Reduced adiposity and improved insulin sensitivity in obese mice with antisense suppression of 4E-BP2 expression

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The prevalence of obesity has been steadily increasing over the last decade and has become a world-wide health problem. Obesity is the primary risk factor associated with development of a cluster of metabolic disorders, including type 2 diabetes, fatty liver, dyslipidemia, and cardiovascular disease. However, effective drugs for the treatment of obesity and associated disorders are largely elusive. Therefore, an urgent need continues to exist for identifying new therapeutic targets and drug platforms for these disorders.

Recent studies indicate that eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4E-BPs) play an important role in the regulation of fat metabolism. 4E-BPs inhibit cap-dependent translation initiation through sequestering eIF4E from the eIF4F complex (17, 25). Three 4E-BPs, 4E-BP1, -2, and -3, have been found in mammals. 4E-BP1 is highly expressed in fat and muscle, whereas 4E-BP2 is ubiquitously expressed with high levels in fat, liver, and central nervous system (CNS) and very low levels in muscle (14, 22, 29). The physiological role of 4E-BP1 has been studied in different laboratories. Although no gross growth or development defects were found in mice lacking 4E-BP1, reduced body weight and body fat content that were associated with increased UCPI expression and increased metabolic rate were observed (2, 5, 29). Lehr et al. (19) found that β3-adrenoreceptor-mediated increases in UCP1 expression (indicating increased thermogenesis) in brown adipose tissues (BAT) occurs in part through suppression of 4E-BP1 expression. Teleman et al. (28) found that increasing 4E-BP activity resulted in whole body increases in fat accumulation, whereas reducing its activity lead to an increased rate of fat burn in Drosophila. However, although the level of 4E-BP1-eIF4E complex was found to vary as a function of nutritional status (30, 31), neither 4E-BP1-null mice nor 4E-BP-null Drosophila demonstrated abnormal tissue or whole body growth and development, suggesting that 4E-BP1 deficiency may only affect the translation of a subset of mRNAs (e.g., fat metabolism-related mRNAs).

Limited data are available on the function of 4E-BP2 and -3 in vivo. Findings by Groll et al. (12) indicate that 4E-BP1 and -2 are differentially regulated. Gene knockout studies suggest that 4E-BP2 is critical for eIF4F complex formation, synaptic plasticity, and memory in CNS and for normal behavior (4). However, the role of 4E-BP2 in modulating metabolic processes was not reported in those studies. Interestingly, the same laboratory recently reported that mice lacking both 4E-BP1 and 4E-BP2 had increased sensitivity to diet-induced obesity and insulin resistance; the former was due to increased adipogenesis coupled with alteration in fat metabolism and energy expenditure; the latter was due to increased ribosomal S6 kinase (S6K) activity in the major metabolic tissues (18).

To explore the possible role of 4E-BP2 in energy metabolism, we specifically reduced its expression in liver and fat in vivo. Findings by Groll et al. (12) indicate that 4E-BP1 and -2 are differentially regulated. Gene knockout studies suggest that 4E-BP2 is critical for eIF4F complex formation, synaptic plasticity, and memory in CNS and for normal behavior (4). However, the role of 4E-BP2 in modulating metabolic processes was not reported in those studies. Interestingly, the same laboratory recently reported that mice lacking both 4E-BP1 and 4E-BP2 had increased sensitivity to diet-induced obesity and insulin resistance; the former was due to increased adipogenesis coupled with alteration in fat metabolism and energy expenditure; the latter was due to increased ribosomal S6 kinase (S6K) activity in the major metabolic tissues (18).

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nied by an increase in whole body metabolic rate, increased insulin signaling activity, and changes in the expression of a variety of genes involved in energy metabolism in both liver and fat. These results demonstrate for the first time that 4E-BP2 plays an important role in energy homeostasis. Therefore, peripheral 4E-BP2 could be a potential therapeutic target for the treatment of obesity and related metabolic disorders.

