A very low carbohydrate ketogenic diet improves glucose tolerance in ob/ob mice independently of weight loss

Michael K. Badman,1 Adam R. Kennedy,2 Andrew C. Adams,1 Pavlos Pissios,1 and Eleftheria Maratos-Flier1

1Division of Endocrinology, Beth Israel Deaconess Medical Center, Center for Life Science, Boston, Massachusetts; and 2The Jackson Laboratory, Bar Harbor, Maine

Submitted 1 June 2009; accepted in final form 2 September 2009

Badman MK, Kennedy AR, Adams AC, Pissios P, Maratos-Flier E. A very low carbohydrate ketogenic diet improves glucose tolerance in ob/ob mice independently of weight loss. Am J Physiol Endocrinol Metab 297: E1197–E1204, 2009. First published September 8, 2009; doi:10.1152/ajpendo.00357.2009.—In mice of normal weight and with diet-induced obesity, a high-fat, low-carbohydrate ketogenic diet (KD) causes weight loss, reduced circulating glucose and lipids, and dramatic changes in hepatic gene expression. Many of the effects of KD are mediated by fibroblast growth factor 21 (FGF21). We tested the effects of KD feeding on ob/ob mice to determine if metabolic effects would occur in obesity secondarily to leptin deficiency. We evaluated the effect of prolonged KD feeding on weight, energy homeostasis, circulating metabolites, glucose homeostasis, and gene expression. Subsequently, we evaluated the effects of leptin and fasting on FGF21 expression in ob/ob mice. KD feeding of ob/ob mice normalized fasting glycemia and substantially reduced insulin and lipid levels in the absence of weight loss. KD feeding was associated with significant increases in lipid oxidative genes and reduced expression of lipid synthetic genes, including stearoyl-CoA desaturase 1, but no change in expression of inflammatory markers. In chow-fed ob/ob mice, FGF21 mRNA was elevated 10-fold compared with wild-type animals, and no increase from this elevated baseline was seen with KD feeding. Administration of leptin to chow-fed ob/ob mice led to a 24-fold induction of FGF21. Fasting also induced hepatic FGF21 in ob/ob mice. Thus, KD feeding improved ob/ob mouse glucose homeostasis without weight loss or altered caloric intake. These data demonstrate that manipulation of dietary macronutrient composition can lead to marked improvements in metabolic profile of leptin-deficient obese mice in the absence of weight loss.

changes are associated with a concerted program of changes in hepatic gene expression. Genes coding for enzymes responsible for lipid oxidation and ketogenic enzymes are increased with a concomitant decrease in lipid synthetic and glucocatabolic genes, including a dramatic suppression of fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1). Gene expression of key regulators of lipid metabolism was profoundly altered in KD-fed mice. Affymetrix analysis revealed a marked induction of fibroblast growth factor (FGF)21 in the liver. This endocrine FGF appears to play a role in mediating the effects of KD, since adenoviral knockdown of FGF21 in KD-fed animals leads to fatty liver, hypertriglyceridemia, and impaired ketosis (3). Recombinant human FGF21 improves both glucose and lipid parameters in rodent and primate models of diabetes (21, 22) and abrogates weight gain and fatty liver in genetic and dietary models of obesity (9, 39).

To further understand the role of KD in treatment of obesity, we examined the effects of KD in a genetically obese rodent, the ob/ob mouse. ob/ob mice are characterized by hyperphagia, reduced energy expenditure, and dysregulated glucose and lipid metabolism (1, 2, 41). Because feeding KD led to dramatic changes in DIO, we hypothesized that feeding of KD to ob/ob mice would significantly improve their metabolic milieu. KD feeding led to substantial improvement in glucose homeostasis in the ob/ob mice in the absence of any weight loss or improvement in lipid profile as seen in wild-type (wt) DIO mice. Studies of hepatic gene expression revealed significant changes, including suppression of enzymes of lipid synthesis and induction of enzymes involved in lipid oxidation in KD-fed groups. However, expression of FGF21, which in part mediates the effects of KD in wt animals, were elevated in chow-fed ob/ob mice and were not further induced by KD feeding. A screen of inflammatory markers associated with decreased insulin sensitivity and impaired glucose homeostasis, including tumor necrosis factor (TNF)-α and interleukin (IL)-6, revealed no change in expression. These data indicate that feeding of KD will improve glucose tolerance in a manner independent of weight loss, leptin, or altered expression of inflammatory markers.

