Central Leptin Regulates Total Ceramide Content and Sterol Regulatory Element Binding Protein-1C Proteolytic Maturation in Rat White Adipose Tissue

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Obesity and type 2 diabetes are associated with insulin and leptin resistance, and increased ceramide contents in target tissues. Because the adipose tissue has become a central focus in these diseases, and leptin-induced increases in insulin sensitivity may be related to effects of leptin on lipid metabolism, we investigated herein whether central leptin was able to regulate total ceramide levels and the expression of enzymes involved in ceramide metabolism in rat white adipose tissue (WAT). After 7 d central leptin treatment, the total content of ceramides was analyzed by quantitative shotgun lipidomics mass spectrometry. The effects of leptin on the expression of several enzymes of the sphingolipid metabolism, sterol regulatory element binding protein (SREBP)-1c, and insulin-induced gene 1 (INSIG-1) in this tissue were studied. Total ceramide levels were also determined after surgical WAT denervation. Central leptin infusion significantly decreased both total ceramide content and the long-chain fatty acid ceramide species in WAT. Concomitant with these results, leptin decreased the mRNA levels of enzymes involved in de novo ceramide synthesis (SPT-1, LASS2, LASS4) and ceramide production from sphingomyelin (SMPD-1/2). The mRNA levels of enzymes of ceramide degradation (Asah1/2) and utilization (sphingomyelin synthase, ceramide kinase, glycosyl-ceramide synthase, GM3 synthase) were also down-regulated. Ceramide-lowering effects of central leptin were prevented by local autonomic nervous system denervation of WAT. Finally, central leptin treatment markedly increased INSIG-1 mRNA expression and impaired SREBP-1c activation in epididymal WAT. These observations indicate that in vivo central leptin, acting through the autonomic nervous system, regulates total ceramide levels and SREBP-1c proteolytic maturation in WAT, probably contributing to improve the overall insulin sensitivity. (Endocrinology 150: 169–178, 2009)
uptake through the inhibition of the enzyme Akt (7, 8), and is involved in the lipotoxicity of the heart (9) and pancreatic β-cells (10). In addition, ceramide is a pivotal substrate for the synthesis of sphingomyelin (SM) and cell surface glycosphingolipids (11).

Recent studies have shown that a low-grade inflammation in adipose tissue contributes to development of obesity related insulin resistance (12). Moreover, increased ceramide content in adipose tissue and inflammation characterize humans with high liver fat content independently of obesity, suggesting that ceramides could contribute to induce both insulin resistance and inflammation (13). In this sense it has been recently reported that ceramide content is increased in adipocytes from insulin-resistant old mice, and suggested that adipocytes are the major contributor to the age-related increase in adipose tissue inflammatory cytokines (14).

A treatment with leptin increases overall insulin sensitivity (15, 16) and down-regulates de novo ceramide synthesis pathway in pancreatic β-cells, by decreasing the expression of serine palmitoyl transferase (SPT) (10). In addition, adenovirus-induced hyperleptinemia prevents ceramide-mediated lipotoxicity in cardiomyocytes from acyl coenzyme A synthase transgenic mice (17). These studies indicate that sphingolipid metabolism could be regulated by central and/or peripheral leptin acting through leptin receptors. Nevertheless, several experimental results suggest that leptin exerts its effects on lipid metabolism predominantly through the hypothalamus, where leptin receptor expression is higher than in other brain regions and tissues (18). In addition, leptin-mediated effects on sphingolipid metabolism in white adipose tissue (WAT) have not been investigated.

Here, we investigated the effects of centrally administered leptin on ceramide contents and on the expression of several enzymes of the sphingolipid metabolism in rat WAT. The role of the autonomic nervous system, as mediator of central leptin effects on WAT, was analyzed. In addition, because changes in the sphingolipid metabolism regulate the activation of the lipogenic transcription factor, sterol regulatory element binding protein (SREBP)-1c (19, 20), we further examined if central leptin regulated SREBP-1c activation, and whether a ceramide-dependent pathway could be involved in this process.

Materials and Methods

Reagents

All chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO) and were of analytical grade. Water and the solvents methanol (both LiChrosolv grade), chloroform (LC grade), acetic acid, and 25% ammonia solution (reagent grade) were from Merck KgA (Darmstadt, Germany). Synthetic lipid standards 1-palmitoyl-2-docosahexanoyl-sn-glycero-3-phosphocholine (PC) (36:6), heptadecanoyl SM (35:12), heptadecanoyl ceramide (Cer 17:0), acetyl ceramide (Cer 2:0), and D6-cholesterol (Chol) were from Avanti Polar Lipids, Inc. (Alabaster, AL). [3-3H]serine was from Amersham Pharmacia Biotech (Uppsala, Sweden).

Animals

Male 3-month-old Wistar rats were housed in climate-controlled quarters with a 12-h light cycle and fed ad libitum standard laboratory diet and water. The animals were handled according to the laws of the European Union and the guidelines of the National Institutes of Health, and the experimental protocols were approved by the institutional committee of bioethics.

