Transgenic mice overexpressing leptin backcrossed to the C57BL/6J genetic background (LepTg) have a lean phenotype, characterized by a 95% reduction in adipose mass; reduced plasma levels of glucose, triglycerides, insulin, and IGF-1; and a 75% decrease in adipocyte size. High-fat diet treatment for 20 wk revealed that the LepTg mice had an increased susceptibility to diet-induced obesity, as demonstrated by their rate of weight gain, higher accumulation of sc white adipose tissue mass, hypertrophy of adipocytes, and normalization of their reduced metabolic parameters. The stromal vascular fraction of white adipose tissue from the LepTg mice was highly cellular and contained cells capable of rapid lipid accumulation in primary cultures. The precipitous diet-induced obesity of the LepTg mice was accompanied with 10-fold and 1.6-fold elevations in insulin and IGF-1, respectively, suggesting that the trophic action of insulin and IGF-1 on the preadipocytes and small adipocytes may have caused them to rapidly differentiate and accumulate triacylglycerol stores. Other contributing factors may involve a shift in insulin sensitivity triggered by hyperleptinemia and a decrease in energy expenditure. These studies demonstrate that a chronic response to hyperleptinemia as in the LepTg mice is a predisposing factor to diet-induced obesity and suggest that individuals who are particularly lean because of increased leptin secretion may develop rapid obesity under conditions of a high-fat diet. (Endocrinology 144: 2865–2869, 2003)

**Materials and Methods**

**Derivation of a congenic line of LepTg mice**

Mice housing, procedures, and handling were in agreement with institutional guidelines and were approved by the University of California-San Francisco Animal Care Committee. We backcrossed the original transgenic mice overexpressing leptin from their mixed (C57BL/6j and DBA/2j) genetic background onto the C57BL/6j background for at least seven generations to generate an N7 congenic line. The transgenic mice overexpressing leptin were identified either by a human leptin RIA (Linco Research, Inc., St. Louis, MO) or by PCR using 10-bp primers that amplify the human leptin transgene only (5’-ATGCAATTGGTGTCAATGAC-3’). The DNA content of epididymal WAT from normal and LepTg mice was determined using a DNA content of white adipose tissue (WAT) assay kit (Sigma, St. Louis, MO) and a set of primers that amplify the human leptin transgene only (5’-GTTGACAAATTGCTTAGATGAC-3’ and 5’-ATGCAATTGGGAAACCTGTG-3’).

**Plasma chemistries, IGF-1, insulin assays, and DNA content of white adipose tissue (WAT)**

Blood was drawn into heparinized tubes from the retro orbital sinus under Avertin anesthesia. The plasma was separated from packed cells and either immediately processed or frozen at −20°C until use. Plasma glucose, triglycerides, and cholesterol levels were determined on a Beckman (Richmond, CA) automated LX-1 clinical chemistry analyzer under Avertin anesthesia. The plasma was separated from packed cells and either immediately processed or frozen at −20°C until use. Plasma glucose, triglycerides, and cholesterol levels were determined on a Beckman (Richmond, CA) automated LX-1 clinical chemistry analyzer and either immediately processed or frozen at −20°C until use. Plasma glucose, triglycerides, and cholesterol levels were determined on a Beckman (Richmond, CA) automated LX-1 clinical chemistry analyzer. IGF-1 and insulin determinations were performed in duplicate with a maximum coefficient of variation of duplicate samples not exceeding 6% for the CFD and 5% for the HFD. Insulin levels were determined with a rat RIA, which cross-reacted 100% with mouse insulin (Linco Research, Inc., St. Charles, MO). The DNA content of epididymal WAT from normal and LepTg mice on the CFD and sc WAT on the HFD was determined using a DNA
extraction kit from QIAGEN (Chatsworth, CA) and normalization of the data to the weights of adipose tissue.

**Histology and determination of adipocytes size**

The WAT was fixed in 10% phosphate-buffered formalin, then processed for paraffin embedding and sectioning according to standard procedures. NIH image software (version 1.62) was used to determine the areas of 200 adipocytes from sections of 4 normal and 4 LepTg mice on the CFD or HFD. The resulting data were statistically evaluated with ANOVA followed by the Tukey’s honest significant difference post hoc test.