MATERIALS AND METHODS

Mouse Maintenance and Diets

All procedures were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. WT male C57BL/6 mice and B6.1-Lepob/J (ob/ob) mice (Jackson Laboratories, Bar Harbor, ME) were housed in groups of four with ad libitum access to food and water unless otherwise stated. All animals were maintained in a temperature-controlled environment at ±24°C under a 12:12-h light-dark cycle and handled at least one time per week.
Dietary intake was either regular chow (F6 Rodent Diet; Harlan Teklad, Madison, WI) comprised of 52% carbohydrate, 18% fat, and 31% protein by calorie or KD (F-3666; Bio-Serv, Frenchtown, NJ) comprised of 0.4% carbohydrate, 95% fat, and 4.5% protein by calorie. For study 1 (long-term effects of KD feeding), four groups of age-matched wt and ob/ob mice were fed chow until the ob/ob cohort reached 45 g, at which point mice were assigned to receive either chow or KD. Dynamic physiological tests were performed after 50 days of feeding, and mice were killed after a 10-day recovery period after a total 60-day period of feeding. In study 2 (short-term leptin administration to ob/ob mice), groups of 10-wk-old wt or ob/ob mice were infused with either saline or leptin (500 ng/h; 12 mg/day) using Alzet micro-osmotic pumps (DURECT, Cupertino, CA) for 3 days. After being filled with either leptin or saline, pumps were primed for 4–6 h at 37°C before implantation in the interscapular region under isoFlurane anesthesia. In study 3 (effects of fasting ob/ob mice), groups of 10-wk-old mice were allowed ad libitum access to food or fasted for 24 h (9:00 A.M. to 9:00 A.M.) before death and dissection. In all cases, euthanasia was carried out by overdose using a pentobarbital sodium anesthesia (Nembutal; Hospira, Lake Forest, IL) and subsequent exsanguination.

**Dynamic Physiological Tests**

Glucose tolerance tests were performed on mice fasted overnight for 16 h. Glucose was determined using a One-Touch glucometer (Abbott Laboratories, Abbott Park, IL) in blood taken from a tail vein nick at 0, 45, 90, and 135 min after intraperitoneal injection of 1 g/kg dextrose. In addition, plasma insulin levels were also determined in tail vein blood collected at 0- and 45-min time points. Insulin tolerance tests were performed on mice fasted for 6 h. Glucose was measured as above at 0, 30, 60, 90, and 120 min after intraperitoneal injection of 0.75 IU/kg regular insulin (Eli Lilly, Indianapolis, IN).

**Indirect Calorimetry**

After 8 wk of feeding KD or chow, core temperatures were measured with a digital rectal thermometer (Physiomed Instruments, Clifton, NJ). The metabolic rate of mice was measured by indirect calorimetry of groups of eight singly housed animals within open-circuit oxymax chambers of the Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). All mice were acclimated to monitoring cages for 48 h before data acquisition.

**Serum Analysis**

Blood was collected from tail vein nick or terminal bleed and stored on ice before centrifugation at 15,000 g for 10 min. Serum was subsequently flash-frozen and stored at −20°C before analysis. Small-scale, linear enzyme colorimetric assays were employed for measurement of duplicate of glucose, β-hydroxybutyrate, triglycerides, cholesterol, and glycerol (StanBio, Boerne, TX). Nonesterified fatty acids (NEFAs) were measured in duplicate spectrophotometrically by an enzymatic colorimetric assay (Wako Chemicals, Richmond, VA). Insulin levels were determined in duplicate by ultrasensitive mouse specific enzyme-linked immunosorbent assay (Crystal Chem, Downers Grove, IL) with intra-assay coefficient of variation (CV) <10% and interassay CV <10%. To derive a surrogate for whole-body insulin sensitivity, quantitative insulin sensitivity check index (QUICKI) was calculated as QUICKI = 1/[log(I0) + log(G0)], where I0 is fasting insulin (µU/ml) and G0 is fasting glucose (mg/dl). Serum glucagon levels were assayed in duplicate (AssayGate, Ijamsville, MD). FGF21 was measured in duplicate by radioimmunoassay (Phoenix Pharmaceuticals, Burlingame, CA) that had 100% cross-reactivity between human and rodent FGF21 with intra-assay CV 5% and interassay CV 7%.

