Original Article

Casitas b-Lineage Lymphoma–Deficient Mice Are Protected Against High-Fat Diet–Induced Obesity and Insulin Resistance

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Casitas b-lineage lymphoma (c-Cbl) is a multiadaptor protein with E3-ubiquitin ligase activity involved in regulating the degradation of receptor tyrosine kinases. We have recently reported that *c-Cbl*$^{-/-}$ mice exhibit a lean phenotype and enhanced peripheral insulin action likely due to elevated energy expenditure. In the study reported here, we examined the effect of a high-fat diet on energy homeostasis and glucose metabolism in these animals. When *c-Cbl*$^{-/-}$ mice were fed a high-fat diet for 4 weeks, they maintained hyperphagia, higher whole-body oxygen consumption (27%), and greater activity (threefold) compared with wild-type animals fed the same diet. In addition, the activity of several enzymes involved in mitochondrial fat oxidation and the phosphorylation of acetyl CoA carboxylase was significantly increased in muscle of high-fat–fed c-Cbl–deficient mice, indicating a greater capacity for fat oxidation in these animals. As a result of these differences, fat-fed *c-Cbl*$^{-/-}$ mice were 30% leaner than wild-type animals and were protected against high-fat diet–induced insulin resistance. These studies are consistent with a role for c-Cbl in regulating nutrient partitioning in skeletal muscle and emphasize the potential of c-Cbl as a therapeutic target in the treatment of obesity and type 2 diabetes.
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The incidence of obesity and type 2 diabetes is increasing throughout the world. This has been ascribed to changes in food intake combined with a more sedentary lifestyle. Regardless of the cause, this emerging health care problem has sparked renewed interest in the study of insulin action and fuel metabolism. In particular, the identification of genes that regulate energy homeostasis in mammals has become a major research interest. Over the past decade, largely through the use of genetically manipulated animal models, a number of genes that result in lean phenotypes have been described. These genes include those that regulate appetite, food absorption, and increased energy expenditure in either muscle or adipose tissue (1). A major advantage of manipulations that increase energy expenditure is that this depletes fat stores not only in adipose tissue but possibly in other cells that are susceptible to lipotoxic damage, thus providing a protective mechanism against the development of insulin resistance and diabetes (2,3).

Genes that are known to regulate whole-body energy expenditure include mitochondrial uncoupling proteins that divert energy stores into heat production (4,5) and lipid handling enzymes, such as acetyl CoA carboxylase (ACC), which regulates the entry of long-chain acyl CoAs into mitochondria (6), and DGAT, a key enzyme in triacylglyceride synthesis (7). Unexpectedly, reduced expression of several molecules that negatively regulate insulin signaling, like the tyrosine phosphatase PTP1b (8), have also been shown to cause a significant increase in whole-body energy expenditure. This provides further evidence for an intimate link between insulin action and energy homeostasis.

We have recently described an unexpected role for Casitas b-lineage lymphoma (c-Cbl) in energy homeostasis. This is of interest because c-Cbl is also a negative regulator of growth factor signaling. c-Cbl was first identified as a cellular homolog of a murine retroviral oncogene that induces pre–β-cell lymphomas and myeloid tumors in mice (9,10). c-Cbl is a multimodular protein with several reported functions, including an E3 ubiquitin ligase activity residing in a RING finger domain that has been reported to negatively regulate growth factor signaling by promoting the ubiquitination and degradation of receptor tyrosine kinases in lysosomes (11,12). Although there has been considerable evidence to implicate a role for c-Cbl in tumorigenesis and hematopoiesis, our recent data were the first to implicate this protein in energy homeostasis. We described that *c-Cbl*$^{-/-}$ mice were both hyperphagic and lean with only 50% as much adipose tissue compared with wild-type control animals. We suggested that this phenotype was due to increased energy expenditure in skeletal muscle (13). These findings were particularly exciting because they provided new clues about the molecular regulation of energy expenditure opening novel avenues for research into the development of diabetes and obesity therapies. A major goal of the present study was to determine whether modifying c-Cbl function could have a protective role against the development of obesity and

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ACC, acetyl CoA carboxylase; c-Cbl, Casitas b-lineage lymphoma; HAD, hydroxyacyl CoA dehydrogenase; IRS-1, insulin receptor substrate-1.

insulin resistance. High-fat feeding is one of the most commonly used laboratory models of insulin resistance. This laboratory model is thought to resemble the change in lifestyle that has occurred in humans over the past 50 years contributing to the obesity/diabetes epidemic. Such diets have been shown to cause increased adiposity and insulin resistance in rodents (14,15). We show that deletion of c-Cbl results in marked protection against increased adiposity and the development of insulin resistance in high-fat feeding. Strikingly these protective mechanisms were evident in the face of increased food intake in the *c-Cbl*$^{-/-}$ mice.

RESEARCH DESIGN AND METHODS

c-Cbl$^{-/-}$ mice were generated as described previously (16). Experiments were performed on mice maintained on the hybrid 129/SvJ × C57BL/6 background. The animals were kept on a 12-h light/dark cycle with free access to food and water. Animals were fed ad libitum for 4 weeks with a Copha vegetable shortening diet (45% of caloric intake from fat [95% saturated fat], 34% from carbohydrates, and 21% from protein) based on Rodent Diet D12451 (Research Diets, New Brunswick, NJ). All of the experiments were carried out in 20- to 24-week-old animals with the approval of the Garvan Institute/St. Vincent's Hospital Animal Experimentation Ethics Committee, following guidelines issued by the National Health and Medical Research Council.

