Insulin Resistance and Lipodystrophy in Mice Lacking Ribosomal S6 Kinase 2

Karim El-Haschimi,[1,2] Scott D. Dufresne,[3] Michael F. Hirshman,[3] Jeffrey S. Flier,[1] Laurie J. Goodyear,[3] and Christian Bjørbæk[1]

The p90 ribosomal S6 kinase 2 (RSK2) is a serine/threonine kinase with high expression levels in adipose tissue. Numerous in vitro studies show that RSK2 is activated by a broad number of cellular stimuli and suggest that RSK2 is involved in the regulation of a variety of cellular processes. However, the physiological role of RSK2 still remains elusive. We therefore generated *rsk2* knockout (KO) mice to better understand the function of RSK2 in vivo. Birth weights of RSK2 KO mice are normal, but the body weight is reduced with age, as compared with wild-type littermates. We found that the difference in body weight was largely caused by a specific loss of white adipose tissue that is accompanied by reduced serum levels of the adipocyte-derived peptide, leptin. KO mice also have impaired glucose tolerance and elevated fasting insulin and glucose levels that are restored following administration of low amounts of leptin, which do not affect food intake. We conclude that RSK2 plays a novel and an important role in regulation of adipose mass in mice and speculate that the reduction in fat tissue may negatively affect insulin sensitivity, as observed in human lipodystrophy, through reduced levels of adipocyte-derived factors, such as leptin. *Diabetes* 52:1340–1346, 2003

RSK2 is a member of a family of growth factor–regulated serine-threonine kinases known as p90rsk (90-kDa ribosomal S6 kinase) or as mitogen-activated protein (MAP) kinase-activated protein kinase 1 (MAPKAP-K1b) (1–6). Several mammalian isoforms of RSK (RSK1–4) have been identified in different species (7–10). In addition, novel proteins that are homologous to RSK have recently been discovered, including mitogen- and stress-activated protein kinase (MSK) and RSK-B (1,11,12). RSK enzymes are activated in response to a variety of cellular stimuli, growth factors, and hormones, including epidermal growth factor, insulin, phorbol-esters, heat shock, and ionizing radiation (1,13–17). The activation is complex and requires phosphorylation at multiple sites by different upstream kinases, including the p42 and p44 MAP kinases, and the 3-phosphoinositide-dependent protein kinase 1 (18,19). RSK proteins contain two distinct kinase domains that set them apart from most other protein kinases (3). After phosphorylation by the upstream kinases, the COOH-terminal kinase domain of RSK autophosphorylates additional sites in the RSK molecule, ultimately resulting in activation of the NH_2-terminal kinase domain, which is responsible for substrate phosphorylation (20–24). RSK enzymes have broad substrate specificity (25), and putative substrates include nuclear proteins and transcription factors such as cAMP-responsive binding-element protein (26,27), lamin-C (28), histone 3B (29), c-*fos* (26), glycogen synthase kinase 3 (30), BAD (31), and IκB/NFκB (32). Based on these findings, RSK proteins are likely to regulate a variety of cellular processes. This fact, combined with their wide but distinct tissue distribution (33), suggests that RSK proteins are involved in the regulation of critical and different functions in the organism. Despite extensive studies during more than a decade, the physiological roles of specific RSK proteins remain elusive.

Recently, it was discovered that mutations in the human *rsk2* gene cause a rare form of the X-linked mental retardation syndrome known as the Coffin-Lowry syndrome (CLS) (34,35). Patients with CLS are characterized by psychomotor retardation, progressive skeletal deformations, and short stature (36–38). The mechanism by which loss of RSK2 function leads to the development of CLS is yet unknown, although recent data suggest that RSK2 is prominently expressed in brain regions essential for cognitive function and learning (33). To better understand the function on RSK2 in vivo, we recently generated mice with targeted disruption of the *rsk2* gene (39) and we described impaired learning capabilities and reduced body length, demonstrating that this mouse can be used as a model for human CLS.