Leptin administration and plasma determinations

Intracerebroventricular rat leptin infusion was performed as previously described (21). Three groups of rats randomly distributed were investigated: 1) infused with leptin (0.2 μg/d), 2) infused with vehicle (PBS) and allowed to eat ad libitum, and 3) infused with vehicle and pair fed to the amount of food consumed by the leptin-infused animals. Body weight and food intake were measured daily during the experiment. After 7 d, rats were killed by decapitation. Visceral epidymal WAT (eWAT) and retroperitoneal WAT (rWAT) fat pads were removed and weighed. Fat pads were then flash frozen in liquid nitrogen and stored at −70 C until use. Plasma leptin, insulin, nonesterified FA, and glucose in overnight-fasted rats were measured as previously described (22).

Glucose tolerance test

Glucose tolerance tests were performed in overnight-fasted rats. Rats were weighed and then injected ip with glucose (2 g/kg body weight). Blood samples were obtained from the tail tip at 0, 15, 30, 60, and 120 min after glucose administration, and glucose levels were measured immediately using an Accutrend Glucose Analyzer (Roche Diagnostics Corp., Indianapolis, IN). Plasma insulin levels were assayed using a specific rat kit as previously described (22). Overall changes in glucose and insulin during the glucose tolerance test were calculated as the area under the curve (AUC) using the GraphPad Prism version 3.03 for Windows (GraphPad Software Inc., San Diego, CA). The ratio of the insulin AUC to glucose AUC was used as an index of whole body insulin sensitivity (23).

Phosphorylated signal transducer and activator of transcription-3 (pSTAT3) immunohistochemistry

Immunohistochemical detection of pSTAT3 was performed as described earlier (21). Briefly, animals treated with intracerebroventricular administration of leptin or saline during 7 d were transcardially perfused with 4% paraformaldehyde. The brain was postfixed and cryoprotected, and 40 μm free-floating sections from the hypothalamus were processed for immunostaining with anti-pSTAT3 (Tyr 705) antibodies (catalog no. 9131; Cell Signaling Technology, Inc., Danvers, MA) diluted 1:100 in PBS-0.02% Triton X-100 following the ABC method (Vector Laboratories, Burlingame, CA) and stained with nickel-enhanced diaminobenzidine. In control sections, omitting the primary antibody, no immunoreaction was seen.

rWAT denervation

For the denervation experiments before leptin treatment, unilateral autonomic nervous system denervation of the retroperitoneal fat (rWAT) was performed according to Kreier et al. (24). Local denervation was achieved by surgically removing nerves running along blood vessels from the diaphragm to the superior tip of the left retroperitoneal fat pad, using the contralateral intact right pad in the same animal as control. As previously described (24), unilateral autonomic denervation of rWAT was verified by the down-regulation of leptin and resistin expression in this tissue (data not shown). Five weeks after the surgery, rats were subjected to central leptin administration as described previously.

Real-time RT-PCR

Total RNA was isolated from eWAT using the TRIZOL reagent (Invitrogen Corp., Carlsbad, CA). The cDNA was synthesized from 1.5 μg deoxyribonuclease-treated RNA as reported (22). Relative quantitation of SPT, longevity assurance homologs (LASS2, 4, and 6), acid (SMPD1) and neutral (SMPD2) sphingomyelinase, SM synthase (SMS), acid (Ash1) and neutral (Ash2) ceramidase, ceramide kinase, glycosylceramide synthase, GM3 synthase, SREBP-1c, insulin-induced gene 1 (IN-SIG-1), leptin, resistin, miRNA expression was performed by TaqMan.
real-time PCR according to the manufacturer’s protocol on an ABI PRISM 7500 using Pre-Developed TaqMan Assay Reagents (PE Applied Biosystem, Foster City, CA). For LASS2, 4, and 6, primers and probe sets were designed using the manufacturer’s software and sequences available in GenBank (M29014). 18S rRNA (TaqMan Assay) with VIC as a real-time reporter was used as a control to normalize gene expression. The ΔΔCT method was used to calculate the relative differences between experimental conditions and control groups as fold change in gene expression (25).

Subcellular membrane fractionation, immunoblot analysis, and SPT assay
eWAT was homogenized in two volumes of isolation medium (250 mM sucrose, 10 mM HEPES (pH 7.4), 1 mM EDTA, 2 mM EGTA, 5 mM NaH2PO4, 5 mM NaF, 1 mM phenylmethylsulfonylfluoride, 2 mM Na3VO4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 μg/ml pepstatin). Purified plasma membrane (PM), Golgi apparatus, and endoplasmic reticulum (ER) fractions were obtained as reported by Simpson et al. (26). Membrane protein concentrations were determined using the bicinchoninic protein assay kit from Pierce (Cultek, Spain), using BSA as standard. β-Actin, Na+/K+-adenosine triphosphatase, GRP78, and TGN38 were used as controls for protein loading of the total tissue extract, PM, ER, and Golgi fractions, respectively (supplemental Fig. 1, which is published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). Samples from all experimental conditions were run on the same gel to allow a direct comparison, immunoblotted, and detected as described previously (27). Antibodies directed toward SREBP-1c, total signal transducer and activator of transcription-3 (STAT3), phospho-STAT3 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and antibodies against β-actin, Na+/K+-adenosine triphosphatase, GRP78, and TGN38 were from Abcam plc (Cambridge, UK).