**Primary cultures of preadipocytes and adipocytes from normal and LepTg mice**

We used the method of adipose precursor cells isolation (11) to recover the stromal vascular fraction of WAT, which contained in the LepTg mice preadipocytes and small adipocytes devoid of lipid droplets. Briefly, epididymal WAT was harvested from freshly killed normal and transgenic male mice. The tissue was washed as much as possible from blood, weighed and cut into 4, 8, 16, 32, 64, 128, and 350 mg for normal mice. Each tissue slice was digested in serum-free DMEM containing 2 mg/ml collagenase II (Sigma) in a 25-ml flask with occasional gentle shaking for approximately 1 h at 37 °C. The resulting cell suspension was then filtered through a 250-μm nylon mesh and the filtrate centrifuged at low speed. The stromal vascular cells in the pellet were resuspended and grown in DMEM containing 10% fetal bovine serum. Cells were plated in 12 multiwell culture plates and maintained at 37 °C in a humidified atmosphere of 5% CO2 in air. The cells were grown in DMEM containing 1 g/liter glucose and 110 mg/liter sodium pyruvate, Ham’s F12 medium, 5 μg/ml bovine insulin, and 10 μg/ml human transferrin. After 2 wk in culture, the cells were fixed with 4% paraformaldehyde in PBS, stained first with 0.6% Oil Red O (ORO), and then with 0.75% Harris hematoxylin. ORO staining was quantitated by a spectrophotometric method (12) using 1 ml of 4% IGEPAL (CA-630) in isopropanol to elute the color off the wells (13). The absorbance of the dye at 520 nm was normalized to the amount of tissue used for the differentiation experiment and expressed as absorbance units per 10 mg of WAT.

**Statistics**

Significant differences between groups were evaluated either by the unpaired Student’s t test or ANOVA followed by the Tukey’s honest significant difference post hoc test with the Statistica version 4.1 computer package (Statsoft, Tulsa, OK) for the Apple Macintosh. All data are expressed in means ± sem. For the comparison of the slopes of weight gain on the HFD, the individual slope of the body weight curve of each mouse on the HFD was obtained from the equation of the linear curve fit. All individual slopes were then entered into an unpaired t test and statistical significance evaluated with Statistica.

**Results**

**Characterization of the C57BL/6J LepTg mice**

The transgenic mice overexpressing leptin (LepTg) were initially bred on a heterogeneously mixed C57BL/6J and DBA/2J genetic background (7). In this study, we backcrossed the leptin transgene for at least seven generations onto the C57BL/6J background to generate a C57BL/6J line of LepTg mice. Monitoring of body weight (Fig. 1A) showed that the LepTg mice were leaner than their wild-type littermates at least until 40 wk of age. Although the LepTg mice ingested 15% less food than their age- and sex-matched normal littermates, the energy intake normalized to body weight to the power 0.75 (10) was similar in both groups (Table 1).

**Treatment of LepTg mice with a HFD**

We found that, despite the initial low body weights of the CFD-fed LepTg mice (17.4 ± 0.4 g compared with 22.6 ± 0.5 g for normal controls; P < 0.01), the HFD-fed LepTg mice gained body weight rapidly surpassing normal mice on the HFD. Computation of the slope of the increase in body weight of each mouse revealed that the body weight gain of the LepTg mice was 1.6-fold faster than that of normal mice.
TABLE 1. Energy intake, weights of brown adipose tissue (BAT) and WAT, DNA content of WAT, and areas of adipocytes in normal and LepTg male mice fed either the CFD or HFD

<table>
<thead>
<tr>
<th></th>
<th>Normal CFD</th>
<th>LepTg CFD</th>
<th>P CFD</th>
<th>Normal HFD</th>
<th>LepTg HFD</th>
<th>P HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy intake (kJ/kg&lt;sup&gt;0.75&lt;/sup&gt;)</td>
<td>58.4 ± 1.8</td>
<td>60.9 ± 1.0</td>
<td>n.s.</td>
<td>44.1 ± 3.0</td>
<td>39.7 ± 1.0</td>
<td>n.s.</td>
</tr>
<tr>
<td>BAT (mg)</td>
<td>161 ± 23</td>
<td>38 ± 5</td>
<td>&lt;0.01</td>
<td>177 ± 17</td>
<td>193 ± 13</td>
<td>n.s.</td>
</tr>
<tr>
<td>WAT (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gonadal</td>
<td>0.20 ± 0.03</td>
<td>0.01 ± 0.002</td>
<td>&lt;0.01</td>
<td>2.2 ± 0.2</td>
<td>1.8 ± 0.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>0.20 ± 0.04</td>
<td>n/d</td>
<td></td>
<td>2.1 ± 0.3</td>
<td>3.2 ± 0.2</td>
<td>0.01</td>
</tr>
<tr>
<td>Perirenal</td>
<td>0.06 ± 0.02</td>
<td>n/d</td>
<td></td>
<td>1.0 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>DNA (μg/mg WAT)</td>
<td>0.5 ± 0.1</td>
<td>2.2 ± 0.5</td>
<td>0.02</td>
<td>0.3 ± 0.06</td>
<td>0.2 ± 0.03</td>
<td>n.s.</td>
</tr>
<tr>
<td>Adipocytes area (px)</td>
<td>48.3 ± 2.6</td>
<td>11.7 ± 0.7</td>
<td>&lt;0.01</td>
<td>132.7 ± 5.8</td>
<td>161.4 ± 5.6</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