Metabolic assays. Glucose tolerance tests (2 g/kg glucose i.p.) were performed in overnight-fasted mice. Blood samples were obtained from the tail tip at the times indicated. Glucose levels were measured using a glucometer (AccuCheck II; Roche). Insulin concentrations were measured using an ultra-sensitive ELISA kit (Mercodia, Uppsala, Sweden). Clearance of the glucose analog [^{3}H]2-deoxyglucose (2-DOG) (2 g/kg glucose i.p., 10 μCi/animal [^{3}H]2-DOG) into glucose-6-phospate and [^{14}C]glucose (10 μCi/animal) clearance into glycogen and triacylglycerides in indicated tissues was measured as described previously (17). Other plasma measurements were performed from blood collected from the chest cavity into tubes containing EDTA and centrifuged at 14,000*g* for 10 min to obtain the plasma. Insulin, leptin, and adiponectin (Linco Research, St. Louis, MO) concentrations were assayed by radioimmunoassay. The concentration of nonesterified fatty acids was determined using a colorimetric kit (Wako Pure Chemical Industries, Osaka, Japan).

Adipocyte size determination. Isolated adipocytes were obtained from excised epididymal fat pads by the collagenase method (18). Cells were then fixed with 2% OsO_4 in PBS overnight at 37°C. Adipocyte diameter from 100 to 200 cells was determined using Adobe Photoshop software from images of adipocyte suspensions obtained by light microscopy.

Pancreatic islet isolation and insulin secretion assay. Mice were anesthetized, and islets were isolated with liberase (Roche) digestion of the pancreas followed by purification with a Ficoll-Paque density gradient (Amersham Biosciences). Batches of five islets were incubated in Krebs Ringer buffer supplemented with either 2.8 or 16.7 mmol/l glucose for 1 h at 37°C. Insulin was measured in an aliquot of the buffer by radioimmunoassay with rat insulin standard.

Indirect calorimetry and ambulatory activity studies. Oxygen consumption (V_{O_2}) was measured using a four-chamber indirect calorimeter (Columbus Instruments, Columbus, OH) with an air flow of 0.5 l/min. Studies were started at 1100 after 1 h of acclimation to the metabolic chamber (31 × 18 × 18 cm). V_{O_2} was measured in individual mice at 15-min intervals over a 24-h period under a consistent environmental temperature (22°C). Ambulatory activity was estimated simultaneously by the number of photobeam breaks occurring in adjacent laser beams using an Opto-Varimex three-sensor system (Columbus Instruments). Cumulative ambulatory activity counts were recorded every 20 min. During the study, mice had ad libitum access to food and water.

Insulin signaling studies. Overnight-fasted male mice were anesthetized, and quadriceps, tibialis cranialis muscles from one leg, and one epididymal fat pad were removed and immediately frozen in liquid nitrogen. Insulin (1 unit/kg) was then injected intravenously, and the second epididymal fat pad and quadriceps and tibialis cranialis muscles from the other leg were excised after 3 min and frozen. Frozen tissues were processed for immunoblotting as described below.

Tissue processing and immunoblotting. Frozen tissues removed from wild-type and *c-Cbl*$^{-/-}$ mice were powdered and resuspended in radioimmunoprecipitation assay buffer (PBS, pH 7.5, 1% Nonidet NP-40, 0.5% sodium deoxy-cholate, and 0.1% SDS), supplemented with protease and phosphatase inhibitors (10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mmol/l Na_3VO_4, 10 mmol/l NaF, and 10 mmol/l Na pyrophosphate), and solubilized for 2 h at 4°C. Aliquots of tissue homogenates were resolved by SDS-PAGE and immunoblotted with antibodies against pSer79-ACC, pSer473-Akt, and total Akt (Cell Signaling Technology, Beverly, MA), ACC (Upstate Biotechnology, Lake Placid, NY), pTyr1162/1163 β-subunit of the insulin receptor (βIR) (Biosource, Camarillo, CA), β-subunit of the insulin receptor (βIR) (Santa Cruz Biotechnology, Santa Cruz, CA), pTyr612 insulin receptor substrate 1 (IRS-1) (Biosource), or IRS-1 (Santa Cruz Biotechnology). Quantitation of immunolabeled bands was performed using a VersaDoc Imaging System (Bio-Rad).

Enzyme activity measurements. Powdered quadriceps muscle samples were homogenized 1:20 (wt/vol) in 50 mmol/l Tris-HCl, 1 mmol/l EDTA, and 0.1% Triton X-100, pH 7.2, using a Polytron instrument (Kinematica, Littau-Lucerne, Switzerland) and were subjected to three freeze-thaw cycles. Enzyme activities were determined at 30°C using a Spectra Max 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). All assays were conducted in duplicate, and reaction rates were linear for ≥2 min. Cytochrome *c* oxidase activity was measured in a reaction mixture containing 100 mmol/l KH_2PO_4/K_2HPO_4 and 0.1 mmol/l cytochrome *c* reduced with sodium hydrosulfite ($Na_2S_2O_4$), pH 7.0. The rate of change in absorbance was monitored at 550 nm ($\epsilon = 19.1\ \mu mol \cdot ml^{-1} \cdot cm^{-1}$). Citrate synthase activity was measured in a reaction mixture containing 100 mmol/l Tris-HCl, 1 mmol/l $MgCl_2$, 1 mmol/l EDTA, 0.2 mmol/l dithio-bis(2-nitrobenzoic acid), 0.3 mmol/l acetyl CoA, and 0.5 mmol/l oxaloacetate (omitted for control), pH 8.2. The rate of change in absorbance was monitored at 412 nm ($\epsilon = 13.6\ \mu mol \cdot ml^{-1} \cdot cm^{-1}$). For β-hydroxyacyl CoA dehydrogenase (HAD), the reaction mixture contained 50 mmol/l imidazole, 0.15 mmol/l NADH, and 0.1 mmol/l acetoacetyl CoA (omitted for control), pH 7.4. βHAD activity was measured at 340 nm by following the disappearance of NADH ($\epsilon = 6.22\ \mu mol \cdot ml^{-1} \cdot cm^{-1}$).