The SPT activity was assayed in microsomal fractions from eWAT by following the incorporation of radiolabel from [3H]serine into chloroform-soluble products according to the methods of Williams et al. (28), with sphinganine as a carrier. Control assays were performed with each sample in the absence of palmityl-coenzyme A. All assays were done in triplicate, and the SPT activity was expressed as pmol/min/mg protein.

Lipid extraction for mass spectrometric analysis
Lipids were extracted from whole eWAT and/or rWAT by Folch et al. (29). Briefly, 20 mg eWAT was homogenized in 0.2 ml methanol and then in 0.4 ml chloroform, in the presence of Cer 17:0 (80 pmol/mg tissue) as internal standard. The suspension was sonicated for 15 min. A total of 0.15 ml water was added, and the mixture was centrifuged for 15 min at 4000 rpm. The organic phase was dried by rotary distillation under a gentle stream of nitrogen. The dried organic phase was taken up in 1 ml MSmix for mass spectrometric analysis.

Chol determination by electrospray ionization tandem mass spectrometry (MS/MS)
Lipid extracts prepared as described previously were acetylated according to Liebisch et al. (30). Afterwards, 20 μl MSmix was added. Cholesterol-acetate was determined by MS/MS in positive ion mode using multiple reaction monitoring scans. Ammonium adducts of molecular ions of O-acetyl Chol with m/z 446.4 (CE 2:0) and of the internal standard O-acetyl D6-Chol with m/z 452.4 (D6-CE 2:0) were selected for quantification by thin-layer chromatography (TLC), as described below. Membrane fractions from eWAT containing 10 μg protein were vortexed in glass tubes with 0.6 ml water, 0.8 ml methanol, and 1.6 ml chloroform, in the presence of internal standards Cer 17:0 (1.575 pmol/μg protein), SM 35:1:2 (17:0) (20 pmol/μg protein), D6-Chol (60 pmol/μg protein), and PC 36:6 (18:3/18:3) (20 pmol/μg protein). The mixture was sonicated for 15 min, centrifuged as described previously, and the dried organic phase was taken up in 1 ml MSmix for mass spectrometric analysis.
infused rats fed 0.5 ml MSmix for the mass spectrometric analysis. A hybrid QSTAR Pulsar method of Folch as indicated in Fig. 1A. The scrapped silica was extracted using the starting point. Strips containing ceramides were excised of the TLC plate consequently, the two parts were aligned. Under these TLC conditions, developed in hexane/ethylacetate (5/1, vol/vol). Developed silica plates performance plates (CAMAG Scientific Inc., Wilmington, NC). Five ml/mg tissue fragments were quantified by positive ion mass spectrometry using Cer 17:0 as internal standard. Values are the means ± sem of three to four separate determinations per group of animals, each one made in duplicate. Different letters indicate significant differences between treatments (P < 0.05, one-way ANOVA followed by Tukey test). Lep, Leptin-infused rats; PF, vehicle-infused pair-fed rats; SS, vehicle-infused rats fed ad libitum.

TLC ceramide purification

Preparative TLC was performed on aluminum-back silica 60 high-performance plates (CAMAG Scientific Inc., Wilmington, NC). Five micrograms (two replicates) of lipid extracts from the whole WAT (eWAT and/or rWAT) in MSmix were loaded as a 1-cm lane on a silica plate and developed in hexane/ethylacetate (5/1, vol/vol). Developed silica plates were dried and cut in half, with one half stained by sulfuric acid. Subsequently, the two parts were aligned. Under these TLC conditions, TAGs and diacylglycerols migrated, whereas ceramides stayed at the starting point. Strips containing ceramides were excised of the TLC plate as indicated in Fig. 1A. The scrapped silica was extracted using the method of Folch et al. (29), and recovered ceramides were redissolved in 0.5 ml MSmix for the mass spectrometric analysis.

