no difference in NF-κB and/or IKKα/β activation in response to ex vivo and/or in vitro metreleptin administration in obese versus 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 C) metreleptin administration has no effects on SHP2 activation in all hPTs. There was no difference in SHP2 activation in response to in vivo metreleptin administration in experiments comparing obese versus lean and male versus female subjects.

Regulation of Differentiation and Lipolysis by In Vitro Metreleptin Administration in hPAs

We observed that in vitro metreleptin administration has no effects on differentiation in hPAs (Fig. 6A and B). By
contrast, we observed that in vitro metreleptin administration induces lipolysis in hPAs (Fig. 6C). There was no difference in differentiation and lipolysis in response to in vitro metreleptin administration in experiments comparing subcutaneous versus omental adipocytes.

**Figure 4**—Regulation of IKKα/β signaling by ex vivo and in vitro metreleptin administration in hPTs. A: Signaling in subcutaneous (SC) and omental (OM) fat from three 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 three female obese subjects. C: Time- and dose-dependent signaling in hPBMCs from three female obese subjects by ex vivo metreleptin administration. D: Signaling in muscle from three female obese subjects before and 30 min after ex vivo metreleptin administration. E: Time- and dose-dependent signaling in primary muscle cells from three female 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. C, control.

Regulation of Cell Growth Signaling and Hypertrophy by In Vitro Metreleptin Administration in Cardiac Myocytes and Uterine Smooth Muscle Cells

In vitro metreleptin administration increased STAT3 activation, but this effect was abolished by coadministration of
STAT3 inhibitor in both cardiac myocytes (CMs) and uterine smooth muscle cells (USMs) (Fig. 7A). Also, we observed that in vitro metreleptin administration increases ERK1/2, JNK, and/or p38 phosphorylation in CMs (Fig. 7B). In vitro metreleptin administration also increased ERK1/2 and/or p38 phosphorylation in USMs (Fig. 7B). By contrast, JNK was not regulated by in vitro metreleptin administration in USMs (Fig. 7B). Hypertrophy was increased by in vitro metreleptin administration in both CMs and USMs (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 in vivo metreleptin signaling in insulin-sensitive tissues such as hAT and hPBMCs, and hMT, and found that leptin activates peripheral STAT3, AMPK, ERK1/2, Akt, mTOR, NF-κB, and/or IKKα/β signaling. Subsequently, we found no difference in in vivo, ex vivo, and/or in vitro metreleptin signaling between male versus female and obese versus lean subjects.

The JAK2/STAT3 pathway is one of the first identified signaling downstream of leptin receptor activation (24,25). STAT3 is a transcription factor that 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 hPBMCs, but not in hMT. Interestingly, unlike our results presented herein, 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 h infusion, the in vivo metreleptin infusion studied herein was 1 min and the signaling was observed 20 min after infusion. Thus, the activation of STAT3 by in vivo metreleptin in hMT needs to be further elucidated in future studies.

**Figure 5** — Regulation of SHP2 signaling by in vivo and ex vivo metreleptin administration in hPTs. 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 three female obese subjects before and 30 min after ex vivo metreleptin administration. C: Time- and dose-dependent signaling in hPBMCs from three 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. C, control.
We have previously shown that leptin-induced ERK/mTOR/Akt signaling is important for cell survival and/or proliferation in hAT and hPBMCs ex vivo (20), and mouse hypothalamic, liver, and muscle cell lines 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 in vivo metreleptin administration activates ERK1/2, mTOR, and Akt signaling in hAT and hPBMCs, 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 hPTs 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 diabetes. 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 be 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 hPTs. 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-γ (IFN-γ) (39). Specifically, metreleptin is closely linked to the activation of inflammatory pathways NF-κB/IKK (40) and increased monocyte chemoattractant protein 1 (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 NF-κB/IKK cascade signaling in hPBMCs, 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 TNF-α 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 versus lean subjects. Also, the effect of leptin on adipocyte function, such as differentiation and lipolysis, was unaltered in adipocytes from obese compared with lean subjects, indicating that the ability to respond to leptin remains unchanged in the obese state. However, all metreleptin signaling pathways studied in hPTs 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, >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 versus 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 central nervous system 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 central nervous system 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 that would mediate energy homeostasis, insulin resistance/metabolism, and immune function in humans and thus add 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 three 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 provides evidence that there is no difference in the signaling between lean versus obese or men versus 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 that 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 hPTs, 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 the development of new therapies for the treatment of insulin resistance syndromes such as type 2 diabetes, inflammation, and obesity.

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Duality of Interest. C.S.M. is a consultant for AstraZeneca. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. H.-S.M. and J.Y.H. wrote the manuscript and participated in the study design, performance, and coordination. P.-O.H., F.D., and B.E.S. participated in the study design, performance, and coordination. C.S.M. wrote the manuscript, participated in the study design, performance, and coordination, and conceived the study. All authors read and approved the final manuscript. H.-S.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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