Statistical analyses. Data are presented as means ± SE, and statistical analyses were performed using an unpaired Student's *t* test. Differences at $P < 0.05$ were considered to be statistically significant.

RESULTS

High-fat–fed c-Cbl–deficient mice are leaner than wild-type animals despite hyperphagia. There was no significant difference in body weight gain during the 4-week feeding period between *c-Cbl*$^{-/-}$ and wild-type male (Fig. 1*A*) and female (Fig. 1*B*) animals. Despite similar weight gain in both groups, the *c-Cbl*$^{-/-}$ mice displayed a 66% increase in food intake in male mice and a 30% increase in female mice (Fig. 1*C*). Consistent with our previous studies in chow-fed animals, the male *c-Cbl*$^{-/-}$ mice on high-fat diet had significantly less adiposity compared with wild-type animals on the high-fat diet (Fig. 1*D*; Table 1). This reduction in fat mass was not restricted to white adipose depots, because brown adipose tissue mass in the intrascapular region was also 40% smaller in *c-Cbl*$^{-/-}$ mice on the high-fat diet (Table 1). A similar difference in white (0.65 ± 0.1, $n = 7$ vs. 1.2 ± 0.1 g, $n = 8$, $P < 0.005$; Fig. 1*D*) and brown (50 ± 4, $n = 7$ vs. 95 ± 5 mg, $n = 8$, $P < 0.001$, respectively) fat mass was observed in female *c-Cbl*$^{-/-}$ mice compared with wild-type mice. The reduction in adiposity exhibited by *c-Cbl*$^{-/-}$ mice was accompanied by a 30% reduction in adipocyte size compared with wild-type animals (Fig. 1*E*). When we examined the adipocyte size distribution, we observed a similar proportion of small cells in both mice strains (Fig. 1*F*). However, wild-type animals had a higher percentage of large (>120 μm in diameter) adipocytes than *c-Cbl*$^{-/-}$ mice (20 and 7%, respectively). Despite the fact that these large-diameter cells represent only 20% of the total number of cells in the population in wild-type animals in regard to cell volume, they actually comprise ~50% of the entire population. In *c-Cbl*$^{-/-}$ mice, the larger diameter cells accounted for only 20% of the total adipose volume.

In agreement with lower adiposity and increased food intake, male *c-Cbl*$^{-/-}$ mice exhibited a 75% reduction in circulating leptin levels compared with wild-type animals (Table 1). However, there was no significant difference in