Quantification of lipid species by quadrupole time-of-flight mass spectrometry (TOF MS)

Mass spectrometric analysis was performed in positive ion mode on a hybrid QSTAR Pulsar i mass spectrometer (MDS Sciex, Concord, Canada) equipped with an automated nanoflow ion source NanoMate HD System (Advion BioSciences, Ithaca, NJ). The instrument was calibrated in MS/MS mode using a synthetic lipid standard, PC as described previously (31). SMs and PCs in eWAT PM were quantified in positive ion mode by precursor ion scanning of the phosphorylcholine head group fragment (m/z 184.07) (supplemental Fig. 2). Spectra were interpreted using prototype Lipid Profiler software (MDS Sciex) (32). Ceramide quantification was performed by MS/MS in positive ion mode using multiple reaction monitoring. Ceramide molecular species detected in eWAT (supplemental Table 1) were fragmented under the collision energy offset of 40 eV. Analytical quadrupole Q1 was operated under the unit mass resolution settings. Peak enhancement was set at m/z 282.3 to increase the detection of the characteristic product ions for the 18-carbon sphingosine base at m/z 252.27, 264.27, and 282.27 (supplemental Fig. 3) (33).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 3.03 for Windows. Significant differences among groups were determined by one-way ANOVA, followed by the Tukey test. For simple comparisons between two groups, an unpaired Student’s t test was performed.

Results

Central leptin infusion reduces total ceramide content in WAT through the autonomic nervous system

Initial mass spectrometric analyses of lipid extracts from whole eWAT showed that acylglycerides, which constitute more than 90% of the lipid contents of this tissue, were interfering with the electrospray ionization MS/MS analysis of the mixture by suppressing the signal (data not shown). Therefore, to determine endogenous ceramides in total WAT ex-

Total ceramide levels and ceramide FA abundance in chloroformic extracts from total eWAT were quantified by positive ion mass spectrometry using Cer 17:0 as internal standard. Values are the means ± sem of three to four separate determinations per group of animals, each one made in duplicate. Different letters indicate significant differences between treatments (P < 0.05, one-way ANOVA followed by Tukey test). Lep, Leptin-infused rats; PF, vehicle-infused pair-fed rats; SS, vehicle-infused rats fed ad libitum.

**TABLE 2.** Effects of central leptin and local autonomic nervous system denervation on ceramide (Cer) contents in rWAT

<table>
<thead>
<tr>
<th>Ceramide species</th>
<th>SS intact</th>
<th>Lep intact</th>
<th>SS denervated</th>
<th>Lep denervated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cer content (pmol/mg tissue)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>11 ± 1</td>
<td>9.3 ± 0.4</td>
<td>9.2 ± 2</td>
<td>13 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0</td>
<td>0.6 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>20:0</td>
<td>0.5 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>22:0</td>
<td>1.4 ± 0.4</td>
<td>1.3 ± 0.3</td>
<td>1.4 ± 0.1</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>24:1</td>
<td>0.8 ± 0.3</td>
<td>0.2 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>24:0</td>
<td>5.5 ± 0.3</td>
<td>4.0 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.1 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.6 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total content (pmol/mg tissue)</td>
<td>20 ± 1</td>
<td>16 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18 ± 0.8</td>
<td>22 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Total ceramide levels and ceramide FA abundance in chloroformic extracts from total eWAT were quantified by positive ion mass spectrometry using Cer 17:0 as internal standard. The same animal, the left retroperitoneal fat pad was denervated, whereas the intact right pad was used as control. Values are the means ± sem of three to four separate determinations per group of animals, each one made in quadruplicate. Lep, Leptin-infused rats; SS, vehicle-infused rats fed ad libitum.

<sup>a</sup> P < 0.05 vs. the respective value in intact vehicle-infused rats.
<sup>b</sup> P < 0.05 vs. the respective value in intact leptin-infused rats, by an unpaired Student’s t test.
TABLE 3. The effect of central leptin administration on body weight, food intake, glucose tolerance, and plasma hormone and metabolite concentrations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SS</th>
<th>PF</th>
<th>Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>353 ± 10a</td>
<td>372 ± 20a</td>
<td>359 ± 13a</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>380 ± 13a</td>
<td>359 ± 11b</td>
<td>344 ± 8b</td>
</tr>
<tr>
<td>Daily food intake (g)</td>
<td>19 ± 1f</td>
<td>13 ± 1f</td>
<td>13 ± 1f</td>
</tr>
<tr>
<td>eWAT weight (g)</td>
<td>5.5 ± 0.3a</td>
<td>5.0 ± 0.4a</td>
<td>3.7 ± 0.6b</td>
</tr>
<tr>
<td>rWAT (g)</td>
<td>4.9 ± 0.4a</td>
<td>4.6 ± 1.1a</td>
<td>2.9 ± 0.4b</td>
</tr>
<tr>
<td>Plasma insulin (μU/ml)</td>
<td>40 ± 12a</td>
<td>35 ± 1.5a</td>
<td>17.5 ± 2.5b</td>
</tr>
<tr>
<td>Plasma leptin (ng/ml)</td>
<td>5.9 ± 0.5a</td>
<td>5.4 ± 1a</td>
<td>5.2 ± 0.8a</td>
</tr>
<tr>
<td>Plasma glucose (mM)</td>
<td>6.5 ± 0.8a</td>
<td>5.9 ± 0.4a</td>
<td>5.9 ± 0.6a</td>
</tr>
<tr>
<td>Plasma NEFA (mM)</td>
<td>0.51 ± 0.06a</td>
<td>0.2 ± 0.03b</td>
<td>0.46 ± 0.04a</td>
</tr>
<tr>
<td>Glucose AUC (mm x min)</td>
<td>2632 ± 291a</td>
<td>2503 ± 423a</td>
<td>2822 ± 119a</td>
</tr>
<tr>
<td>Insulin AUC (μU x min)</td>
<td>71 ± 29a</td>
<td>57 ± 27a</td>
<td>19 ± 1b</td>
</tr>
<tr>
<td>Insulin sensitivity index</td>
<td>0.026 ± 0.008a</td>
<td>0.021 ± 0.007a</td>
<td>0.006 ± 0.001b</td>
</tr>
</tbody>
</table>