We now report the unexpected observation that RSK2-deficient mice have a specific, but partial, loss of adipose tissue, demonstrating a novel role of RSK2 in body weight regulation. The mice are also resistant to weight gain when fed a high-fat diet (HFD). Furthermore, the RSK2-deficient mice exhibit insulin resistance and tendency toward fatty livers, which are characteristic features of human lipodystrophy. Treatment of the RSK2 knockout (KO) mice with low levels of leptin, which do not affect food intake, resulted in a complete normalization of circulating glucose and insulin levels. We conclude that that the RSK2 KO mouse is a novel animal model of lipoatrophic diabetes, and our data support the notion that leptin can regulate

From the [1]Beth Israel Deaconess Medical Center, Boston, Massachusetts; the [2]University Hospital Magdeburg, Magdeburg, Germany; and the [3]Joslin Diabetes Center, Boston, Massachusetts.

Address correspondence and reprint requests to Christian Bjørbæk, Beth Israel Deaconess Medical Center, 330 Brookline Ave., RN 325, Boston, MA 02215. E-mail: cbjorbae@bidmc.harvard.edu.

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CLS, Coffin-Lowry syndrome; HFD, high-fat diet; MAP, mitogen-activated protein; RSK2, ribosomal S6 kinase 2; WAT, white adipose tissue.

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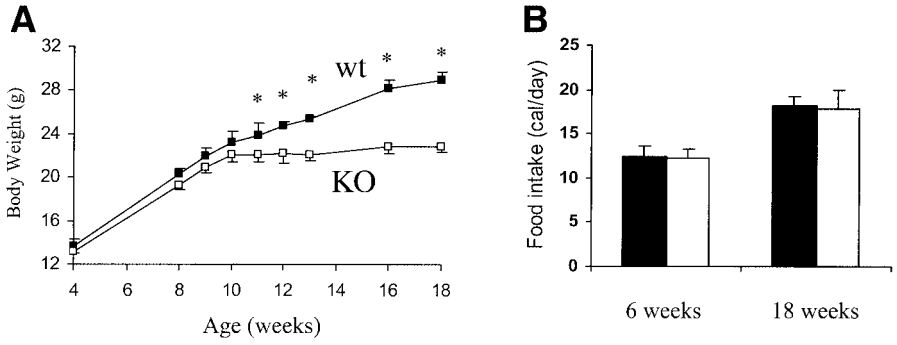

FIG. 1. RSK2 KO mice have reduced body weight with age but normal food intake. *A*: Growth curves on chow diet. Mice were housed individually at the age of 4 weeks, and body weight was measured weekly. ■, wild-type (wt); □, KO. *B*: Food intake on chow diet. Average daily intake was measured over a period of 2 days, at 6 and 20 weeks of age. ■, wild-type animals; □, KO animals. Data are means ± SE. Significance: *$P < 0.01$. Groups of eight animals were studied. These parameters were measured at least three independent times.

glucose disposal and insulin sensitivity independent of the effect on food intake.

RESEARCH DESIGN AND METHODS

Animals. Mice lacking functional *rsk2* genes were generated as described earlier (39). The mice were backcrossed onto the C57BL6 background for more than six generations. All mice used in this study were male offspring from mating between heterozygous females and wild-type males. Because the *rsk2* gene is located on the X chromosome, as in the case of the human *rsk2* gene (24), the genotypes of male offspring were either wild-type (+/Y) or KO (−/Y). The animals and procedures used were in accordance with the guidelines and approval of the Harvard Medical School and Beth Israel Deaconess Institutional Animal Care and Use committees. Unless otherwise stated, animals were housed individually with food and water available ad libitum, in a light- and temperature-controlled environment.

High-fat diet treatment. Male RSK2 KO and wild-type littermates were weaned at the age of 3 weeks and were housed individually with continuous access to chow (Purina Rodent Chow #5008: 6.5% calories as fat; Purina Diets, St. Louis, MO) and water. At the age of 4 weeks, the rodent chow diet was replaced by a HFD (Special Diet #D12451: 45% calories as fat; Research Diets, NB, NJ) and body weight and food intake was measured weekly for 10 consecutive weeks. After 10 weeks, the food was removed at the beginning of the dark cycle and the animals were killed in the first 2 h of the following light cycle. Blood was collected for hormone measurement. Serum was immediately isolated and stored at −80°C until assayed. The body composition was assessed by whole carcass analysis as described earlier (40). Briefly, eviscerated carcasses were digested by alcoholic potassium hydroxide hydrolysis at 60°C over 2 days with saponification of all fats, neutralization, and then enzymatic determination of glycerol. Body lipid was determined by multiplying moles of glycerol per mouse with the average relative molecular mass of a triglyceride molecule (860 g/mol).