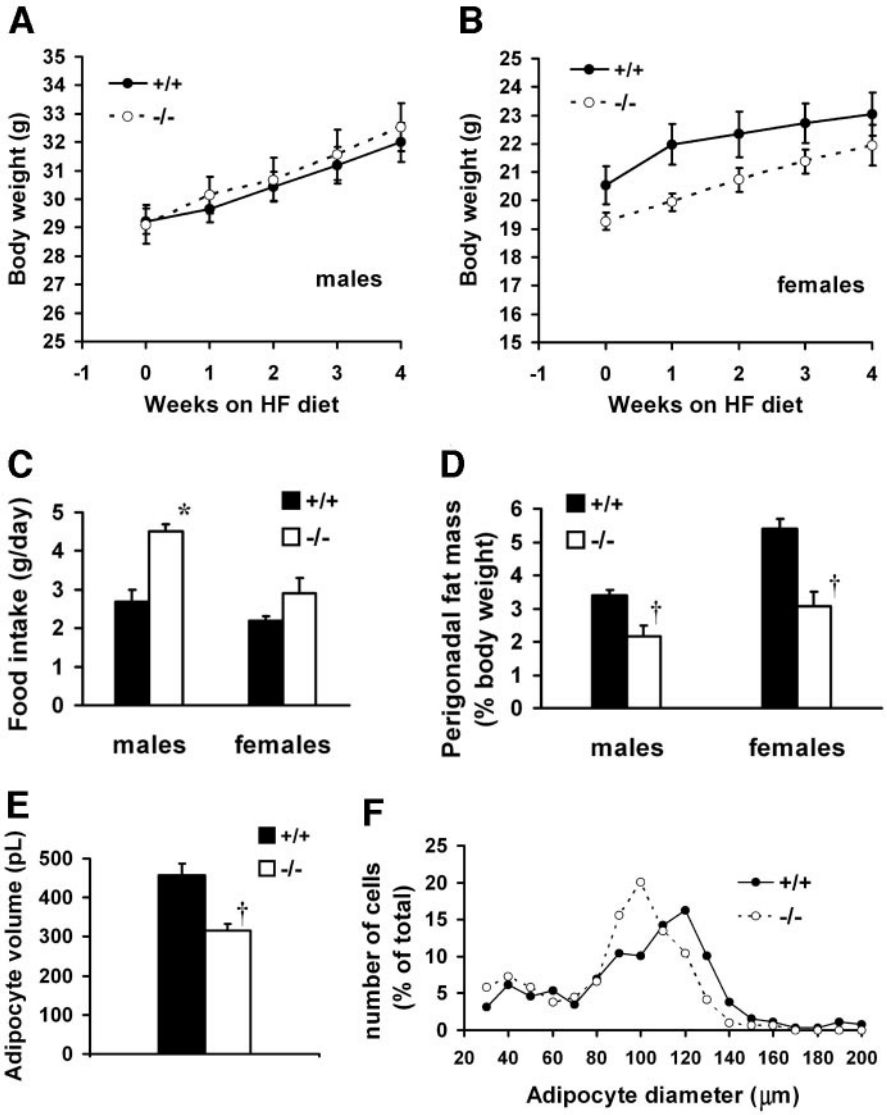

FIG. 1. Effect of high-fat diet on body weight, food intake, and adiposity on age-matched wild-type (+/+, ● and ■) and c-Cbl–deficient (−/−, ○ and □) mice. Graphs show the body weight during 4 weeks feeding with high-fat diet in male (*A*) and female (*B*) mice. Food intake (*C*) and male epididymal and female ovarian fat content (*D*) of mice, expressed as percent of total body weight, after 4 weeks of feeding with high-fat diet. Average adipocyte volume (*E*) and representative adipocyte size distribution (*F*) of cells isolated from epididymal fat pads. Data represent the means ± SE of 6–10 animals per group, except *E* and *F*, in which we examined 100–200 isolated adipocytes from two animals per group. **P* < 0.05; †*P* < 0.01.

plasma adiponectin levels, another cytokine secreted by fat cells, between wild-type and *c-Cbl*$^{-/-}$ mice (Table 1). A similar reduction in plasma leptin levels was observed in female *c-Cbl*$^{-/-}$ mice compared with wild-type mice (17.8 ± 2.3 vs. 4.5 ± 1.1 ng/ml, $n = 7$ for each group, $P < 0.001$, wild-type vs. *c-Cbl*$^{-/-}$ mice, respectively). In view of the similar phenotype observed in male and female *c-Cbl*$^{-/-}$ mice compared with wild-type mice, we have tended to focus the remainder of our studies on male animals.

High-fat–fed c-Cbl–deficient mice exhibit improved glucose tolerance compared with wild-type animals fed a high-fat diet. High-fat–fed wild-type and *c-Cbl*$^{-/-}$ mice exhibited similar fasting glucose levels (Table 1). However, *c-Cbl*$^{-/-}$ mice displayed significantly lower (2.5-fold) fasting circulating insulin levels compared with wild-type animals (Table 1). We performed a glucose tolerance test as an index of whole-body insulin action in these animals. Consistent with previous studies, high-fat feeding caused significantly impaired glucose clearance in wild-type animals (Fig. 2*A*). Strikingly, the *c-Cbl*$^{-/-}$ mice fed a high-fat diet displayed a significantly better glucose tolerance compared with high-fat–fed wild-type animals (Fig.

TABLE 1
Summary of parameters of high-fat–fed wild-type and *c-Cbl*$^{-/-}$ mice

	c-Cbl$^{+/+}$	*c-Cbl*$^{-/-}$
Body weight (g)	31.4 ± 0.3	31.7 ± 0.9
Epididymal white fat mass (g)	1.1 ± 0.1	0.7 ± 0.1*
Brown fat mass (mg)	147 ± 13	92 ± 10†
Plasma parameters		
Fasting glucose (mmol/l)	6.3 ± 0.3	7.8 ± 1.2
Fasting insulin (pmol/l)	224 ± 46	89.2 ± 9.3*
Fasting leptin (ng/ml)	16.3 ± 2	4.1 ± 1.2†
Fasting adiponectin (μg/ml)	1.6 ± 0.2	1.3 ± 0.3
Indirect calorimetry studies		
V_{O_2} (ml · g^{-1} · h^{-1})		
Light phase	1.68 ± 0.05	2.08 ± 0.07‡
Dark phase	1.85 ± 0.07	2.40 ± 0.05‡
Ambulatory activity (*n* events)		
Light phase	1,042 ± 127	2,971 ± 459†
Dark phase	1,366 ± 101	4,543 ± 64†

Data are means ± SE (n = 6–12 male mice/group). **P* < 0.05; †*P* < 0.01; ‡*P* < 0.001.