After 7 d central leptin (Lep) 0.2 μg/d or vehicle (SS or PF) infusion, fasting hormonal and metabolic parameters were assessed in plasma. Results are the mean ± SEM (n = 6–10 rats per group of animals, made in triplicate). For the ip glucose tolerance test, blood glucose and serum insulin levels were determined in duplicate (n = 3–4 rats per group of animals). The average AUC of glucose and insulin, and insulin sensitivity index were calculated as indicated in Materials and Methods. Different letters indicate significant differences among treatments (P < 0.05, one-way ANOVA followed by Tukey test). Lep, Leptin-infused rats; NEFA, nonesterified FA; PF, vehicle-infused pair-fed rats; SS, vehicle-infused rats fed ad libitum.

FIG. 2. Changes in tyrosine phosphorylation of STAT3 after central leptin administration. A–D, Representative microphotographs of STAT3-PY immunohistochemistry of hypothalamic sections from saline-infused and leptin-infused (0.2 μg/d) rats for 7 d. After 7-d infusion of leptin, there is an increase in the staining of STAT3-PY (see arrows) in the paraventricular nucleus (PVN) (B) and arcuate nucleus (ARC) (D) respect to saline-infused (A and C) rats. E, A representative immunoblot showing total STAT3 and STAT3-PY in 70 μg total extracts from eWAT after saline or leptin treatment from three individual experiments per group of animals. Bands were quantified by scanning densitometry, and data were expressed as ratio of phosphorylated to total STAT3. There were not differences in the eWAT data between groups of rats after central saline and/or leptin infusion (data not shown). Scale bars, 150 μm. Leptin, Leptin-infused rats; PF, vehicle-infused rats pair-fed to the amount of food consumed by the leptin-infused animals; SS, vehicle-infused rats fed ad libitum-fed rats.

The total ceramide content in eWAT from saline-infused ad libitum and pair-fed control rats was 36 ± 3 pmol/mg tissue (Table 1). Upon central leptin infusion, the total ceramide content in eWAT was significantly reduced by 31%, with the largest and significant decreases of approximately 31–37% being observed for the most abundant Cer 16:0, Cer 24:1, and Cer 24:0 ceramides.

The levels of endogenous ceramides in rWAT extracts quantitated by mass spectrometry were comparable to the ones obtained in eWAT (Table 2). Besides, total ceramide content and the long-chain FA ceramides in rWAT were also significantly decreased after central leptin administration.

To investigate if central leptin effects on WAT were mediated by neural circuits and/or elicited directly after binding to leptin receptors in WAT, we performed unilateral autonomic nervous denervation of the rWAT. As shown in Table 2, local denervation abolished central leptin lowering effects on cellular ceramides in rWAT. Moreover, although central leptin treatment significantly decreased food intake and visceral fat weight, no differences were found in plasma leptin levels (Table 3). Finally, central leptin treatment increased the STAT3 tyrosine phosphorylation levels in the paraventricular and arcuate nuclei (Fig. 2, B and D), regions of the hypothalamus known to express high levels of leptin receptors, but there was not leptin effect on STAT3 tyrosine phosphorylation in eWAT (Fig. 2E). Collectively, these results suggest that central leptin effects on ceramide contents in WAT are indirect and mediated through hypothalamic neural circuits and the autonomic nervous system.

trants, we purified them by TLC before mass spectrometric analysis. A representative survey (TOF MS) spectrum acquired by direct infusion of TLC-purified lipid extracts from rat eWAT in positive ion mode is shown in Fig. 1A. Although in TOF MS major signals were ions from background, in MS/MS spectra acquired from ceramide precursors, the specific fragments m/z 252.27, 264.27, and 282.27 were clearly detectable (Fig. 1B) (33). These reporter ions were subsequently used for ceramide quantification.