**Liver Triglyceride Analysis**

Mice were killed by lethal overdose of anesthesia, and tissues were rapidly dissected and flash-frozen in liquid nitrogen. Liver triglyceride was estimated by homogenization of 100 µg of tissue in 1 ml of extract buffer containing 0.1% Triton X-100 and incubated at 60°C for 1 h. Triglyceride content of unfractionated homogenate (NEFAs) were measured in duplicate spectrophotometrically by an enzymatic assay (Pierce, Rockford, IL) with intra-assay coefficient of variation (CV) <5% and interassay CV <10%.

**RNA Extraction and Quantitative Real-Time PCR**

Hepatic RNA was extracted with the RNAeasy tissue mini kit with column DNase digestion (Qiagen, Valencia, CA). cDNA was synthesized from 1 µg of total liver RNA with Ambion RETROscript (Qiagen) using random decamer primers. Quantitative real-time PCR
was performed in duplicate in a total reaction volume of 20 µl using either SYBR Green master mix (Applied Biosystems, Foster City, CA) with primers as previously published for metabolic and regulatory genes (3, 20) or Taqman gene expression assays and master mix (Applied Biosystems) for inflammatory markers using a MX3000p instrument (Stratagene, La Jolla, CA). Results are shown as relative expression compared with ribosomal RNAs.

Data Analysis

Data shown are means ± SE. Normal distribution of data was assumed and parametric statistical tests were used throughout. Comparisons were made with a two-tailed t-test with assumption of equal variance.

RESULTS

Study 1: Long-Term Effects of KD Feeding

KD feeding results in weight loss in wt but not ob/ob mice. Consistent with our previous findings, after 60 days of study, KD-fed wt mice weighed significantly less than chow-fed controls. Age-matched ob/ob mice initially weighed an average of 21.6 g more than wt controls. In contrast to the wt groups, KD feeding did not lead to a significant change in weight of ob/ob mice when fed KD (Fig. 1A). Chow-fed ob/ob mice had higher caloric intake than chow-fed wt controls (Fig. 1B). However, the core temperature in both chow- and KD-fed ob/ob groups were significantly lower than wt mice (Fig. 1C).

Furthermore, ob/ob groups had a significantly lower weight-adjusted VO2 (Fig. 1D) although comparison of heat production over a 72-h period of indirect calorimetry revealed no significant difference between chow- and KD-fed ob/ob mice (Fig. 1E). The respiratory exchange ratio approached 0.7 in KD-fed wt and ob/ob mice (Fig. 1F), consistent with a predominantly lipid fuel-based metabolism during KD feeding.

KD improves glucose metabolism in ob/ob mice. Following 50 days of KD feeding, there was significant improvement in insulin sensitivity of ob/ob mice despite the absence of weight change. Fasting glucose levels fell from 251 ± 38 mg/dl in chow-fed ob/ob mice to 110 ± 17 mg/dl in KD-fed ob/ob mice (Fig. 2A). Similarly, glucose excursions during the intraperito-neal glucose tolerance test were attenuated by KD feeding of ob/ob mice (Fig. 2A). KD-fed groups exhibited reduced fasted insulin levels in both wt (0.87 ± 0.13 ng/ml chow vs. 0.23 ± 0.09 ng/ml KD) and ob/ob (4.89 ± 0.37 ng/ml chow vs. 2.83 ± 0.36 ng/ml) mouse groups (Fig. 2B). Furthermore, the glucose-induced increment in circulating insulin in response to glucose challenge was restored in KD-fed ob/ob mice (Fig. 2B). As previously shown, chow-fed ob/ob mice were remarkably resistant to insulin treatment, with a maximal decrease in circulating glucose to 85 ± 7% basal levels. In contrast, glucose levels fell to 55 ± 6% basal levels following insulin administration in KD-fed ob/ob mice (Fig. 2C). Improved glycemia in ob/ob mice was seen in the context of a 75% fall of insulin levels. Calculation of QUICKI, a surrogate marker of insulin sensitivity (19–23), revealed that this metric was increased from 0.31 ± 0.01 in chow-fed to 0.41 ± 0.03 in KD-fed wt and from 0.23 ± 0.00 in chow-fed to 0.27 ± 0.01 in KD-fed ob/ob mice (Fig. 2D).

KD feeding alters lipid profile. KD feeding led to a 22% reduction in fed serum triglycerides in wt mice. Although there was a 13% trend toward lower triglycerides in KD-fed ob/ob mice (Fig. 2D).