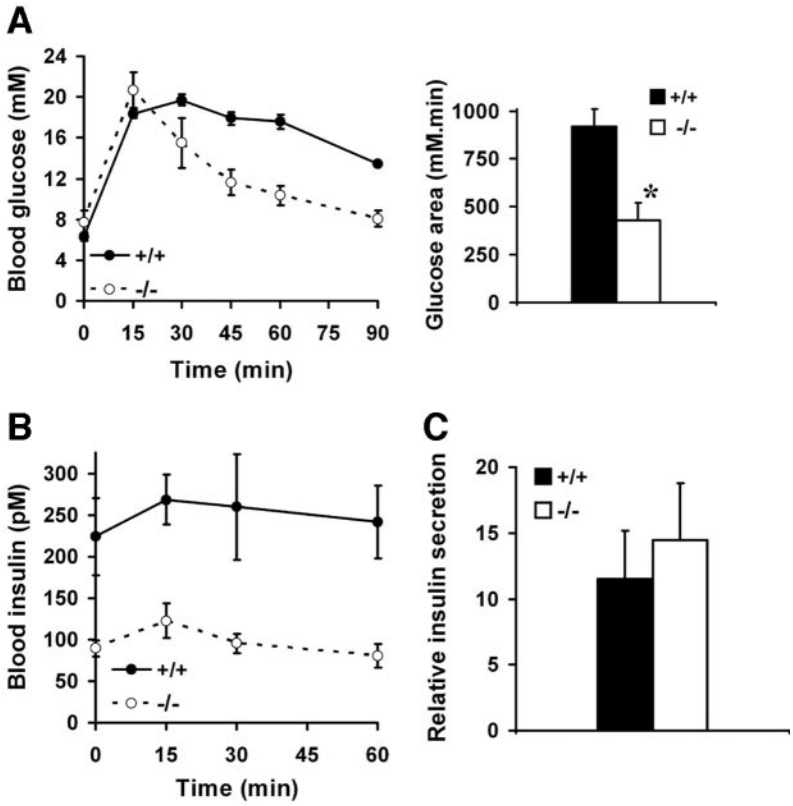

FIG. 2. Glucose metabolism in fat-fed wild-type (+/+, ● and ■) and c-Cbl–deficient (−/−, ○ and □) mice in vivo. *A*: Glucose tolerance test (2 g/kg) in age-matched, high-fat–fed mice. Histogram on the *right* shows the area under the curve. *B*: Circulating insulin levels during the glucose tolerance test. Data represent the means ± SE of five *c-Cbl*$^{-/-}$ and six wild-type animals. **P* < 0.01. *C*: Insulin secretion from pancreatic islets isolated from wild-type and *c-Cbl*$^{-/-}$ mice. Data are expressed as insulin detected in the media in the presence of 16.7 mmol/l glucose relative to insulin detected in the presence of 2.8 mmol/l glucose. Data represent the means ± SE of four mice per group.

2*A*). In addition, the improved glucose tolerance in the high-fat–fed *c-Cbl*$^{-/-}$ mice was observed in the face of a significantly lower plasma insulin concentration compared with wild-type mice (Fig. 2*B*; Table 1). To determine whether the lower insulin levels detected in *c-Cbl*$^{-/-}$ mice were due to a defect in insulin secretion, we performed insulin secretion assays using pancreatic islets isolated from both wild-type and *c-Cbl*$^{-/-}$ mice. Islets isolated from high-fat–fed wild-type and *c-Cbl*$^{-/-}$ mice exhibited a similar insulin secretion response when incubated with a glucose concentration similar to that detected during the glucose tolerance test (Fig. 2*C*). This demonstrated that the lower circulating insulin level in *c-Cbl*$^{-/-}$ mice was not the result of any defect in glucose-stimulated insulin release from islets.

Elevated skeletal muscle glucose clearance in c-Cbl–deficient mice. The increased glucose tolerance in the face of reduced insulin levels observed in high-fat–fed *c-Cbl*$^{-/-}$ mice suggests that these mice have enhanced insulin action in peripheral tissues. To determine the contribution of different tissues to the improved glucose clearance observed in *c-Cbl*$^{-/-}$ animals, we examined the clearance of [^{3}H]2-DOG administered with the glucose load during the glucose tolerance test. As shown in Fig. 3*A*, total [^{3}H]2-DOG uptake was higher in skeletal muscle (2.5-fold) and heart (4.5-fold) from high-fat–fed *c-Cbl*$^{-/-}$ mice compared with high-fat–fed wild-type animals. However, no significant difference in total [^{3}H]2-DOG clearance into white or brown adipose depots was observed between *c-Cbl*$^{-/-}$ and wild-type mice. We also co-administered [^{14}C]glucose with the glucose load during the glucose tolerance test to determine the fate of glucose in different tissues. We first examined [^{14}C]glucose incorporation into glycogen in muscle and liver. [^{14}C]glucose incorporation into muscle glycogen was 2.5-fold higher in *c-Cbl*$^{-/-}$ mice fed with the high-fat diet compared with wild-type animals (Fig. 3*B*, *left*). In contrast to the situation in muscle, [^{14}C]glucose clearance into glycogen in liver from *c-Cbl*$^{-/-}$ animals was reduced by 75% compared with wild-type mice (Fig. 3*B*, *right*), probably because a larger proportion of the glucose load was cleared by muscle in high-fat–fed *c-Cbl*$^{-/-}$ mice. We also observed a reduction in triacylglyceride stores in liver from high-fat–fed *c-Cbl*$^{-/-}$ mice compared with high-fat–fed wild-type mice (14.4 ± 1.7, $n = 5$ vs. 32.7 ± 3.5 μmol triacylglyceride/g tissue, $n = 6$, respectively, $P < 0.002$). Although we also observed lower triacylglyceride content in skeletal muscle from high-fat–fed *c-Cbl*$^{-/-}$ compared with high-fat–fed wild-type animals, this difference was not statistically significant (19.5 ± 2.5, $n = 13$ vs. 24.3 ± 2.1, $n = 14$, respectively, $P = 0.087$). In addition, circulating nonesterified fatty acids levels were not significantly different in high-fat–fed wild-type and *Cbl*$^{-/-}$ animals (0.6 ± 0.08 vs. 0.63 ± 0.04 mmol/l ($n = 16$), respectively).