MATERIALS AND METHODS

Selection of 4E-BP2 ASOs. Rapid throughput screens of ~80 ASOs against 4E-BP2 were performed in bEND and EMT-6 cells, and the reduction of target gene expression was analyzed with real-time quantitative RT-PCR after transfection of the cells with 150 nM of the ASOs for 24 h. Four ASOs that showed the greatest potency in both cell lines were further screened in mouse primary hepatocytes by transfecting the cells with the ASOs at different concentrations. Based on IC_{50} values, three potent ASOs were selected, and their in vivo activity was further confirmed in lean C57BL/6J mice. Two ASOs were finally selected for this study on the basis of the reduction of tissue 4E-BP2 mRNA levels in lean mice after short-term dosing: ISIS 232828 (4E-BP2 ASO #2) was used in in vitro experiments for confirmation of the effects caused by ISIS 232828 (see below). All the ASOs have a uniform phosphorothioate backbone and a 20-base chimeric design containing 2′-O-(methoxy)-ethyl (2′-MOE) modification on the first five and last five bases. This modification enhances their binding affinity to complementary sequences and their resistance to the action of nucleases. A negative control ASO (ISIS 141923), which has the same chemical composition as the 4E-BP2 ASOs but no complementarity to any known gene sequence, was also included in the study.

Animal care and treatments. All experiments were performed in accordance with Institutional Animal Care and Use Committee guidelines. Male, 4-wk-old C57BL/6J mice were purchased from the Jackson Laboratory and maintained on a 12:12-h light-dark cycle with free access to food and water. The mice were fed a high-fat (HF) diet containing 60 kcal% fat (Research Diet D12492; Research Diets, New Brunswick, NJ) for 15 wk to induce obesity and insulin resistance. The animals were then divided into different groups (n = 7) and treated (sc injection) with 4E-BP2 ASO or control ASO (dissolved in saline) at a dose of 25 mg/kg body wt, or with a similar volume of saline, twice a week for 6 wk. The HF diet was fed to all the groups throughout the study. A group of lean C57BL/6J mice fed normal rodent chow and injected with saline served as normal controls. During the study, body weight and weekly accumulated food intake were monitored. At the end of the study, the mice were euthanized, and different tissues were collected and quickly frozen in liquid N_{2} for further analysis.

For the insulin challenge studies, DIO mice were treated with 4E-BP2 ASO or control ASO at a dose of 37.5 mg/kg body wt, or with a similar volume of saline, twice a week for 2 wk. The mice were then fasted overnight and given a bolus intraperitoneal injection of insulin at 2 U/kg body wt or vehicle. Ten minutes later the animals were euthanized, and liver and epididymal fat were collected and quickly frozen in liquid N_{2} for further analysis.

Body composition analysis. Body composition of the mice was measured with an Echo MRI whole body composition analyzer (Echo Medical System, Houston, TX) at the beginning and at the end of the study.

Metabolic rate measurement. Metabolic rate after 5 wk of treatment was measured using indirect calorimetry (Oxymax System; Columbus Instruments, Columbus, OH). Animals were acclimated to metabolic chambers for 24 h before initiation of the measurement. For each treatment group, six animals were measured for a 24-h period.

Glucose tolerance tests. Glucose tolerance tests (GTT) were conducted after 5.5 wk of treatment. Prior to GTT, the mice were fasted...
overnight and were subsequently injected intraperitoneally with glucose at a dose of 1.0 g/kg body wt. Blood glucose levels were measured before glucose injection (0 min, baseline values) and at 30, 60, 90, and 120 min after injection using a Glucometer (Abbott Laboratories, Bedford, MA).

Biochemical analysis. Plasma insulin levels were measured with a commercial Elisa kit (ALPCO Diagnostics, Manufactured by Mercodia Sweden) according to the manufacturer’s instructions. Plasma glucose, triglycerides (TG), cholesterol, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured with an Olympus Analyzer AU400 (Melville, NY). Liver TG content was measured as described previously (9).

Isolation and transfection of primary mouse hepatocytes. Isolation and transfection of mouse primary hepatocytes were conducted as described previously (36). Briefly, the cells with >85% viability were seeded onto 60-mm culture plates or 25-cm² flasks at 10⁶ cells per plate or flask in culture medium (Williams’ medium E with 10% fetal bovine serum, and 10 nM insulin plus penicillin and streptomycin) and cultured overnight. For transfection, the hepatocytes were incubated for 4 h with 1.5 ml of transfection mixture, which contained 150 nM control ASO or 4E-BP2 ASO and 4.5 μg lipofectin (Invitrogen, CA) in Williams’ medium E. The mixture was then aspirated and replaced with the culture medium to allow the cells to recover.