Fig. 2. Effects of KD feeding on glucose metabolism. A: KD feeding normalized fasting glucose levels and reduced glucose excursions in ob/ob mice. Open circles, chow-fed wt; filled circles, KD-fed wt; open squares, chow-fed ob/ob mice; filled squares, KD. *P < 0.001 chow- vs. KD-fed ob/ob. B: KD feeding reduced fasting insulin levels. Filled bars, fasted; open bars, 45 min insulin. +P < 0.05 chow- vs. KD-fed wt mice. *P < 0.001 chow- vs. KD-fed ob/ob mice. Increment in circulating insulin in response to glucose challenge returned in KD-fed ob/ob mice. #P < 0.01 wt chow-fed basal vs. 45 min and ob/ob KD-fed basal vs. 45 min. C: insulin tolerance test revealed increased sensitivity in KD-fed ob/ob mice. Filled circles, chow-fed wt; open circles, KD-fed wt; filled squares, chow-fed ob/ob mice; open squares, KD. *P < 0.001 chow- vs. KD-fed ob/ob mice. D: quantitative insulin sensitivity check index (QUICKI) metric was greatly improved by KD feeding of ob/ob mice. Filled bars, chow; open bars, KD. #P < 0.01 chow- vs. KD-fed wt mice. *P < 0.001 chow- vs. KD-fed ob/ob mice.
mice, this effect did not reach statistical significance (Table 1). NEFA were elevated in response to KD feeding in wt mice; no increment was seen in the already elevated NEFA levels in KD-fed ob/ob mice. Of note, KD-fed wt mice became overtly ketogenic, whereas there was only a small, albeit significant, change in the circulating β-hydroxybutyrate concentration in KD-fed ob/ob mice. There was a 48% increase in circulating cholesterol in KD-fed wt mice; however, the 7% trend toward increased circulating cholesterol in KD-fed ob/ob mice did not reach statistical significance (Table 1). Hepatic triglyceride was increased in KD-fed wt mice compared with chow-fed wt controls. However, there was no additional accumulation in the already fatty livers of KD-fed ob/ob mice (Table 1).

**KD feeding alters hepatic gene expression in wt and ob/ob mice.** Analysis of metabolic enzyme gene expression profiles in KD-fed ob/ob mice showed changes previously noted in wt mice consuming KD. Increases in hepatic expression of gluconeogenic enzymes glyceraldehyde-3-phosphate dehydrogenase and the rat-limiting phosphoenolpyruvate carboxykinase failed to reach statistical significance in ob/ob mice over wt controls (data not shown). As expected, expression of enzymes of lipid synthesis FAS and SCD1 were grossly elevated by 6.3- and 8.5-fold, respectively, in chow-fed ob/ob mice compared with wt controls (Fig. 3, A and B). Notably, feeding KD led to a marked suppression of FAS and SCD1 to 12.6 and 0.5% of chow-fed values, respectively, in ob/ob mice despite the absence of weight loss. Expression of genes required for fatty acid transport and oxidation also showed marked changes; the fatty acid translocase CD36 was upregulated in chow-fed ob/ob mice compared with wt controls (P < 0.005), and there was further induction of CD36 in KD-fed ob/ob mice (Fig. 3C). There was no difference in expression of enzymes of β-oxidation, long-chain acyl-coenzyme A dehydrogenase and 3-hydroxyacyl-coenzyme A dehydrogenase (HADH), between chow-fed wt or chow-fed ob/ob mice, and both were induced approximately twofold by KD feeding in mice of both genotype (Fig. 3, D and E). Notably, another hepatic PPARα target, uncoupling protein 2 (UCP2), was also upregulated by KD feeding by 12-fold in wt and 3.6-fold in ob/ob mice (Fig. 3F). Genes of ketosis, including 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (HMGS2) and 3-hydroxybutyrate dehydrogenase (HBDH) were also both induced approximately twofold by KD feeding from similar baseline levels in wt and ob/ob mice (Fig. 3, G and H).