We next investigated whether the higher glucose clearance into muscle of high-fat–fed *c-Cbl*$^{-/-}$ mice compared with high-fat wild-type mice was the result of differences in insulin signaling in this tissue. Intravenous injection of 1 unit/kg insulin elicited >10-fold increase in the phosphorylation of insulin receptor, IRS-1, and Akt in quadriceps muscle from high-fat wild-type mice (Fig. 4). The insulin-stimulated tyrosine phosphorylation of IRS-1 was 60% higher in muscle from high-fat–fed *c-Cbl*$^{-/-}$ mice compared with wild-type animals ($P < 0.05$, $n = 12$ for each group). We observed no significant difference in the phosphorylation of either insulin receptor ($P = 0.24$, $n = 12$ for each group) or Akt ($P = 0.13$, $n = 12$ for each group) between high-fat–fed wild-type and *c-Cbl*$^{-/-}$ mice.

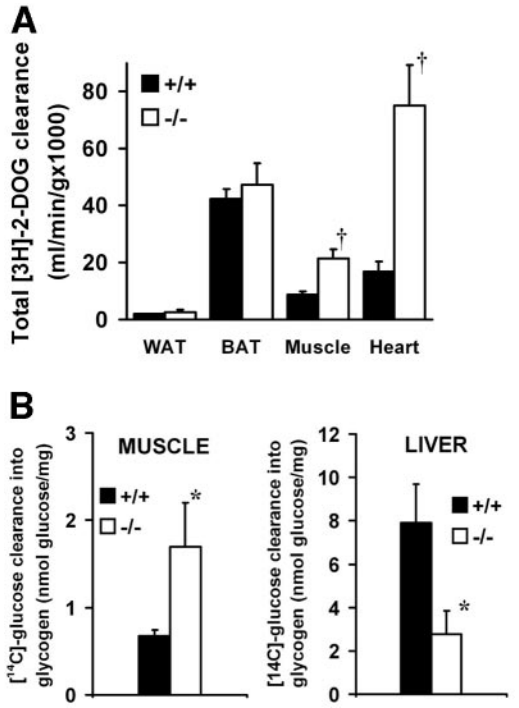

FIG. 3. *A*: Clearance of the glucose analog [^{3}H]2-DOG into glucose-6-phospate in white adipose tissue (WAT), brown adipose tissue (BAT), skeletal muscle, and heart during a glucose tolerance test. *B*: Clearance of [^{14}C]glucose into glycogen in muscle (*left*) and liver (*right*) during a glucose tolerance test. Data represent the means ± SE of six wild-type (■) and five *c-Cbl*$^{-/-}$ (□) mice. **P* < 0.05; †*P* < 0.01.

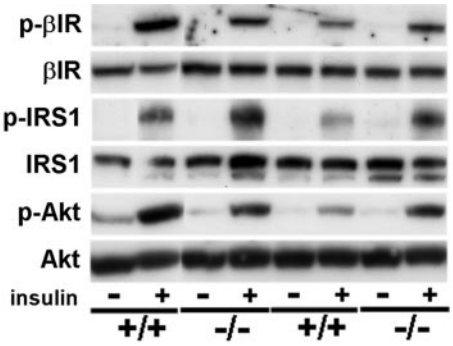

FIG. 4. Insulin signaling studies in skeletal muscle from fat-fed mice. Quadriceps muscles from wild-type (+/+) and *c-Cbl*$^{-/-}$ (−/−) mice, were removed before (−insulin) and 3 min after (+insulin) an intravenous insulin injection. Tissue homogenates (15 μg) were immunoblotted with antibodies specific for the insulin receptor (βIR) and pTyr1162/1,163-βIR (p-βIR), IRS-1 and pTyr612-IRS-1 (*p*-IRS-1), or Akt and pSer473-Akt (pAkt). Two representative animals per group are shown. Relative insulin-stimulated IRS-1 phosphorylation (calculated as phospho/total amount) was significantly higher in *c-Cbl*$^{-/-}$ mice compared with wild-type animals (*P* < 0.05, *n* = 12 for each group). No significant difference was observed in the relative phosphorylation of either insulin receptor or Akt between both groups.

High-fat–fed *c-Cbl*$^{-/-}$ mice exhibit increased ambulatory activity and Vo_2. We have shown above that *c-Cbl*$^{-/-}$ mice fed high-fat diet are lean and hyperphagic compared with high-fat–fed wild-type animals (Fig. 1). These results suggest that *c-Cbl*$^{-/-}$ mice fed with a fat-rich diet exhibit higher energy expenditure than wild-type animals. To test this hypothesis, we examined the whole-body Vo_2 rate of these animals for 24 h by indirect calorimetry. Wild-type and *c-Cbl*$^{-/-}$ mice exhibited a typical circadian variation in Vo_2 with higher consumption rates during the dark phase (Fig. 5*A*), coinciding with the greater feeding and activity of these animals at night. *c-Cbl*$^{-/-}$ mice exhibited higher overall Vo_2 (27%) compared with wild-type animals (2.23 ± 0.06, $n = 7$ vs. 1.76 ± 0.06 ml · g^{-1} · h^{-1}, $n = 8$, $P < 0.0001$, respectively). This phenotype was observed at all times of the day (Fig. 5*A*), although the difference in Vo_2 between wild-type and *c-Cbl*$^{-/-}$ mice was slightly greater during the dark phase of the cycle (Table 1). We also observed higher ambulatory activity in high-fat–fed *c-Cbl*$^{-/-}$ mice (threefold) compared with wild-type mice (Fig. 5*B*). This was observed during both the light and dark phases of the cycle (Table 1).