There are 4–7 mice in each group except for 10 mice for BAT and WAT weights in the HFD groups. DNA content of WAT was determined from epidydimal fat depots on the CFD and sc fat depots on the HFD. Adipocytes areas are expressed in pixels (px). P values were computed between groups on the CFD and HFD. n.s., Not significant; n/d, not detectable.

(P < 0.01; Fig. 1B). At the end of the HFD treatment, the LepTg mouse had an increase in body weight consisting of 128 ± 9% compared with 70 ± 6.5% in normal mice. Energy intake remained not statistically different between the two groups.

Fat mass, leptin levels, histology, and DNA content of WAT

At 9 wk of age, the WAT of the LepTg mice was reduced by approximately 95% on the CFD and limited to a minute epididymal mass. No other fat depot could be detected in the LepTg mice at this age. Although in the LepTg mice all fat depots had expanded as a result of the HFD and were similar to those of normal mice, only their sc WAT mass was 52% larger than that of normal mice (Table 1). On the CFD, the DNA content of the epidydimal WAT of the LepTg mice was 4-fold higher than that of normal mice, whereas on the HFD, the sc WAT DNA was similar in both groups (Table 1).

Endogenous and transgenic leptin levels were markedly elevated in CFD and HFD LepTg mice (Fig. 1C). On the CFD, the endogenous leptin levels were almost identical between normal and transgenic mice (3.1 ± 0.4 ng/ml vs. 3.8 ± 0.4 ng/ml; n = 10 in each group), whereas in the LepTg mice, human leptin circulated at 30.9 ng/ml representing an 8-fold elevation over their own endogenous leptin levels. The combined leptin levels of the LepTg mice, however, were 11-fold over those of normal mice. At the end of the HFD treatment, endogenous leptin had increased by approximately 12-fold over CFD levels in the normal and LepTg mice attaining 36.9 ± 4.2 ng/ml and 41 ± 2.8 ng/ml, respectively. However, human leptin levels were increased by 2.3-fold over CFD levels reaching 72.6 ± 4.2 ng/ml on the HFD. Overall, the combined leptin levels of the LepTg mice on the HFD remained 3.2-fold higher than those of normal mice on the HFD.

Histological examination of WAT (Fig. 2) revealed that the CFD-fed LepTg mice had adipocytes, which were 76% smaller than those of normal mice and that contained either no visible or minute lipid droplets. However, on the HFD, these adipocytes had hypertrophied 22% more than those of normal HFD-fed mice (Table 1).

Primary cultures of adipocytes

To determine whether the preadipocytes and small adipocytes of the LepTg mice were capable of differentiation and growth into lipid filled adipocytes, we recovered the stromal vascular cell fraction of WAT from normal and LepTg mice and cultured it in vitro to induce differentiation and growth. We found that, compared with normal mice, the small adipose mass of the LepTg mice had an increased cellularity, which resulted in substantial accumulation of triacylglycerols as evidenced by ORO staining (Fig. 3A). Quantitation of the ORO dye, a measure of triglyceride content of differentiated adipocytes, revealed that the LepTg adipocytes had accumulated 4.6-fold more lipid than normal adipocytes (P < 0.01; Fig. 3B).

Metabolic parameters, insulin, and IGF-1 levels

On the CFD, plasma levels of glucose and triglycerides were decreased by 33% (P < 0.01) and 76% (P < 0.01), respectively, in ad libitum-fed LepTg mice compared with controls. Cholesterol and β-hydroxybutyrate were unchanged. Both plasma glucose and triglycerides levels increased in the LepTg mice from the CFD to the HFD but were not significantly different anymore compared with normal mice on the HFD (Table 2).