**Table 1. Effects of KD feeding on wt and ob/ob mice**

<table>
<thead>
<tr>
<th></th>
<th>Chow</th>
<th>KD</th>
<th>Chow</th>
<th>KD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type</td>
<td>ob/ob</td>
<td>Wild Type</td>
<td>ob/ob</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>2.16±0.48*†‡</td>
<td>1.32±0.08†‡</td>
<td>19.6±5.0*§</td>
<td>5.4±1.3*‡§</td>
</tr>
<tr>
<td>Glucagon, pmol/l</td>
<td>15.1±0.91†‡</td>
<td>17.7±2.5†‡</td>
<td>38.6±9.1*§</td>
<td>9.7±0.71*§</td>
</tr>
<tr>
<td>NEFA, meq/l</td>
<td>0.51±0.02†‡</td>
<td>0.61±0.04§</td>
<td>0.64±0.06§</td>
<td>0.66±0.07§</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>248±9*†‡</td>
<td>155±16†‡</td>
<td>296±12*§</td>
<td>205±17*‡§</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>87.2±6.7*†‡</td>
<td>68.2±2.5†‡</td>
<td>135±28*</td>
<td>117±14*§</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>94.0±3.3*†‡</td>
<td>139±4.4†‡</td>
<td>174±9.3*‡</td>
<td>187±5.0*§</td>
</tr>
<tr>
<td>Liver triglyceride, mg/g</td>
<td>3.50±0.10*†‡</td>
<td>8.94±0.28‡</td>
<td>8.81±0.72§</td>
<td>8.10±0.82§</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE; n = 5–8 mice. NEFA, nonesterified fatty acid. Wild-type (wt) or ob/ob mice fed ketogenic diet (KD) for 60 days. Mice were compared with age- and initial weight-matched chow-fed control groups. Circulating hormones and metabolites were measured in blood collected at the time of death (9:00 A.M.) while mice were allowed access to food and water ad libitum. Liver triglyceride was calculated from assay of extract from liver flash-frozen at the time of death. P < 0.05 compared with wt chow (§)-, wt KD (*)-, ob/ob chow (+)-, and ob/ob KD (‡)-fed groups.

**Fig. 3. Effects of KD feeding on expression of enzymes of hepatic metabolism.** A and B: enzymes of lipid synthesis [fatty acid synthase (FAS) and 3-hydroxyacyl-coenzyme A desaturase 1 (SCD1)]. C–E: genes required for fatty acid transport and oxidation [fatty acid translocase (CD36) and long-chain acyl-coenzyme A dehydrogenase (ACADL)] and 3-hydroxyacyl-coenzyme A dehydrogenase (HADH). F: peroxisome proliferator-activated receptor (PPARα) target gene uncoupling protein-2 (UCP2). G and H: genes of ketosis [3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (HMGS2) and 3-hydroxybutyrate dehydrogenase (HBDH)]. Filled bars, chow fed; open bars, KD fed. +P < 0.05, #P < 0.01, and *P < 0.001 chow vs. KD fed.
Hepatic FGF21 is induced by KD feeding in wt but not ob/ob mice. Because hepatic FGF21 plays a vital role in mediating the effects of KD, we examined expression in ob/ob mice. Hepatic FGF21 mRNA levels were induced 10-fold in chow-fed ob/ob mice compared with wt controls. There was no further induction above already elevated levels by KD feeding of ob/ob mice, in contrast to wt mice in which KD feeding led to marked induction of FGF21 (Fig. 4A). Consistent with elevated activity of peroxisome proliferator-activator receptor (PPAR)α, expression of PPARα was itself increased twofold in chow-fed ob/ob mice. Expression of PPARγ was also elevated in chow-fed ob/ob mice, and, in common with PPARα, there was no significant difference between chow- and KD-fed groups of ob/ob mice (Fig. 4, B and C). Similarly, liver expression of PPARγ coactivator-1β, a key regulator of hepatic lipid metabolism, was upregulated in ob/ob mice but showed no interaction with KD feeding (Fig. 4D). In contrast, expression of sterol-regulatory element-binding protein-1c (SREBP1c) was downregulated by KD feeding in wt mice, a suppression that was blunted in ob/ob mice (Fig. 4E). Further analysis of expression of FGF21 in adipose tissue revealed increased expression in chow-fed ob/ob mice but no changes with KD feeding (Table 2).

Analysis of inflammatory markers [IL-1, IL-6, TNF-α, suppressor of cytokine signaling 3, chemokine (C-C motif) ligand 2 in both liver and adipose tissue, and serine (or cysteine) peptidase inhibitor] in adipose tissues alone revealed no improvements associated with KD feeding (Table 2).