***c-Cbl*$^{-/-}$ mice fed with high-fat diet exhibit greater mitochondrial oxidative capacity in skeletal muscle.** To further investigate the higher energy expenditure phenotype observed in *c-Cbl*$^{-/-}$ mice, we examined the activity of key enzymes indicative of the mitochondrial oxidative capacity in muscle homogenates from fat-fed wild-type and *c-Cbl*$^{-/-}$ mice. Both cytochrome *c* oxidase and citrate synthase activities were ~40% higher in quadriceps from *c-Cbl*$^{-/-}$ mice compared with wild-type animals (Fig. 6*A* and *B*). The activity of βHAD, a key enzyme of fatty acid oxidation, was also higher (85%) in muscle from *c-Cbl*$^{-/-}$ mice (Fig. 6*C*). Phosphorylation of ACC inhibits its activity (19), reducing intracellular malonyl CoA levels and allowing free fatty acids to enter the mitochondria through the carnitine-acyl transferase shuttle (20). ACC phosphorylation was increased by 2.5-fold in quadriceps muscle from fat-fed *c-Cbl*$^{-/-}$ mice compared with wild-type animals, whereas there was no change in the total amount of the ACC enzyme (Fig. 6*D*).

DISCUSSION

In this study, we have shown that mice lacking expression of the E3 ubiquitin ligase c-Cbl are protected against the development of insulin resistance and increased adiposity that normally accompanies consumption of a high-fat diet. Fat-fed *c-Cbl*$^{-/-}$ mice had significantly less adipose tissue, lower liver triglycerides, and increased glucose disposal compared with wild-type mice fed the same diet despite the fact that the knockout mice had 50% higher food intake during the 4-week feeding period. The improved glucose tolerance in the *c-Cbl*$^{-/-}$ mice was accompanied by increased insulin action and signal transduction in skeletal muscle. The *c-Cbl*$^{-/-}$ mice also maintained higher energy expenditure and greater ambulatory activity than wild-type animals. Furthermore, there was a significant increase in the activity of key enzymes of fatty acid oxidation as well as the phosphorylation of ACC in skeletal muscle in *c-Cbl*$^{-/-}$ mice.

We propose that enhanced whole-body energy expenditure is the major cause of the reduced fat stores in *c-Cbl*$^{-/-}$ mice. As a consequence of the decreased adiposity, *c-Cbl*$^{-/-}$ mice are protected against the deleterious effects of fat oversupply on insulin action and signal transduction that has previously been observed in both humans and rodents (14,15,21,22). Similar to *c-Cbl*$^{-/-}$ animals, mice deficient in other negative regulatory proteins of growth factor action, like PTP1b and SHIP2, exhibit a phenotype of improved insulin action and leanness (8,23,24). However, because these animal models have reduced adipose tissue as well as enhanced insulin action, it is difficult to dissect the relationship between changes in insulin action and energy expendi-

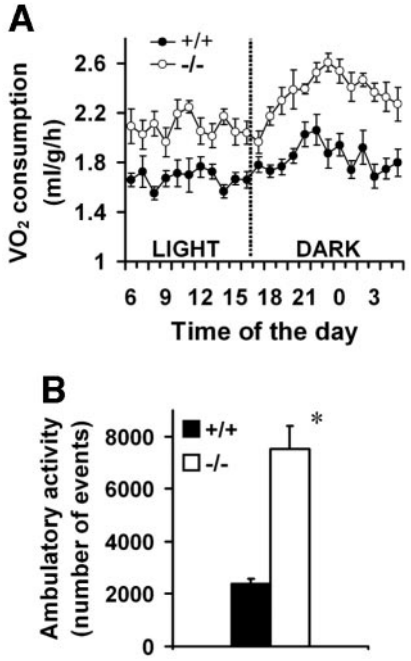

FIG. 5. Respirometry studies in mice fed with high-fat–fed diet. *A*: 24-h Vo_2 rate; *B*: ambulatory activity. Data represent the means ± SE of eight wild-type (● and ■) and seven *c-Cbl*$^{-/-}$ (○ and □) mice. **P* < 0.001. LIGHT and DARK correspond to the light phase (0600 to 1800) and the dark phase (1800 to 0600) of the cycle, respectively.