Determination of TG synthesis and fatty acid oxidation in transfected mouse hepatocytes in vitro. Twenty-four hours after transfection, TG synthesis in mouse hepatocytes was determined by measuring the incorporation of [3H]glycerol into TG (7, 36). Fatty acid oxidation was determined by measuring the oxidation of [14C]oleate into acid-soluble products and CO₂, as previously described (35, 36).

Insulin challenge in transfected mouse hepatocytes in vitro. For insulin signaling activity analysis, the transfected hepatocytes were incubated overnight in William’s E medium containing only 0.1% BSA and then were challenged with 2 nM insulin for 2 min.

Gene expression analysis. For gene expression analysis, total RNA from hepatocytes was isolated using a Qiagen RNA easy kit, and total RNA from animal tissues was isolated by homogenizing tissues in RLT buffer (Qiagen) followed by centrifugation with cesium chloride gradient. Real-time quantitative RT-PCR was performed with custom-made RT-PCR enzymes and reagent kit (Invitrogen Life Technology, Carlsbad, CA), primer and probe sets (Table 1) designed with Primer Express software (PE Applied Bioscience, Foster city, CA), and an ABI Prism 7700 Sequence Detector (PE Applied Biosciences). For the analysis, 50 or 100 ng of total RNA was used for each reaction. Each sample was run in duplicate, and the mean values were used to calculate the mRNA levels and gene expression. The expression was

Fig. 1. Eukaryotic initiation factor 4E-binding protein-2 (4E-BP2) antisense specifically reduced 4E-BP2 expression in liver and fat. Diet-induced obese (DIO) mice were treated with 4E-BP2 antisense oligonucleotide (ASO) or control ASO at 25 mg/kg body wt or with a similar volume of saline twice a week, for 6 wk. Total RNA was prepared from liver, epididymal white fat (WAT), and interscapular brown fat (BAT) and used for real-time quantitative RT-PCR analysis to evaluate expression of 4E-BP2. Tissue lysates prepared from liver and WAT were used for Western analysis on the protein levels of 4E-BP1, 4E-BP2, and eIF4E. 4E-BP2 ASO dramatically reduced 4E-BP2 mRNA in liver, WAT, and BAT (A), which resulted in similar degree of reduction in its protein levels (B and C). However, the ASO did not cause changes in the protein levels of 4E-BP1 or eIF4E. Data are expressed as means ± SE; n ≥ 5. **P < 0.01 vs. controls.
normalized with the amount of total RNA loaded that was determined with a Ribogreen assay.

Western blot analysis. To investigate whether reduction of 4E-BP2 caused changes in related proteins such as 4E-BP1 and eIF4E, or whether the reduction affected insulin signaling activity or the activities of the proteins involved in related pathways, Western blot analysis was conducted. Tissues were homogenized or cells were lysed in a lysis buffer (150 mM NaCl, 50 mmol/l Tris, pH 7.5, 1% triton X-100, 0.5% NP-40, 0.25% sodium deoxycholate, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mM NaOV, 1 mM NaF) containing Protease Inhibitor Cocktail I (Calbiochem). The lysates were cleared by centrifugation for 15 min at 12,000 g. Equal amounts of total protein for different samples were separated on gradient SDS-PAGE gels (Bio-Rad, Hercules, CA) under reduced conditions and then transferred onto PVDF membranes. The blots were then incubated with primary antibody against eIF4E (BD Biosciences, San Jose, CA), transferrin receptor (Cell Signaling, Danvers, MA), UCP1 (Sigma, St. Louis, MO) or PPARα coactivator-1 (PGC-1; Cayman, Ann Arbor, MI). The signals were then detected using horseradish peroxidase-conjugated secondary antibody and ECL detection reagents (Amersham Biosciences).