Study 2: Effects of Leptin Administration and Fasting of FGF21 Expression in ob/ob Mice

Leptin treatment induces hepatic FGF21 expression in ob/ob mice. To establish the possible role of leptin deficiency in mediating increased FGF21 expression seen in chow-fed ob/ob mice, leptin was infused in ob/ob mice and wt controls with free

Table 2. Effects of KD feeding on tissue inflammatory markers in wt and ob/ob mice

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>KD</th>
<th>ob/ob</th>
<th>KD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chow</td>
<td>KD</td>
<td>Chow</td>
<td>KD</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1</td>
<td>1.00±0.14†‡</td>
<td>1.84±0.28§</td>
<td>1.66±0.27§</td>
<td>1.96±0.39§</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.00±0.22</td>
<td>1.98±0.54</td>
<td>1.28±0.23</td>
<td>1.11±0.19</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.00±0.44†‡</td>
<td>2.82±0.44§</td>
<td>3.5±0.75§</td>
<td>3.74±0.78§</td>
</tr>
<tr>
<td>SOCS3</td>
<td>1.00±0.41†‡</td>
<td>1.33±0.35</td>
<td>1.26±0.38</td>
<td>2.74±0.62§</td>
</tr>
<tr>
<td>Ccl2</td>
<td>1.00±0.30†‡</td>
<td>8.59±0.55§</td>
<td>8.55±0.81§</td>
<td>8.17±0.83§</td>
</tr>
<tr>
<td>Perigonadal adipose tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF21</td>
<td>1.00±0.21†‡</td>
<td>1.69±0.48†‡</td>
<td>4.9±1.00§</td>
<td>5.97±0.37§</td>
</tr>
<tr>
<td>IL-1</td>
<td>1.00±0.12†</td>
<td>1.45±0.19</td>
<td>1.62±0.26§</td>
<td>1.31±0.16</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.00±0.12†‡</td>
<td>0.63±0.11†‡§</td>
<td>2.45±0.51§</td>
<td>2.14±0.34§</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.00±0.18</td>
<td>0.81±0.09</td>
<td>4.00±0.87§</td>
<td>4.95±1.11§</td>
</tr>
<tr>
<td>SOCS3</td>
<td>1.00±0.19†‡</td>
<td>0.82±0.14††</td>
<td>2.12±0.50§</td>
<td>2.04±0.28§</td>
</tr>
<tr>
<td>Ccl2/MCP1</td>
<td>1.00±0.15†‡</td>
<td>0.69±0.11†‡</td>
<td>10.00±2.41§</td>
<td>7.73±0.37§</td>
</tr>
<tr>
<td>Serpine1/PAI</td>
<td>1.00±0.16†‡</td>
<td>2.23±0.57†‡§</td>
<td>4.07±0.88§</td>
<td>4.81±0.35§</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE; n = 5–8 mice. IL, interleukin; TNF, tumor necrosis factor; SOCS, suppressor of cytokine signaling; Ccl, chemokine (C-C motif) ligand; FGF, fibroblast growth factor; MCP, monocyte chemotactic protein; PAI, plasminogen activator inhibitor. Wild-type or ob/ob mice were fed KD for 60 days. Mice were compared with age- and initial weight-matched chow-fed control groups. Liver and perigonadal adipose tissues were harvested and flash-frozen at the time of death. Assays were performed by quantitative PCR using Taqman gene expression assays and master mix. *P < 0.05 compared with wt chow (§), wt KD (+), ob/ob chow (†), and ob/ob KD (‡)-fed groups. KD feeding was not associated with reduced expression of inflammatory markers in the ob/ob mice.
access to chow. Administration of leptin to ob/ob mice led to a 24-fold induction of hepatic FGF21. However, this was associated with a 12% weight loss secondary to a marked reduction in food intake. In leptin-treated wt mice, there was also decreased food intake, but mice did not lose as much weight and failed to induce hepatic FGF21 (Fig. 5, A and B).

Fasting for 24 h induces hepatic FGF21 expression in ob/ob mice. To rule out the possibility that the rise in FGF21 with leptin treatment in ob/ob animals was secondary to decreased food intake as opposed to a direct effect of leptin, we fasted ob/ob mice for 24 h and found that fasting was associated with an additional induction of hepatic FGF21 expression that was reflected in circulating FGF21 levels (Fig. 5, C and D). Combined fasting and leptin treatment did not lead to any additional increase in FGF21 expression in fasted animals (data not shown). In contrast to KD feeding alone, fasting led to a significant increase in circulating ketones in both fasted wt (0.95 ± 0.08 vs. 7.23 ± 0.37 mmol/l) and fasted ob/ob (1.54 ± 0.44 vs. 12.5 ± 2.1 mmol/l) mice.