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Central leptin infusion increases overall insulin sensitivity

Recent evidence demonstrated that decreasing ceramide synthesis, using pharmacological and genetics approaches, improved insulin sensitivity in rodent models of insulin resistance (34). Thus, to determine whether the decrease in ceramide content in WAT after central leptin infusion was associated with changes in overall insulin sensitivity, we performed an ip glucose tolerance test. As can be seen in Table 3, the AUC for glucose was similar for the three groups of rats. In contrast, the AUC for insulin in response to the glucose load was much lower in leptin-treated than in saline-infused ad libitum and pair-fed control rats (Table 3). Thus, the insulin sensitivity index, calculated as the ratio of the insulin AUC to glucose AUC, was improved significantly in leptin-treated rats. These data might suggest that leptin lowering effects on ceramides in WAT could contribute, at least in part, to increase the overall insulin sensitivity.

Leptin regulates the expression of enzymes involved in ceramide metabolism

Because in mammalian tissues ceramides are mainly produced by de novo synthesis, we analyzed the expression pattern and the enzymatic activity of SPT, the rate-limiting enzyme in this pathway, as well as expression of the three (dihydro)ceramide synthase isoforms (LASS2, 4, and 6) (35) detected by us for the first time in eWAT.

Central leptin infusion repressed the gene expression of SPT by 29% compared with the pair feeding (Fig. 3). Moreover, SPT activity in eWAT parallels mRNA expression and was significantly reduced in leptin-treated compared with saline-infused pair-fed control rats (12.2 ± 1.4 vs. 15.7 ± 0.8 pmol/mg · min, respectively; n = 6; P < 0.05). The mRNA levels of LASS2 and LASS4 were down-regulated by 57 and 31%, respectively, compared with the pair-fed animals, whereas LASS6 mRNA levels did not change (Fig. 3).

Ceramides are also formed through SM hydrolysis by acid (SMPD-1) and/or neutral (SMPD-2) sphingomyelinas. Central leptin treatment down-regulated SMPD-1 and SMPD-2 by 57 and 44%, respectively, compared with the pair feeding (Fig. 3). These results suggest a decrease in ceramide production from SM in eWAT after central leptin treatment.

Cellular ceramide levels also depend on the activity of enzymes of ceramide degradation (Asah1/2) and of ceramide utilization for the synthesis of more complex sphingolipids (SMS, ceramide kinase, glycosyl-ceramide synthase, GM3 synthase). Central leptin reduced the gene expression of these enzymes compared with the pair-fed rats (Fig. 3).

Leptin treatment reduces total ceramide content in the Golgi apparatus from eWAT

According to our data, total ceramide content in Golgi membrane fraction from ad libitum and pair-fed rats was 1.5 ± 0.2 pmol/µg protein (Table 4). Central leptin infusion significantly reduced total ceramide levels in the Golgi fraction by 33%, compared with the pair-fed group of animals. In addition, leptin changed the FA ceramide profile increasing the relative abundance of the medium-chain FA Cer 16:0 and decreasing the unsaturated long-chain FA Cer 24:1 (Table 4). These results agree well with the changes elicited by leptin on LASS isoforms expression in eWAT (Fig. 3).

Central leptin administration inhibits SM synthesis and decreases the SM and free Chol contents at the PM of eWAT

Lower ceramide levels in the Golgi membrane fraction (Table 4), together with a down-regulated SMS gene expression in leptin-treated rats (Fig. 3), the enzyme that catalyzes the production of SM from ceramide in the Golgi apparatus, suggested a decreased SM synthesis in eWAT after leptin treatment. To confirm this hypothesis, we measured the SM levels at the PM, the subcellular membrane fraction with the highest SM concentration (36).

In line with our hypothesis, total SM levels at the PM decreased significantly after leptin treatment compared with the pair-fed control animals (313 ± 14 vs. 403 ± 25 pmol/µg protein) (Table 5), being the most abundant the SM 16:0, which aligns with the substrate preference of ceramide transport protein (CERT), the protein that transfers ceramides from the ER to the Golgi apparatus for SM synthesis (37). In addition, the amount of free Chol at the PM,
Central leptin administration impairs SREBP-1c proteolytic maturation in the ER

Leptin administration inhibits lipogenesis in lean tissues (22, 40) and WAT (22, 41, 42). In lean tissues this effect was exerted through down-regulation of SREBP-1c mRNA levels (22, 40). However, in agreement with our previous observations (22), SREBP-1c mRNA levels in eWAT were not affected by central leptin treatment (Fig. 3). Considering that SREBP is also regulated posttranscriptionally, and that ceramide synthesis regulates SREBP cleavage in Chinese hamster ovary cells (20), we studied the effects of central leptin on the mechanism of SREBP-1c activation in eWAT.

In leptin-infused rats, the inactive precursor form of SREBP-1c protein (125 kDa) significantly increased in ER (Fig. 3), whereas the amount of the mature transcriptionally active form of SREBP-1c (65 kDa) decreased, compared with the pair-fed control rats (Fig. 4A, lower panel). Moreover, although total ceramide (5.4 ± 0.6 pmol/µg protein) and free Chol (133 ± 11 pmol/µg protein) levels in the ER of eWAT were unaltered by central leptin (data not shown), the mRNA levels of INSIG-1, an ER protein involved in SREBP-1c activation (43), were markedly up-regulated (Fig. 3), compared with the pair feeding. Therefore, our results suggest that leptin inhibits SREBP-1c proteolytic maturation by retaining the precursor form of this transcription factor in the ER of eWAT, probably, through INSIG-1 induction.