We also determined plasma IGF-1 levels in genetically heterogeneous (C57BL/6J-DBA/2J; Ref. 7) and C57BL/6J congenic LepTg mice (this study) on the CFD and found them to be significantly depressed compared with normal
Fig. 3. In vitro growth and differentiation of preadipocytes from normal and LepTg mice. A, Photomicrograph (×100) of cells recovered from the stromal vascular fraction of 4 mg of WAT from normal and LepTg mice. The cells were grown and differentiated in vitro before staining with ORO for lipid content. Note the increased cellularity and the prominent staining of lipids from LepTg mice compared with normal mice. B, Quantitation of Oil Red O staining in primary adipocytes cultures from normal (N) and LepTg mice. **, P < 0.01.

**TABLE 2.** Metabolic parameters of normal and LepTg mice on the CFD and HFD diets

<table>
<thead>
<tr>
<th>Glucose (mmol/liter)</th>
<th>Normal CFD</th>
<th>LepTg CFD</th>
<th>P CFD</th>
<th>Normal HFD</th>
<th>LepTg HFD</th>
<th>P HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.7 ± 0.5</td>
<td>7.8 ± 0.6</td>
<td>&lt;0.01</td>
<td>15.2 ± 1.4</td>
<td>15.7 ± 0.8</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Triglycerides (mmol/liter)</th>
<th>Normal CFD</th>
<th>LepTg CFD</th>
<th>P CFD</th>
<th>Normal HFD</th>
<th>LepTg HFD</th>
<th>P HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.51 ± 0.06</td>
<td>0.12 ± 0.01</td>
<td>&lt;0.01</td>
<td>0.42 ± 0.05</td>
<td>0.4 ± 0.05</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cholesterol (mmol/liter)</th>
<th>Normal CFD</th>
<th>LepTg CFD</th>
<th>P CFD</th>
<th>Normal HFD</th>
<th>LepTg HFD</th>
<th>P HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>n.s.</td>
<td>3.7 ± 0.3</td>
<td>3.9 ± 0.2</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>β-Hydroxybutyrate (μmol/liter)</th>
<th>Normal CFD</th>
<th>LepTg CFD</th>
<th>P CFD</th>
<th>Normal HFD</th>
<th>LepTg HFD</th>
<th>P HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.9 ± 0.7</td>
<td>2.2 ± 0.6</td>
<td>n.s.</td>
<td>2.2 ± 0.3</td>
<td>1.5 ± 0.4</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Insulin (pmol/liter)</th>
<th>Normal CFD</th>
<th>LepTg CFD</th>
<th>P CFD</th>
<th>Normal HFD</th>
<th>LepTg HFD</th>
<th>P HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 ± 30</td>
<td>45 ± 7.5</td>
<td>&lt;0.01</td>
<td>330 ± 60</td>
<td>480 ± 210</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IGF-1 (nmol/liter)</th>
<th>Normal CFD</th>
<th>LepTg CFD</th>
<th>P CFD</th>
<th>Normal HFD</th>
<th>LepTg HFD</th>
<th>P HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>55.3 ± 3.2</td>
<td>43.3 ± 2.7</td>
<td>0.01</td>
<td>72.3 ± 3.5</td>
<td>69 ± 3.0</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>

Control littersmates in both LepTg lines. For example, at 11–15 wk of age, the IGF-1 levels in genetically heterogeneous normal (n = 14) and LepTg (n = 11) males were 26% lower than normal mice (57.2 ± 3.0 mmol/liter vs. 42.5 ± 2.0 mmol/liter; P < 0.01). Similarly, at 11–16 wk of age, IGF-1 levels were 22% lower in the LepTg mice on the CFD than normal mice on the C57BL/6j genetic background (Table 2). Elevations in IGF-1 levels from the CFD to the HFD consisted of 31% for normal mice (P < 0.01) and 59% for the LepTg mice (P < 0.01). Insulin levels were also significantly modulated from the CFD to the HFD, accounting for a 2.2-fold increase in normal mice (P < 0.01) and a 10.7-fold increase in LepTg mice (P < 0.01; Table 2). Both insulin and IGF-1 were not significantly different between normal and LepTg mice on the HFD alone.