**DISCUSSION**

In humans, consumption of KD is associated with weight loss and improvement of metabolic parameters in obese individuals and those with type 2 diabetes (10, 29, 31, 40). We have previously shown that, in wt mice, the rodent version of KD (6), KD reverses DIO and is associated with profound improvements in both circulating lipid profile and glucose tolerance (20). In wt mice with DIO, weight loss is the result of increased energy expenditure in the context of increased leptin levels that normalize as fat mass decreases (20). Weight gain is also prevented by KD in other rodents (7, 35, 42), and it has been suggested that, in juvenile rats, the metabolic effects of KD feeding may be mediated by leptin (37). We therefore undertook an evaluation of the potential effectiveness of KD in a genetic model of obesity and insulin resistance, the ob/ob mouse.

In contrast to DIO animals, KD-fed ob/ob did not lose weight over a 60-day period and maintained the same weight as the chow-fed ob/ob group. We confirmed our previous findings that KD-fed WT mice had an elevated V˙O2 although notably V˙O2 was not altered in KD-fed ob/ob mice in either the light or dark periods. Nevertheless, there was a substantial improvement in glucose homeostasis in ob/ob mice eating KD as assessed by a number of parameters. First, in the ad libitum-fed state, ob/ob mice on KD had circulating glucose levels that were 25% lower than ob/ob mice eating chow. Second, KD-fed ob/ob mice had fasting glucose levels that were as low as those seen in wt animals. Third, there was a substantial difference of ~75 and 50%, respectively, in fed and fasted insulin levels between chow- and KD-fed ob/ob groups. The QUICKI metric was markedly improved in KD-fed ob/ob mice, providing a further surrogate marker of increased insulin sensitivity. Dynamic tests of glucose and insulin tolerance were also markedly improved. Of note, glucose excursion during glucose tolerance tests was reduced in KD-fed ob/ob mice, and, although not normalized, there was significant reduction in the area under the glucose curve. This fits with the improvements in insulin sensitivity seen during insulin tolerance testing in which glucose levels fell by 40% in ob/ob mice eating KD compared with <20% in mice eating chow, which is consistent with increased but not completely normalized insulin sensitivity. These results were notable given the absence of weight loss in the ob/ob mice and comparable caloric intake and energy expenditure in fed ob/ob animals. Changes in insulin sensitivity were seen in the absence of any changes in inflammatory markers. However, there were marked differences in serum glucagon levels between chow- and KD-fed ob/ob mouse groups. This contrasts to our previous findings in WT mice, which, as reiterated in this study, have no difference in circulating glucagon levels in chow- and KD-fed states. This decrease in glucagon may contribute significantly to the improved insulin insensitivity seen in the KD-fed ob/ob groups. Whether this is a direct effect of KD on islet glucagon secretion or mediated through another endocrine feedback loop potentially involving FGF21 provides scope for further study.

KD feeding leads to major changes in hepatic gene expression in wt mice with DIO. We found similar changes, including a marked elevation in expression of lipid oxidative and suppression of lipid synthetic genes, in KD-fed ob/ob mice, which occurred in the absence of weight loss. In particular, KD feeding suppressed SCD1 expression in common with other lipid-synthetic genes, including FAS (3, 20). This is notable, since ob/ob mice express hepatic SCD1 at levels over and above those seen in DIO mice (5, 8). The association of improved metabolic status with reduced SCD1 expression may be particularly important given the putative role of SCD1 and products thereof in the regulation of insulin sensitivity and glucose disposal (28, 30, 32, 33). It is possible that KD-mediated downregulation of SCD1 expression is at least in part responsible for improved glucose handling in the ob/ob mice. Expression of SCD1 is regulated by SREBP1c, which also regulates expression of FAS (4, 24, 36), in contrast to wt mice

**Fig. 5.** Leptin treatment and fasting induces FGF21 in ob/ob mice. A: weight loss following 72-h administration of saline (filled bars) or leptin (open bars) to wt or ob/ob mice. *P < 0.001 leptin treatment vs. saline control. B: induction of FGF21 gene expression resulting from leptin-mediated anorexia. +P < 0.05. C: FGF21 is induced by fasting in ob/ob mice. Filled bars, fed; open bars, fasted. +P < 0.05 and *P < 0.001 fasted vs. fed control. D: circulating levels of FGF21 reflect FGF21 gene expression in ob/ob mice fed (filled bars) and fasted (open bars). +P < 0.05 and *P < 0.001 fasted vs. fed control.

*AJP-Endocrinol Metab • VOL 297 • NOVEMBER 2009 • www.ajpendo.org*
where KD feeding results in reduced SREBP1c expression in both lean and DIO wt mice (20) concomitant with reduced expression of FAS and SCD1. In ob/ob mice, reduction of SREBP1c expression by KD was minimal, perhaps reflecting the high levels of circulating insulin levels.

