Induction by leptin of uncoupling protein-2 and enzymes of fatty acid oxidation

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ABSTRACT We have studied mechanisms by which leptin overexpression, which reduces body weight via anorexic and thermogenic actions, induces triglyceride depletion in adipocytes and nonadipocytes. Here we show that leptin alters in pancreatic islets the mRNA of the genes encoding enzymes of free fatty acid metabolism and uncoupling protein-2 (UCP-2). In animals infused with a recombinant adenovirus containing the leptin cDNA, the levels of mRNAs encoding enzymes of mitochondrial and peroxisomal oxidation rose 2- to 3-fold, whereas mRNA encoding an enzyme of esterification declined in islets from hyperleptinemic rats. Islet UCP-2 mRNA rose 6-fold. All in vitro changes occurred in vitro in normal islets cultured with recombinant leptin, indicating direct extraneuronal effects. Leptin overexpression increased UCP-2 mRNA by more than 10-fold in epididymal, retroperitoneal, and subcutaneous fat tissue of normal, but not of leptin–receptor-defective obese rats. By directly regulating the expression of enzymes of free fatty acid metabolism and of UCP-2, leptin controls intracellular triglyceride content of certain nonadipocytes, as well as adipocytes.

Overexpression of leptin, the adipocyte hormone that regulates body composition through its effects on food intake and energy metabolism (1–5), causes the rapid disappearance of all grossly visible body fat, usually within 1 week (6). In addition, the triglyceride (TG) content of nonadipocytes such as the pancreatic islets is profoundly reduced (7). Because these changes in body fat are unaccompanied by an increase in plasma levels of free fatty acids (FFA) and β-hydroxybutyrate or by ketonuria, we have concluded that the TG must have been undergone internal hydrolysis and oxidation within the individual cells (8). This conclusion is supported by in vitro studies showing that leptin lowers the TG content of isolated islets by reducing esterification and by increasing oxidation of FFA (8). It is also consistent with a report that leptin decreases the TG content of isolated islets from hyperleptinemic rats. By directly regulating the expression of enzymes of fatty acid metabolism and of UCP-2, leptin controls intracellular triglyceride content of certain nonadipocytes, as well as adipocytes.

MATERIALS AND METHODS

Animals. Lean wild-type (+/+ ) male Zucker diabetic fatty (ZDF) rats and obese homozygous (fa/fa) male ZDF rats were bred in our laboratory from [ZDF/Drt-fa[F10]] rats purchased from R. Peterson (University of Indiana School of Medicine, Indianapolis). Their genotype was confirmed using the method of Phillips et al. (15). Some of the rats were made hyperleptinemic by the previously described method of leptin gene transfer (6), in which a recombinant adenovirus containing the rat leptin cDNA under control of the cytomegalovirus (CMV) promoter (AdCMV–leptin) or virus containing the bacterial β-galactosidase gene (AdCMV–β-gal) was administered. Two milliliters of AdCMV–leptin or AdCMV–β-gal containing a total of 1 × 1012 plaque-forming units were infused into lean male homozygous ZDF (+/+ ) rats over a 30-min period. A third group of rats was infused with only saline. A fourth group of rats was pairfed to the hyperleptinemic rats. Animals were studied in individual metabolic cages, and food intake and body weight were measured daily. On the fourth post-infusion day they were sacrificed. At that time, food intake and body weight in AdCMV–leptin-infused rats were significantly below AdCMV–β-gal and saline-infused controls, but body fat had not yet disappeared.

Extraction of Total RNA and Reverse Transcriptase (RT)-PCR Quantitation. Fat tissue was obtained from epididymal, retroperitoneal, and subcutaneous sites. Pancreatic islets were isolated according to the method of Naber et al. (16). Total RNA was extracted with the TRIzol isolation method (Life Technologies, Gaithersburg, MD) from about 100 isolated islets of individual rats. RNA was treated with RNase-free DNase (Promega), and first-strand cDNA was generated from 1 μg of RNA in a 20 μl volume using the oligo(dT) primer in the 1st-strand cDNA synthesis kit (CLONTECH). One microliter of the reverse transcription reaction mix was amplified with primers specific for rat acyl-CoA oxidase (ACO), carnitine palmitoyltransfase (CPT-I), ACC, glycerol-3-phosphate acyltransferase (GPAT), and UCP-2 in a total of 50 μl. All sequences were from the GenBank. Accession numbers and primer and probe sequences are shown in Table 1. Linearity of the PCR reaction was tested by amplification of 200 ng of total RNA per reaction from 15–50 cycles. The linear range was found to be between 15 and 40 cycles. In no case did the amount of RNA used for PCR reaction exceed 200 ng per reaction. The samples were amplified for 25–28 cycles using the following parameters: 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min. β-actin primers were used as a control. Levels of mRNA were expressed as the ratio of signal intensity for the
RESULTS