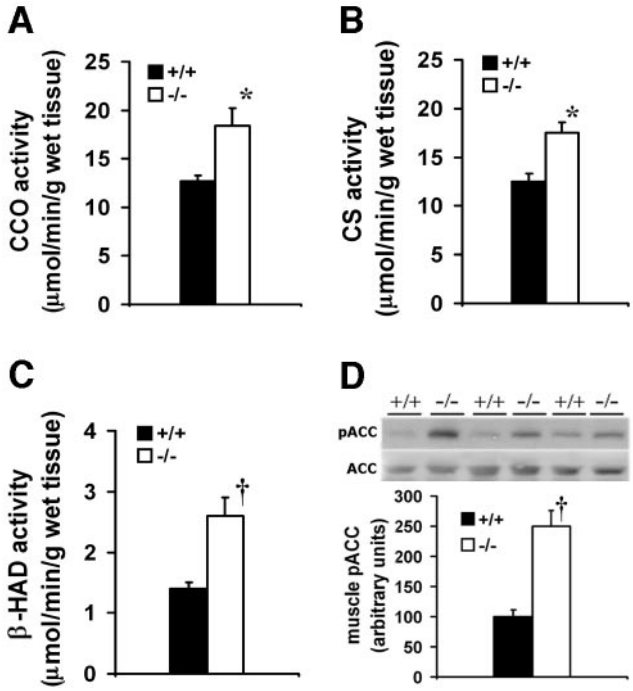

FIG. 6. Fuel oxidation studies in skeletal muscle from fat-fed mice. Cytochrome *c* oxidase (CCO) (*A*), citrate synthase (CS) (*B*), and βHAD (*C*) enzymatic activities were assayed in homogenates from quadriceps muscles from wild-type (+/+) and *c-Cbl*$^{-/-}$ (−/−) mice as described in RESEARCH DESIGN AND METHODS. *D*: Histograms show means ± SE of seven to eight animals. Quadriceps muscle homogenates (15 μg total protein) were immunoblotted with antibodies specific for either ACC or pSer79-ACC (pACC). *Top panel*: Muscle lysates from three different wild-type (+/+, ■) and *c-Cbl*$^{-/-}$ (−/−, □) mice. Histogram shows the means ± SE. ACC phosphorylation of six animals in each group. **P* < 0.01; †*P* < 0.001.

ture. On the one hand, it is well established that increased energy expenditure leading to reduced fat accumulation results in improved insulin action (25–27). On the other hand, a mechanism whereby improved insulin action leads to increased energy expenditure and less lipid accumulation, although not excluded, is more difficult to rationalize. Consistent with this, several mouse models, like APS- or Grb14-deficient mice or mice expressing reduced levels of the regulatory subunits of phosphatidylinositol 3-kinase, exhibit improved insulin action and/or signal transduction without any concomitant change in adipose mass (17,28,29). Data reported here also support the concept that increased insulin action is unlikely to be upstream of increased energy expenditure because the fat-fed *c-Cbl*$^{-/-}$ mice in the current study had similar glucose tolerance, insulin action, and fat content to chow-fed, wild-type animals (13) while continuing to consume considerably more calories to support their increased energy expenditure. Therefore, we suggest that it is the decreased lipid accumulation as a result of the increased energy expenditure that is the major contributor to improved insulin action in fat-fed *c-Cbl*$^{-/-}$ animals.

A key question arising from these studies is what causes the increased energy expenditure in the *c-Cbl*$^{-/-}$ mice? Brown adipose tissue and skeletal muscle have previously been described to be major regulators of whole-body energy expenditure in rodents (30). We have observed no major changes in brown adipose tissue mass or morphology that would be consistent with changes in thermogenesis in this tissue (13). On the other hand, we have observed changes in a number of parameters, implicating a major role for skeletal muscle in increased energy expenditure in *c-Cbl*$^{-/-}$ mice. We have previously described that muscle mitochondrial diameter is increased in *c-Cbl*$^{-/-}$ mice (13), and in the present study, we show that the activity of citrate synthase, cytochrome *c* oxidase and βHAD, key regulatory enzymes involved in muscle mitochondrial energy homeostasis, are significantly increased in high-fat–fed *c-Cbl*$^{-/-}$ mice (Fig. 6). The magnitude of the changes observed in the activity of these enzymes is comparable with that previously described in skeletal muscle from chronically exercise-trained animals (31,32). In addition, muscle from both chow-fed and fat-fed *c-Cbl*$^{-/-}$ mice exhibited a substantial increase in ACC phosphorylation (Fig. 6) (13). Collectively, these data are consistent with a model whereby both nutrient flux into muscle mitochondria and muscle mitochondrial oxidative capacity are upregulated in *c-Cbl*$^{-/-}$ mice, contributing to a major role for this tissue in facilitating the increased energy expenditure observed in these animals. This model is consistent with studies in other animal models such as the ACC2 knockout mouse, which, like *c-Cbl*$^{-/-}$ mice, display a lean phenotype despite being hyperphagic and are also protected against high-fat diet–induced insulin resistance (6,33).

We cannot exclude a role for adipose or other tissues in contributing to the changes in muscle mitochondrial energetics in *c-Cbl*$^{-/-}$ mice. A number of adipocyte-specific secretory factors have been shown to influence muscle metabolism either acutely or chronically (34). Furthermore, adipose-specific deletion of GLUT4 resulted in impaired insulin action in skeletal muscle (35). The brain has also been shown to play a major role in regulating whole-body fuel metabolism. Manipulations that modify the levels of malonyl CoA or fatty acid oxidation in the

hypothalamus have been shown to lead to alterations in food intake and body weight in rodents (36–38). However, in these studies, manipulating metabolic parameters in the brain was shown to have a significant effect on fuel metabolism in the liver but not in skeletal muscle (36,39). This emphasizes the need to study tissue-specific c-Cbl knockout mice to pinpoint the major tissue(s) responsible for the increased energy expenditure.

Based on the present studies, we suggest that c-Cbl may play an important role as part of the energy homeostat in mammals. Like PTP1b, c-Cbl may act as a clamp on energy expenditure, and therefore, regulating c-Cbl expression or c-Cbl activity may be a way of therapeutically regulating energy expenditure. Further studies will be required to map which function of the c-Cbl protein is responsible for the lean phenotype observed in *c-Cbl*$^{-/-}$ mice. For example, if the ubiquitin ligase activity located in the RING finger domain of c-Cbl is essential for this phenotype, this would suggest that c-Cbl may control the expression of a protein or proteins that in turn regulate energy expenditure. Whatever the mechanism(s) involved, the current study clearly demonstrates that the increased energy expenditure observed in *c-Cbl*$^{-/-}$ mice is preserved when the mice are challenged with a high-fat diet and that lack of c-Cbl protects the mice from the development of obesity and insulin resistance.

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