FGF21 is a metabolic regulator that plays a critical role in mediating the effects of KD in wt animals. In wt mice, we and others have shown that hepatic FGF21 is primarily regulated by PPARα (3, 18). As reported by Lundasen and coworkers (25) we have found that FGF21 expression is increased in the livers of ob/ob mice. In the livers of ob/ob mice, expression of both PPARα and -γ are elevated (11, 27), and it is likely that PPARα is activated by increased circulating endogenous fatty acids in this model. Furthermore, hepatic PPARα target genes CD36, UCP2, and HMGC2 were robustly elevated in chow-fed ob/ob mouse liver. Because FGF21 potentiates hepatic fatty acid metabolism, basal induction indicates a degree of hormesis in response to endogenous fatty acids. We extended these observations by exposing ob/ob mice to KD, which in wt mice activates PPARα (3). As in wt mice, ob/ob mouse hepatic PPARα target genes, including CD36, ACDL, HADH, UCP2, HMGC2, and HBHD, were upregulated by KD feeding. In contrast, there was no significant increment in FGF21 beyond the already elevated basal level.

To understand the contribution of leptin deficiency to the increase in hepatic FGF21 expression, we treated ob/ob mice with leptin at doses just above those required to reverse the metabolic phenotype of ob/ob mice (13) and which led to weight loss in wt and ob/ob mice (16). Surprisingly, leptin infusion was associated with a significant additional upregulation of FGF21 in ob/ob mice but not in wt mice. This induction of FGF21 in ob/ob occurs in the context of levels that are already high. The elevation of FGF21 mRNA was observed in the context of leptin-induced hypoglycemia; this suggests that, in mice with lower food intake, FGF21 rises in a setting where increased β-oxidation of fatty acids from endogenous fuel supplies is required. This is a key role for FGF21 (3, 18). To distinguish between a primary effect of leptin or reduction in food intake, we directly examined the effect of fasting on FGF21 expression and found that, when mice fasted, FGF21 was greatly induced in the ob/ob mice; furthermore, there was no additional increase when mice were fasted and treated with leptin. This indicates that it is reduced food intake, through induction of endogenous ligands such as PPARα, rather than leptin that leads to the increased expression of FGF21 in ob/ob mice with reduced food intake.

In conclusion, we have demonstrated that KD feeding improved the metabolic phenotype of ob/ob mice in the absence of weight loss. Previously, it has been unclear whether beneficial effects of KD feeding on glucose homeostasis were the result of carbohydrate restriction or weight loss. Importantly, in this study with ob/ob mice, we show that effects on glycemia and insulin sensitivity are independent of weight loss. Furthermore, these effects are independent of leptin and occur in the absence of any change in inflammatory markers in either the liver or perigonadal white adipose tissue. It is possible that some of the effects are mediated by altered SCD1 expression, which is known to affect glucose sensitivity and is markedly suppressed by KD feeding. However, effects occur in the absence of any further induction of hepatic FGF21. The elevated basal levels of hepatic FGF21 expression noted in ob/ob mice is likely the result of increased basal PPAR activation. Curiously, KD feeding of ob/ob mice increases expression of a number of PPARα target genes but does not further induce FGF21. However, this effect is not because of maximal expression in the ob/ob model, since reduced caloric intake due to fasting or leptin treatment leads to further induction of FGF21 from baseline. This may be due to lipolysis that occurs under fasting or leptin-treated conditions, yielding increased levels of endogenous PPARα ligands over and above what is generated by KD feeding. Thus further studies are required to fully understand the remarkable effect of KD feeding and its effects on obesity and glucose intolerance.

ACKNOWLEDGMENTS

We are grateful for the technical assistance of Fen Fen Liu, Frank Marino, and Huali Yin in the production of this paper. We thank Drs. Carl Grunfeld and Joyce Repa for discussions relating to this work.

This work was presented in part as a poster form at the 2008 Keystone Symposium on Diabetes Mellitus, Insulin Action and Resistance, Breckenridge, Colorado.

GRANTS

This work has been supported by generous contributions from the Picower Foundation (E. Maratos-Flier and M. K. Badman), NIDDK Grants DK-069983-01 and DK-56116 (E. Maratos-Flier), and BONRC Pilot and Feasibility Grant DK-46200-15 (M. K. Badman).

REFERENCES