**FFA Metabolism in Hyperleptinemic Islets.** Recombinant leptin has been shown to reduce the TG content of islets by increasing oxidation and reducing esterification of radiolabeled palmitate in *vitro* (8). While chronic hyperleptinemia has been demonstrated to reduce the TG content of islets to unmeasurable values in *vivo* (7, 8), effects on long-chain fatty acid metabolism in islets had not been studied in this model. Consequently, islets were isolated from hyperleptinemic rats and their euleptinemic control groups and cultured in 0.1 mM [3H]palmitate for 24 hr. The esterification rate per 24 hr in islets of hyperleptinemic rats was 33% below pairfed controls, while the rate of oxidation was 92% higher (Table 2). These metabolic changes resemble those induced in cultured islets by recombinant leptin (8), and may account for the marked depletion of fat in the islets of the hyperleptinemic rats. Moreover, they suggested that leptin might deplete TG by up-regulating enzymes of FFA oxidation, and increase thermogenesis by uncoupling oxidation from energy-consuming processes.

**mRNA Levels of Enzymes Involved in FFA Oxidation and Esterification.** To determine if the metabolic effects of hyperleptinemia were associated with appropriate changes in the expression of the enzymes of long-chain fatty acid metabolism, the ratio of the mRNA encoding each enzyme of interest to that for β-actin mRNA was determined in all groups of rats. The enzymes of interest included two enzymes of FFA oxidation, CPT-I and ACC. The GPAT, which catalyzes the first step in FFA esterification to TG. In freshly isolated islets from leptin-overexpressing rats CPT-I and ACC, the product of which, malonyl-CoA, inhibits CPT-I (10), and GPAT, which catalyzes the first step in FFA esterification to TG. In freshly isolated islets from leptin-overexpressing rats CPT-I and ACC, the product of which, malonyl-CoA, inhibits CPT-I (10), and GPAT, which catalyzes the first step in FFA esterification to TG.

**Table 1. Sequences of PCR primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer (5’-3’-3’-5’)</th>
<th>Antisense primer (5’-3’-3’-5’)</th>
<th>Internal primers (5’-3’, 30-mers)</th>
<th>Size of cDNA, bp</th>
<th>Nucleotide no.</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>TTGATACCACTGGGACGATAGG</td>
<td>GAGTCTGACCTGCTGTGGTCAAG</td>
<td>GGCAGACATTCGTACATGTCGAG</td>
<td>764</td>
<td>1552-2991</td>
<td>J00691</td>
</tr>
<tr>
<td>CPT-I</td>
<td>TATGGAGAAGTGCTGCTCCT</td>
<td>CTCGAGAGCATGCTGCTGTC</td>
<td>ATCCTGGGTTAATGCTGCTGTTGG</td>
<td>629</td>
<td>3094-3722</td>
<td>L07736</td>
</tr>
<tr>
<td>ACO</td>
<td>GCCCTGACCTGTGCTGATAC</td>
<td>AGGAACTGCTCTGAACTGTGCGC</td>
<td>GCCTGACCTGCTGCTGCTGCTG</td>
<td>634</td>
<td>2891-3324</td>
<td>J02752</td>
</tr>
<tr>
<td>ACC</td>
<td>TCTGCTGACCTGCTGCTGTC</td>
<td>AGTGCACCTGCTGCTGCTGCTG</td>
<td>AGTGCACCTGCTGCTGCTGCTG</td>
<td>535</td>
<td>4666-5180</td>
<td>J03808</td>
</tr>
<tr>
<td>GPAT</td>
<td>TGGATACCACTGGGACGATAGG</td>
<td>GAGTCTGACCTGCTGTGGTCAAG</td>
<td>GGCAGACATTCGTACATGTCGAG</td>
<td>504</td>
<td>1827-2350</td>
<td>M79803</td>
</tr>
<tr>
<td>UCP-2</td>
<td>AACATGCTCTACACAGGCGGG</td>
<td>AGTACGCTGACCTGCTGCTG</td>
<td>GTCAATGCTCATTGACTGCTGCTG</td>
<td>471</td>
<td>311-782</td>
<td>J069135</td>
</tr>
</tbody>
</table>