For analysis of GLUT4 abundance in plasma membrane, subcellular fractionation of white adipose tissue (WAT) was performed as described (15, 34), and the plasma membrane fraction was used for immunoblotting with GLUT4 antibody (Cell Signaling). To investigate whether reduction of 4E-BP2 affects eIF4F complex formation, the phosphorylation levels of eIF4E were evaluated by measuring phosphorylation levels at Ser209, the major complex formation, the phosphorylation levels of eIF4E were evaluated by measuring phosphorylation levels at Ser209, the major phosphorylation site of the protein (11, 33), by immunoblotting with primary antibody against eIF4E (BD Biosciences, San Jose, CA), transferred onto PVDF membranes. The blots were then incubated with primary antibody against eIF4E (BD Biosciences, San Jose, CA), Akt, Ser\textsuperscript{73}-phosphorylated Akt (p\textsuperscript{AKT}\textsuperscript{S73}), ribosomal protein S6 (S6), Ser\textsuperscript{240/244}-phosphorylated S6 (pS6\textsuperscript{240/244}), Thr\textsuperscript{389}-phosphorylated S6K1 (pS6K1\textsuperscript{T389}) (Cell Signaling, Danvers, MA), UCP1 (Sigma, St. Louis, MO) or PPARα coactivator-1 (PGC-1; Cayman, Ann Arbor, MI). The signals were then detected using horseradish peroxidase-conjugated secondary antibody and ECL detection reagents (Amersham Biosciences).

Table 2. Food intake and anthropometric and plasma biochemical parameters measured in mice before, during, or after different treatments

<table>
<thead>
<tr>
<th>Variables</th>
<th>Measurement Time</th>
<th>Saline (n = 7)</th>
<th>Control ASO (n = 7)</th>
<th>4E-BP2 ASO (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weekly food intake, g</td>
<td>During</td>
<td>18.6 ± 0.56</td>
<td>18.13 ± 0.84</td>
<td>17.57 ± 0.35</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>Before</td>
<td>39.8 ± 1.3</td>
<td>39.4 ± 0.6</td>
<td>40.7 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>39.9 ± 1.4</td>
<td>39.2 ± 1.0</td>
<td>37.8 ± 1.3†</td>
</tr>
<tr>
<td>Total body fat content, g</td>
<td>Before</td>
<td>13.4 ± 0.9</td>
<td>12.4 ± 0.6</td>
<td>13.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>14.5 ± 1.1</td>
<td>13.8 ± 0.8</td>
<td>11.1 ± 1.1†</td>
</tr>
<tr>
<td>Percent body fat</td>
<td>Before</td>
<td>34.0 ± 1.5</td>
<td>33.2 ± 1.2</td>
<td>34.1 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>35.4 ± 1.5</td>
<td>34.9 ± 1.3</td>
<td>29.0 ± 1.99‡</td>
</tr>
<tr>
<td>Epididymal fat pad, g</td>
<td>After</td>
<td>2.25 ± 0.14</td>
<td>2.30 ± 0.15</td>
<td>1.86 ± 0.11*</td>
</tr>
<tr>
<td>Interscapular brown fat pad, g</td>
<td>After</td>
<td>0.28 ± 0.04</td>
<td>0.26 ± 0.02</td>
<td>0.17 ± 0.02‡</td>
</tr>
<tr>
<td>Body lean mass, g</td>
<td>Before</td>
<td>23.1 ± 0.4</td>
<td>22.8 ± 0.5</td>
<td>23.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>22.3 ± 0.5</td>
<td>22.2 ± 0.4</td>
<td>22.7 ± 0.4</td>
</tr>
<tr>
<td>Plasma ALT, U/l</td>
<td>After</td>
<td>40.7 ± 5.7</td>
<td>39.4 ± 4.9</td>
<td>47.7 ± 3.5</td>
</tr>
<tr>
<td>Plasma AST, U/l</td>
<td>After</td>
<td>62.4 ± 7.3</td>
<td>64.1 ± 4.3</td>
<td>76.1 ± 3.6</td>
</tr>
<tr>
<td>Plasma cholesterol, mg/dl</td>
<td>After</td>
<td>183.7 ± 6.7</td>
<td>181.4 ± 7.3</td>
<td>163.8 ± 7.4</td>
</tr>
<tr>
<td>Plasma triglycerides, mg/dl</td>
<td>After</td>
<td>63.1 ± 2.1</td>
<td>58.7 ± 3.6</td>
<td>53.0 ± 3.9</td>
</tr>
<tr>
<td>Plasma FFA, mEq/l</td>
<td>After</td>
<td>0.61 ± 0.03</td>
<td>0.60 ± 0.02</td>
<td>0.62 ± 0.06</td>
</tr>
<tr>
<td>Plasma adiponectin, µg/ml</td>
<td>After</td>
<td>2.83 ± 0.04</td>
<td>2.85 ± 0.01</td>
<td>2.61 ± 0.25</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. FFA, free fatty acids; AST, aspartate aminotransaminase; ALT, alanine aminotransaminase. †P < 0.05 vs. before treatment; *P < 0.05, ‡P < 0.01 vs. controls.
body weight in animals treated with 4E-BP2 ASO was attributable to a decrease in body fat content.