Discussion

Leptin affects body weight and food intake acting through neuroendocrine pathways and autonomic nerves, which transmit leptin signal to the periphery. However, the neural pathways and the neurochemical phenotypes of the neurons comprising the autonomic nervous circuits to WAT are largely unknown (44). The results presented here demonstrate that central leptin administration decreased total ceramide levels in WAT acting through the autonomic nervous system. Although we have not addressed the pathway for central leptin action on WAT ceramide metabolism, the fact that plasma leptin and STAT3 phosphorylation in eWAT and also decreased upon leptin infusion compared with the pair-fed control rats (38, 39), was similar to the one of SM according to previous reports (38, 39), was similar to the one of SM and also decreased upon leptin infusion compared with the pair-fed control animals (358 ± 4 vs. 435 ± 10 pmol/µg protein; n = 3–4; P < 0.05). Finally, in this subcellular fraction, total ceramide (11 ± 1.0 pmol/µg protein) or PC (741 ± 21 pmol/µg protein) levels did not change after central leptin treatment (data not shown).

<table>
<thead>
<tr>
<th>Ceramide species</th>
<th>SS (pmol/µg protein)</th>
<th>PF (pmol/µg protein)</th>
<th>Leptin (pmol/µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>0.51 ± 0.05</td>
<td>0.50 ± 0.09</td>
<td>0.27 ± 0.07</td>
</tr>
<tr>
<td>18:0</td>
<td>0.062 ± 0.005</td>
<td>0.05 ± 0.01</td>
<td>0.040 ± 0.007</td>
</tr>
<tr>
<td>20:0</td>
<td>0.11 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>22:0</td>
<td>0.065 ± 0.006</td>
<td>0.05 ± 0.01</td>
<td>0.030 ± 0.005</td>
</tr>
<tr>
<td>24:1</td>
<td>0.44 ± 0.05</td>
<td>0.44 ± 0.06</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>24:0</td>
<td>0.39 ± 0.06</td>
<td>0.37 ± 0.05</td>
<td>0.23 ± 0.04</td>
</tr>
<tr>
<td>Total content (pmol/µg protein)</td>
<td>1.6 ± 0.2 a</td>
<td>1.5 ± 0.2 a</td>
<td>1.0 ± 0.2 b</td>
</tr>
</tbody>
</table>

Total ceramide levels and ceramide FA relative abundance in the Golgi fraction of eWAT were quantified by positive ion mass spectrometry using Cer 17:0 as internal standard. Values are the means ± SEM of three to four separate determinations per group of animals, each one made in duplicate. Different letters indicate significant differences between treatments (P < 0.05, one-way ANOVA followed by Tukey test). Lep, Leptin-infused rats; PF, vehicle-infused pair-fed rats; SS, vehicle-infused rats fed ad libitum.
erating effects on cellular ceramides in rWAT were suppressed by local surgical autonomic nervous denervation of WAT. Thus, leptin, acting through hypothalamic neural circuits and the autonomic nervous system, decreases total ceramide levels in WAT. The decrease in ceramide levels in both fat pads approximately 31% after central leptin infusion, are similar to the levels found in several in vitro studies reporting that induc- ing comparable changes in endogenous ceramide contents is sufficient to elicit effects on insulin signaling (45, 46). More- over, the reported ceramide increase in rodent insulin-resis- tant lean tissues is also modest (26% in liver and 32% in skeletal muscle) (5). These observations strongly suggest that the leptin-induced decrease in WAT ceramide levels may be sufficient to play a role in the effects of the hormone on this tissue.

The decrease in total ceramide levels after central leptin treat- ment were consistent with the reduced expression of genes in- volved in de novo ceramide synthesis and/or formation such as SPT, LASS2 and 4, SMPD1, and SMPD2. The expression of the genes involved in ceramide degradation and utilization was also reduced by leptin, suggesting that this could constitute a mechanism for maintaining the cellular levels of this biologically active lipid within a physiological range. Although the mechanisms regulating SPT or LASS gene expression are largely unknown, the data presented herein provide for the first time evidence of a link between leptin and these genes in eWAT.

The activity of the SPT, the rate-limiting enzyme in de novo ceramide synthesis, is strongly enhanced by the availability of FA in vitro (47). In this sense, we have previously suggested that an elevated lipolysis and suppression of glyceroneogenesis in eWAT could be the explanation of the higher plasma FA levels observed in leptin-treated rats (Table 3) (22) and of the decrease in visceral fat content reported herein, compared with the pair-fed group. In addition, there is evidence reporting that leptin administration decreased 3-fold the lipoprotein lipase activity in eWAT (48) and down-regulated the mRNA levels of FAT/CD36 in isolated adipocytes (49). Collectively, these data suggest a decreased FA uptake by WAT after leptin treat- ment. The reduction in the FA supply to WAT and the significant decrease in SPT activity reported herein could contribute to explain the decrease in total ceramide levels in this tissue after central leptin infusion.