**Table 2. Rates of oxidation and esterification of 9,10-[3H]palmitate in islets isolated from AdCMV–β-gal and AdCMV–leptin-infused rats and paired controls**

<table>
<thead>
<tr>
<th></th>
<th>AdCMV–β-gal</th>
<th>AdCMV–leptin</th>
<th>Paired</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation (3H2O nmol/hr per islet)</td>
<td>0.050 ± 0.008</td>
<td>0.114 ± 0.025†</td>
<td>0.060 ± 0.008</td>
</tr>
<tr>
<td>Esterification ([3H]palmitate nmol/hr per islet)</td>
<td>0.500 ± 0.034</td>
<td>0.383 ± 0.038†</td>
<td>0.568 ± 0.036</td>
</tr>
<tr>
<td>Oxidation/esterification, %</td>
<td>10.4 ± 2.4</td>
<td>31.5 ± 9.0†</td>
<td>10.4 ± 0.9</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM of three experiments.

*P < 0.05 vs. AdCMV–β-gal.

†P < 0.05 vs. paired. Unlabeled palmitate are 0.1 mM in the media.
food intake and also by a mechanism independent of food intake.

Representative Southern blots of RT-PCR products appear in Fig. 2.

In Vitro Effects of Leptin on Enzymes of FFA Oxidation and Esterification in Pancreatic Islets. To determine if the foregoing changes that occurred in vivo were, at least in part, independent of the hypothalamic pathway of leptin action, we cultured islets with or without recombinant leptin in the medium. The 20 ng/ml leptin concentration employed was similar to the plasma level in hyperleptinemic rats, which averaged 20 ng/ml. Qualitatively identical results were observed in normal islets cultured for 2 days in the presence or absence of 20 ng/ml of recombinant leptin (Fig. 1B). The mRNA ratio for ACO and CPT-I were both significantly increased \( (P < 0.001) \), whereas those for ACC and GPAT were significantly reduced \( (P < 0.001) \).

mRNA Levels of UCP-2 in Hyperleptinemia. There was no evidence that energy generated in the hyperleptinemic rats by the increased oxidation of fat was being channeled into energy-requiring processes (6, 8). This suggested that the leptin-stimulated increase in FFA oxidation was uncoupled. Since we found no evidence for ectopic expression of the UCP-1 of brown adipose tissue in islets or other tissues (data not shown), we searched for increased levels of uncoupling protein-2 (UCP-2), the recently discovered and widely expressed protein with a 59% amino acid homology to UCP-1 (13). It has been presumed that UCP-2, like UCP-1, forms a pathway for proton flux from cytosol to mitochondrial matrix. UCP-2 mRNA in islets of hyperleptinemic rats was over 10 times greater than in islets of paired controls and over 6 times...
4.6-fold increase in the mRNA ratio of UCP-2 to mRNA.

fourth day while it was still identifiable, and measured UCP-2 expression to be significantly increased; ACO mRNA was more than 10 times as high in the islets of hyperleptinemic rats as in free-feeding AdCMV–β-gal-infused controls. However, it was also reduced in islets of paired rats to 23% of controls. This indicates that dietary restriction reduces ACC expression. Nevertheless, the in vitro studies demonstrate a direct down-regulation of ACC by leptin that may have been concealed in vivo by dietary down-regulation of ACC mRNA to undetectable levels.

Table 3. Comparison of UCP-2 mRNA expression in pancreatic islets isolated from saline, AdCMV–β-gal, and AdCMV–leptin infused rats and pairfed controls

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Saline</th>
<th>AdCMV–β-gal</th>
<th>AdCMV–leptin</th>
<th>Pairfed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean +/+ ZDF</td>
<td>0.3 ± 0.7 (3)</td>
<td>0.3 ± 0.04 (3)</td>
<td>1.9 ± 0.2 (5)*†</td>
<td>0.2 ± 0.02 (3)</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SEM. Number of experiments are in parentheses.