**Discussion**

Experimentally induced hyperleptinemia in rodents via exogenous leptin treatment results in a lean phenotype caused by a decrease in food intake and an increase in energy expenditure (14–17). However, the hyperleptinemia characteristic of most forms of obesity, whether in animal models or individuals (9), fails to result in similar effects and is thus attributed to leptin resistance. In a previous study (7), we had shown that LepTg mice on a mixed C57BL/6j-DBA/2j genetic background showed an age-related increase in body weight and adiposity caused by a late onset leptin resistance. On the C57BL/6j genetic background, this effect is more subtle suggesting a causative role of modifier genes from the DBA/2j genome on the onset of leptin resistance. Ongoing backcrosses of the leptin transgene onto the DBA/2j genetic background will reveal the contribution of this genome to the phenotype of leptin resistance.

It has been shown that feeding normal mice with a HFD induced obesity and elevated leptin levels, thus establishing a leptin resistant state (18). It was surprising and counter-intuitive to find out that, whereas the LepTg mice were lean on the CFD, their continuous response to hyperleptinemia on the CFD did not prevent nor delay their DIO. On the contrary, and despite continuous secretion of transgenic leptin, DIO was accelerated in the LepTg mice, suggesting that their lean hyperleptinemic state could have been a precipitating factor to their DIO. Although adipocyte hypertrophy accounted for their adipose mass expansion, a disruption in leptin transport across the blood brain barrier may underlie their leptin resistance. For example, C57BL/6j mice fed a HFD were insensitive to peripherally administered leptin, but showed only decreased sensitivity to its central infusions (19–21). Thus, it is likely that a similar mechanism may interfere with the central action of leptin in HFD-fed LepTg mice. However, the effectors leading to a rapid accumulation of WAT mass must lie in the periphery, likely at the level of preadipocytes differentiation and accumulation of triacylglycerols in their small adipocytes. Although the low levels of insulin and IGF-1 in the LepTg mice on the CFD may be secondary to their phenotype, they could fail to promote a favorable environment for preadipocyte differentiation and triacylglycerol accumulation. The subsequent increase in insulin and IGF-1 from the HFD would then be consistent with increased adipocyte differentiation and triacylglycerols accumulation. It is well known that insulin and more specifically IGF-1 play a critical role in preadipocyte differentiation and are necessary for the in vitro differentiation of 3T3-L1 preadipocytes to adipocytes (22, 23).

Another contributing factor to the diet-induced obesity of the LepTg mice may be a decrease in energy expenditure. Although short-term leptin infusions in normal mice have resulted in an increase in energy expenditure (14, 24) and it would be logical to assume the same for the LepTg mice, the effect of a chronic response to hyperleptinemia on energy expenditure has not yet been determined.
assessment of energy expenditure of the LepTg mice on the HFD will reveal the extent to which energy expenditure plays a role in the diet-induced obesity phenotype of the LepTg mice.

Collectively, our findings suggest that the small adipose mass of the LepTg mice may be peripherally caused by both impaired differentiation of preadipocytes and intracellular accumulation of triacylglycerols. Alternatively, it is interesting to note that, even though the energy intake of the LepTg mice was comparable to that of normal mice, the amplified effects of the HFD and advancing age on their phenotype may have resulted from a shift in insulin sensitivity triggered by hyperleptinemia.

The finding of a significant attenuation in circulatory IGF-1 levels in the LepTg mice raises the possibility that growth hormone (GH), which is the main stimulator of IGF-1 release from the liver, may also be decreased. Additional studies will be needed to define the role of GH with respect to IGF-1 in animals chronically responsive to hyperleptinemia.

The extension and relevance of these findings to human obesity hint that, if a subset of individuals are lean because of increased leptin secretion, they may develop an abundance of preadipocytes and small adipocytes, which when exposed to rich-fat diets would undergo rapid differentiation and expansion resulting in severe obesity. Other possibilities include a shift in insulin sensitivity and/or decreased energy expenditure. In either case, the unraveling of lean individuals with elevated leptin levels might identify them as a new class at increased risk for diet-induced obesity.

Acknowledgments

Received December 20, 2002. Accepted March 27, 2003.

Address all correspondence and requests for reprints to: F. Chehab, 505 Parnassus Avenue, University of California, Department of Laboratory Medicine, San Francisco, California 94143-0134. E-mail: chehabf@labmed2.ucsf.edu.

References