Obesity is accompanied by an increased proinflammatory cy- tokine secretion either by adipocytes or by macrophages infiltr- ating the fat bed (50), and ceramides have mediated the in-
flammatory response of several cytokines (51). In fact, it has recently been reported that treatment of 3T3-L1 adipocytes with C6 ceramide induced gene expression of the proinflammatory molecules TNF-α, IL-6, and monocyte chemoattractant protein-1 to various extents (52). Moreover, recent evidence demon- strated that decreasing ceramides synthesis, using pharmaco- logical and genetic approaches, improves insulin sensitivity in rodent models of insulin resistance, obesity, and diabetes (34). In addition, these authors hypothesized that ceramide generated in and secreted from visceral WAT could contribute to the insulin resistance in lean tissues. With this regard, central leptin administration decreases both TNF-α protein levels (53) and mRNA expression (unpublished observations) in eWAT. Thus, the decrease in WAT ceramide content by central leptin re- ported herein would contribute to impair the buildup of the chronic inflammatory state that characterizes obesity and to increase overall insulin sensitivity. This conclusion is sup- ported by studies in mice showing that reducing ceramide levels in isolated adipocytes decreased TNF-α, and IL-6 produc- tion (14) and by the improved insulin sensitivity index observed herein after central leptin administration (Table 3). Neverthe- less, although in this study we cannot establish a direct association between the decrease in WAT ceramide content and the improved insulin sensitivity after central leptin infusion, our data agree with previous findings reporting that decreasing ceramide levels ameliorates insulin resistance and improves whole body insulin sensitivity (34).

Ceramide is one of the substrates for SM synthesis. Thus, lower ceramide levels in WAT, together with a decreased INS gene expression in this tissue of leptin-treated rats, could result in reduced SM contents. In line with this hypothesis, the PM, the subcellular organelle with the highest SM content (36), shows decreased SM levels upon central leptin infusion, whereas the contents of PC, the lipid that provides the choline head group for SM synthesis, do not change. In addition, ceramide levels in the Golgi fraction, the main site for SM synthesis, are lowered by
leptin infusion. All these observations, together with the down-regulation of sphingomyelinases gene expression in eWAT in leptin-infused rats, support the notion that the lower SM levels in PM are due to decreased ceramide availability for SM synthesis and not to enhanced SM hydrolysis.

Decreased SM synthesis has inhibited SREBP-1c maturation in Chinese hamster ovary cells (20). The mechanism proposed was that decreasing the PM SM content would result in a higher free Chol partitioning to intracellular membranes, such as ER, preventing the SREBP cleavage-activating protein (SCAP)-SREBP complex translocation from ER to Golgi for SREBP proteolytic activation. However, despite the leptin-induced decrease in SM and free Chol contents at the eWAT PM, free Chol levels do not change in ER in this tissue, suggesting that the inhibition of SREBP-1c ER-to-Golgi translocation in leptin-treated rats is probably due to a high content of INSIG-1 in the ER fraction. Moreover, when insulin levels are low, as in the case of leptin-treated rats, interactions between INSIGs and SCAP are facilitated, and the SCAP-SREBP-1c-complex is retained in the ER membranes (54).

Central leptin elicits an almost a 300% increase in INSIG-1 gene expression, the ER anchor of the SCAP-SREBP complex (43). This could enhance the accumulation of SREBP in the ER, decreasing its arrival to the Golgi apparatus for proteolytic activation, and thereby contributing to decrease lipogenesis in WAT. Our findings agree well with the reports of Yang et al. (43), who obtained a Chol-independent retention of the SREBP-SCAP complex in the ER by overexpressing INSIG-1. However, the mechanism of centrally mediated leptin induction of INSIG-1 in eWAT needs further investigation.

Finally, although SREBP-1c is well known for playing a central role in the regulation of lipogenic genes expression, the regulation of its activity alone by leptin does not seem to account fully for the inhibition of lipogenic gene expression because SREBP-1c-knockout mice present only a 50% reduction in FA synthesis (55). In fact, our recently published data showed that the lipogenic transcription factor carbohydrate response element binding protein is also involved in the leptin-mediated down-regulation of lipogenic gene expression in WAT (22).

In conclusion, the results presented here demonstrate that leptin, acting only within the brain and through the autonomic nervous system, reduces total ceramide levels in WAT. On the other hand, most likely through INSIG-1 gene up-regulation, central leptin prevents SREBP-1c maturation in eWAT. Although in this study we cannot establish a direct association between central leptin effects and insulin sensitivity, our data suggest that leptin could contribute to improve the whole body insulin sensitivity regulating the sphingolipid metabolism. Nevertheless, additional studies have to be done before a final conclusion may be drawn.

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