Lower body weight and fat content coupled with unchanged food intake in 4E-BP2 ASO-treated mice is indicative of a higher metabolic rate. This was confirmed through indirect calorimetric measurements, which showed an increase in O2 consumption rate, being ~10% higher (P < 0.05) during the dark phase and ~9% higher (P < 0.05) during the light phase (Fig. 2A) with an unchanged respiratory exchange ratio (Fig. 2B) in the 4E-BP2 ASO-treated group vs. either control group. Gene expression analysis found that 4E-BP2 ASO treatment increased β3-adrenoceptor (β3-AR) mRNA levels by >45% in WAT (P < 0.05, Fig. 2C and ~32% in BAT, P > 0.05, Fig. 2D). In addition, treatment increased UCP1 levels in BAT (Fig. 2, E and F), although it did not change its mRNA levels (data not shown) in this tissue. These data are consistent with increased metabolic rate. However, in contrast to the findings in 4E-BP1-null mice, no changes in UCP1 mRNA or protein levels in WAT or in PGC-1 mRNA or protein levels in BAT or WAT were found in the 4E-BP2 ASO-treated group (data not shown). Furthermore, 4E-BP2 ASO treatment resulted in a 40–55% decrease in the expression of mitochondrial dicarboxylate carrier (mDIC), fatty acid synthase (FAS), and diacylglycerol acytyltransferase 2 (DGAT2) genes in WAT (Fig. 2C), indicating decreased lipogenesis in this tissue. In vitro studies demonstrated that reduction of 4E-BP2 expression resulted in a decrease in TG synthesis rate (P < 0.05) and an increase in fatty acid oxidation rate in mouse primary hepatocytes.

Fig. 2. 4E-BP2 antisense treatment increased metabolic rate in DIO mice. Indirect calorimetric measurement found that treatment with 4E-BP2 ASO increased VO2 in both dark and light phases (A) but did not change respiratory exchange ratio (RER; B). Real-time quantitative RT-PCR analysis showed higher expression of β3-adrenergic receptor (AR-β3) in WAT (C) and BAT (D), and lower expression of mitochondrial dicarboxylate carrier (mDIC), fatty acid synthase (FAS), and diacylglycerol acytyltransferase 2 (DGAT2) in WAT from 4E-BP2 ASO-treated mice (C). Immunoblotting analysis demonstrated increased UCP1 levels in WAT with unchanged PGC-1 levels in BAT from 4E-BP2 ASO-treated mice (E and F). NS, nonspecific band. Data are expressed as means ± SE; n ≥ 4. *P < 0.05, **P < 0.01 vs. controls.
Reduction of 4E-BP2 expression improved liver steatosis. HF-diet feeding caused severe liver steatosis, as shown by an increase in liver TG content of more than fivefold relative to lean chow fed mice (Fig. 3A). Treatment with control ASO did not affect liver TG content (Fig. 3A). However, 4E-BP2 ASO treatment reduced liver TG content by >50% compared with either the saline treatment group or the control ASO treatment group (P < 0.01; Fig. 3A). No liver toxicity as assessed by measuring plasma AST and ALT activities (Table 2), nor was hepatomegaly observed in any of the treatment groups (Fig. 3B). To investigate the mechanism by which 4E-BP2 ASO treatment improved liver steatosis, mouse hepatocytes were treated with the ASO, and then TG synthesis and fatty acid oxidation rates were measured. A significant decrease in TG synthesis rate that was associated with an increase in fatty acid oxidation rate was observed in hepatocytes treated with 4E-BP2 ASO (Fig. 3, C, D, and E), indicating that the improved liver steatosis could be due to decreased lipogenesis and increased fat burn in this tissue. ASO treatment did not cause changes in plasma cholesterol levels, TG levels, free fatty acid levels, or adiponectin levels (Table 2).