Effect of Leptin on UCP-2 mRNA of White Adipocytes in Vivo. Because of the interest in the diabetogenic effects of obesity (20–22), we have focused on the pancreatic islets as a target tissue for direct leptin action. But since the most dramatic component of the hyperleptinemic phenotype is the disappearance of all identifiable fat tissue within 7 days of the infusion of the AdCMV–leptin (6), we also collected epididymal, retroperitoneal, and subcutaneous white fat tissue on the fourth day while it was still identifiable, and measured UCP-2 mRNA.

We therefore searched for leptin-induced changes in the expression of genes encoding the enzymes involved in oxidation and esterification of FFA and of a gene suspected to be involved in thermogenesis. mRNA for enzymes of FFA oxidation was significantly increased; ACO mRNA was more than 4 times as high in islets of hyperleptinemic rats as in free-feeding AdCMV–β-gal-infused controls and 2.6 times the level in pairfed controls, and CPT-I mRNA was almost 3 times as high. Induction by leptin of these two key enzymes of FFA oxidation was clearly independent of the reduction in caloric intake.

DISCUSSION

Recent studies (6, 8) suggest that nonadipocytes normally contain a small quantity of TG. This TG supply may be a vestige of a period in phylogeny before the evolution of adipocytes when each cell carried its own TG reservoir. While the evolution of adipocytes may have rendered superfluous the internal source of FFA for various intracellular signaling functions. For example, normal islets contain approximately 100 ng of TG per islet (20); in rats with hyperleptinemia islet TG is zero (7, 8).

The rapid disappearance of body fat without a rise in plasma FFA, β-hydroxybutyrate or urine ketones in hyperleptinemia (8), is in contrast to the rise in FFA and ketones that accompanies fat loss caused by starvation or insulin deficiency (10). This difference pointed to leptin-regulated internal control of the intracellular fat pool in adipocytes and nonadipocytes alike. If this is correct, tissues of hyperleptinemic rats should exhibit an increased rate of FFA oxidation and a low rate of esterification, as had been reported when normal islets were cultured in the presence of leptin (8). Such changes were indeed found in islets from hyperleptinemic rats. Since they did not occur in islets of pairfed rats, the changes in FFA metabolism were independent of the reduction in caloric intake.

Table 4. Comparison of UCP-2 mRNA expression in fat tissues isolated from saline, AdCMV–β-gal, and AdCMV–leptin infused rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Saline</th>
<th>AdCMV–β-gal</th>
<th>AdCMV–leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean +/+ ZDF</td>
<td>0.6 ± 0.15 (3)</td>
<td>0.5 ± 0.06 (3)</td>
<td>2.6 ± 0.3 (3)*</td>
</tr>
<tr>
<td>Epididymal</td>
<td>0.5 ± 0.06 (3)</td>
<td>0.5 ± 0.1 (3)</td>
<td>2.5 ± 0.4 (3)*</td>
</tr>
<tr>
<td>Retroperitoneal</td>
<td>0.5 ± 0.11 (3)</td>
<td>0.5 ± 0.06 (3)</td>
<td>2.5 ± 0.2 (3)*</td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>0.498 (2)</td>
<td>0.377 (1)</td>
<td>0.407 ± 0.065 (3)</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SEM. Number of experiments are in parentheses.
orexical action of leptin. Qualitatively identical induction of UCP-2 by leptin was observed in vitro in cultured islets from normal rats. This suggests that, at least in this unphysiologic model of leptin overexpression, the effects of leptin observed in vivo may in large part represent direct actions not mediated via the hypothalamus. Finally, UCP-2 mRNA was increased in the subcutaneous retroperitoneal and epididymal fat tissue of hyperleptinemic lean rats, but not of leptin-resistant obese ZDF (fa/fa) rats. These findings observed by RT-PCR were confirmed by RNA blot hybridization (data not shown).

Still to be elucidated are the mechanisms by which leptin induces these changes. Because FFA may serve as a ligand for peroxisome proliferator-activated receptors, one must entertain the possibility that leptin first activates an unidentified intracellular lipase that increases intracellular FFA and exerts its effects on gene expression via these nuclear receptors. This idea is supported by the fact that troglitazone, reported to increase UCP-2 mRNA in islets (M.S., Y.-T.Z., and R.H.U., unpublished data).

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