Reduction of 4E-BP2 expression lowered blood glucose levels and improved insulin sensitivity. Compared with chow-fed mice, HF diet feeding caused not only hyperinsulinemia but also hyperglycemia, both of which worsened over time as their HF diet continued (Fig. 4, A and B). Treatment with control ASO did not cause any changes in these two parameters. However, 4E-BP2 ASO resulted in normalization of both plasma insulin and glucose levels after 6 wk of treatment (Fig. 4, A and B). These data indicated that 4E-BP2 ASO treatment improved insulin sensitivity. To further confirm this, GTT was carried out. The glucose excursion curve in 4E-BP2 ASO-treated mice was significantly improved relative to control groups (P < 0.01; Fig. 4C). Gene expression analysis found that the expression of hepatic glucose-6-phosphatase (G-6-Pase) was lowered by 60% (P < 0.01) and phosphoenolpyruvate carboxykinase (PEPCK) was lowered by 29%, whereas the expression of hepatic glycogen synthase was increased by 27% (P < 0.01) in 4E-BP2 ASO-treated mice (Fig. 4D), implying a decrease in hepatic glucose output in these mice. Immunoblotting analysis found that GLUT4 abundance in plasma membrane of WAT was increased in 4E-BP2 ASO-treated mice in 4E-BP2 ASO-treated mice (Fig. 4, E and F), indicating an increase in glucose uptake.

Reduction of 4E-BP2 expression increased downstream insulin signaling activity. To further investigate the mechanism by which 4E-BP2 ASO treatment improved insulin sensitivity, 4E-BP2 ASO-treated mice and control mice were challenged with insulin, and the activities of the key enzymes involved in insulin signaling were examined in both liver and WAT. Insulin challenge caused a greater increase in phosphorylation of AktSer473 in both WAT (P < 0.01) and liver (P < 0.05) of 4E-BP2 ASO-treated mice relative to either saline-treated or control ASO-treated mice (Fig. 5, A and B). These data are consistent with increased levels of plasma membrane GLUT4 in WAT in 4E-BP2 ASO-treated mice. However, insulin challenge did not change the levels of pIRβDyr1162/1163 (Fig. 5A for WAT data; liver data not shown) in these mice. These data

Fig. 3. 4E-BP2 antisense treatment improved liver steatosis in DIO mice. Treatment with 4E-BP2 ASO lowered liver triglyceride (TG) content (A) with no effect on liver weight (B). In vitro assays showed that 4E-BP2 ASO-caused reduction of 4E-BP2 expression in mouse primary hepatocytes (C) was accompanied by a decreased TG synthesis rate (D) and increased fatty acid oxidation (E). For the in vitro assays, isolated mouse hepatocytes were seeded in 60-mm plates or 25-cm² flasks. Cells were then transfected with a 4E-BP2 ASO or a control ASO for 4 h. After 24-h recovery, incorporation of glycerol into TG or oxidation of [14C]oleate into CO2 and acid-soluble products was determined. Data are expressed as means ± SE; n = 7 for in vivo assays, n = 3 for in vitro assays. *P < 0.05, **P < 0.01 vs. controls.
indicated that 4E-BP2 ASO caused improvement in insulin sensitivity by augmenting downstream insulin signaling activity. To confirm that the enhancement of pAkt (Ser473) in response to insulin challenge is caused by a reduction of 4E-BP2 ASO expression, mouse primary hepatocytes were also transfected with another 4E-BP2 ASO (4E-BP2 ASO #2), which targets a different area of the 4E-BP2 mRNA, along with the 4E-BP2 ASO used for the above in vivo experiments, and the cells were then challenged with insulin. Both ASOs dramatically reduced 4E-BP2 protein levels and further enhanced the phosphorylation levels of Akt (Ser473) in response to insulin challenge compared with controls (Fig. 5C). These data demonstrate that reduction of 4E-BP2 expression improves downstream insulin signaling both in vivo and in vitro.

To investigate whether reduction of 4E-BP2 expression affected phosphorylation levels of eIF4E and S6K, Western blotting assays were done with WAT and liver samples from ASO-treated mice with (Fig. 5D) or without (data not shown) insulin challenge. In contrast to the finding in 4E-BP1-null mice (29), reduction of 4E-BP2 expression with antisense did not affect the phosphorylation levels of eIF4E (p-eIF4E (Ser209)) in either liver or WAT under either condition. Also, in contrast to the findings in 4E-BP1 and 4E-BP2 double-knockout mice (18), reduction of 4E-BP2 expression with antisense did not
affect the phosphorylation levels of S6K (pS6K1 Thr389) in either WAT or liver (Fig. 5D). These results further indicate that antisense reduction of 4E-BP2 causes different intracellular effects than genetic reduction of 4E-BP1 alone or of 4E-BP1 and 4E-BP2 together.

DISCUSSION

In this study, we used antisense technology to specifically reduce 4E-BP2 expression in liver and fat in DIO mice to investigate the possible role of 4E-BP2 in energy metabolism. Genetically, global deletion of 4E-BP2 results in altered behavior and hippocampus-dependent synaptic plasticity and memory in mice (3, 4), thereby precluding any evaluation of the role of 4E-BP2 in peripheral tissues. In contrast, antisense reduction of 4E-BP2 expression in the peripheral tissues did not cause any apparent abnormal behavior or overt toxicity. Reduction of 4E-BP2 levels also did not cause changes in food intake. However, 4E-BP2 reduction decreased body weight and body adiposity, improved liver steatosis, lowered plasma glucose and insulin levels, and improved insulin sensitivity in DIO mice. These results demonstrate for the first time that 4E-BP2 plays an important role in metabolism and energy homeostasis.

Decreased body weight and body adiposity after 4E-BP2 ASO treatment were attributable to both increased metabolic rate and decreased lipogenesis. This conclusion is supported by the following findings. First, indirect calorimetric measure-
ments demonstrated increased whole body energy expenditure in 4E-BP2 ASO-treated mice. Second, gene expression analysis found increased expression of β3-AR, the key adrenergic receptor for the epinephrine/norepinephrine system-mediated thermogenesis, in fat tissues, and decreased expression of mDIC, FAS, and DGAT2 in WAT, key enzymes in lipogenesis in this tissue. Third, immunoblotting analysis directly demonstrated increased UCP1, the most important thermogenic player in rodents, in BAT of the 4E-BP2 ASO-treated mice. Last, reduction of 4E-BP2 expression in mouse hepatocytes in vitro not only decreased TG synthesis but also increased fatty acid oxidation. These findings are consistent with the increased fat burn rate found in 4E-BP-null Drosophila during starvation (28). Unchanged mRNA levels but increased protein levels of UCP1 in the BAT of 4E-BP2 ASO-treated mice indicate that 4E-BP2 plays a regulatory role in UCP1 translation in BAT of rodents. However, in contrast to the findings in 4E-BP1-null mice (29), no changes in UCP1 or PGC-1 were detected in WAT of 4E-BP2 ASO-treated mice. Therefore, although increased energy expenditure was found in both 4E-BP1-null mice (29) and 4E-BP2 ASO-treated mice, the underlying molecular mechanism appears to be different.

It is well known that obesity not only causes increased adiposity but also often causes ectopic fat deposition in a variety of tissues, which can result in decreased insulin secretion from β-cells and insulin resistance in other peripheral tissues such as the liver (6, 20). Consistent with this notion, reductions in body weight and adiposity in mice treated with 4E-BP2 ASO were accompanied by a decrease in liver TG content and an improvement in insulin sensitivity. Lower body fat content accompanied by decreased plasma glucose levels were also observed in 4E-BP1-null mice (29). However, no data were reported on the effects of 4E-BP1 deficiency on insulin sensitivity and related insulin signaling activity. Here, we found that reduction of 4E-BP2 expression caused decreased expression of hepatic G-6-Pase and PEPCK and increased expression of GS. G-6-Pase is the final “gate” for release of hepatic glucose from both gluconeogenesis and glycolysis pathways; PEPCK is the limiting enzyme for glycogenolysis, whereas GS is the rate-limiting enzyme for hepatic glycogen synthesis. Therefore, the changes in the expression of these three hepatic genes suggested a decrease in hepatic glucose output as a result of antisense reduction of 4E-BP2 levels and a consequent lowering of plasma glucose levels.

In conclusion, long-term overfeeding results in obesity, which often causes insulin resistance, type 2 diabetes, fatty liver disease, hypertension, and other cardiovascular problems. Data from the current study demonstrate that peripheral reduction of 4E-BP2 expression can reduce body weight and adiposity and improve liver steatosis and insulin sensitivity by increasing energy expenditure and decreasing lipogenesis. Therefore, peripheral 4E-BP2 could be a promising therapeutic target for the treatment of obesity and related metabolic disorders.

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REFERENCES

12. Grolleau A, Sonenberg N, Wietzerbin J, Beretta L. Differential regulation of 4E-BP1 and 4E-BP2, two repressors of translation initiation,