Glucose tolerance test. Mice were fasted overnight for ~16 h before injections. Glucose (20% wt/vol solution in sterile 0.9% NaCl) was then injected intraperitoneally (1 mg glucose/g body wt). Blood glucose concentrations were measured before and 15, 30, 60, and 120 min after the glucose injection (41).

Measurements of glucose and hormones. Plasma leptin and insulin were determined by radioimmunoassay (mouse leptin and rat insulin kit; Linco Research Institute, St. Louis, MO). Glucose levels were measured using a blood glucose meter (Profile Touch II; LifeScan, Milpitas, CA).

Leptin treatment. Male RSK2 KO mice and wild-type littermates were studied at 20–22 weeks of age and were housed individually with continuous access to food and water. Body weight and food intake were measured every day at 0900. After 10 days of baseline measurement of body weight and food intake, each group of mice was divided in two weight-matched subgroups, and an Alzet micro-osmotic pump (model 1002, mean pumping rate 0.21 μl/h for >14 days; Alza Corporation, Palo Alto, CA) was inserted subcutaneously in the back of each mouse (42). Recombinant mouse leptin (Eli Lilly, Indianapolis, IN) was dissolved in sterile PBS (pH 7.4) (Dulbecco's PBS; Life Technologies) containing 0.1% BSA before loading the micro-osmotic pumps. The pumps delivered 0 or 5 μg recombinant mouse leptin per day in a total volume of 100 μl. After pump insertion, measurement of body weight and food intake was continued. Fourteen days after inserting the pumps, food was removed at the beginning of the dark cycle, and all mice were killed in the first 2 h of the following light cycle.

Statistical analysis. Individual variations were analyzed by a Fisher's protected least significant difference test or a *t* test.

RESULTS

We have earlier described the generation and characterization of RSK2 KO mice (39). In that study, we found evidence of impaired brain function and decreased body length, consistent with the mental retardation and reduced growth characteristics of humans with CLS who lack functional RSK2 proteins (34). This suggests that the RSK2 KO mouse may be a useful model for the study of this disease.

During our earlier studies, we noted that the animals lacking RSK2 exhibited a lower body weight than control wild-type littermates. To investigate this more carefully, we first crossed the RSK2 KO mice onto the C57BL6 background for more than six generations. This was done with the purpose to obtain data comparable to published studies of energy homeostasis in mice. Because the *rsk2* gene is located on the X-chromosome (7,10), the genotypes of male offspring of heterozygous females and wild-type males were either wild-type (+/Y) or KO (−/Y). These animals were used for the present studies. During the breeding process, we noted that inter-mating of heterozygous animals, or with wild-type animals, produced litters with expected frequencies of the different genotypes. However, mating with either homozygous males or females yielded no pups, suggesting that RSK2 plays a role in fertility or in embryonic development. The mechanism underlying this phenotype is unknown and was not investigated further but deserves additional studies.

After breeding onto the C57BL6 background, we obtained growth curves of wild-type and RSK2 KO mice when given a regular chow diet (Fig. 1*A*). Although the birth weights of KO mice are normal, body weights are relatively lower later in life compared with wild-type littermates. At 18 weeks of age, KO mice weigh 21% less than controls. The lean phenotype was not caused by less energy intake because measurements of food consumption revealed no differences between the genotypes at 6 or 18 weeks of age (Fig. 1*B*). These results imply a role of RSK2 in bodyweight regulation, which has not been reported earlier.

Closer examination of body composition revealed a marked reduction of white adipose tissue (WAT) in KO mice. As shown in Fig. 2*A* and *B*, 1-year-old KO animals exhibit a marked 50–80% reduction of the size of fat depots, without any changes in the weight of other tested tissues, including brown adipose tissue, heart, testis, or kidneys. The relative loss of WAT becomes more significant with increased age (not shown). Consistent with

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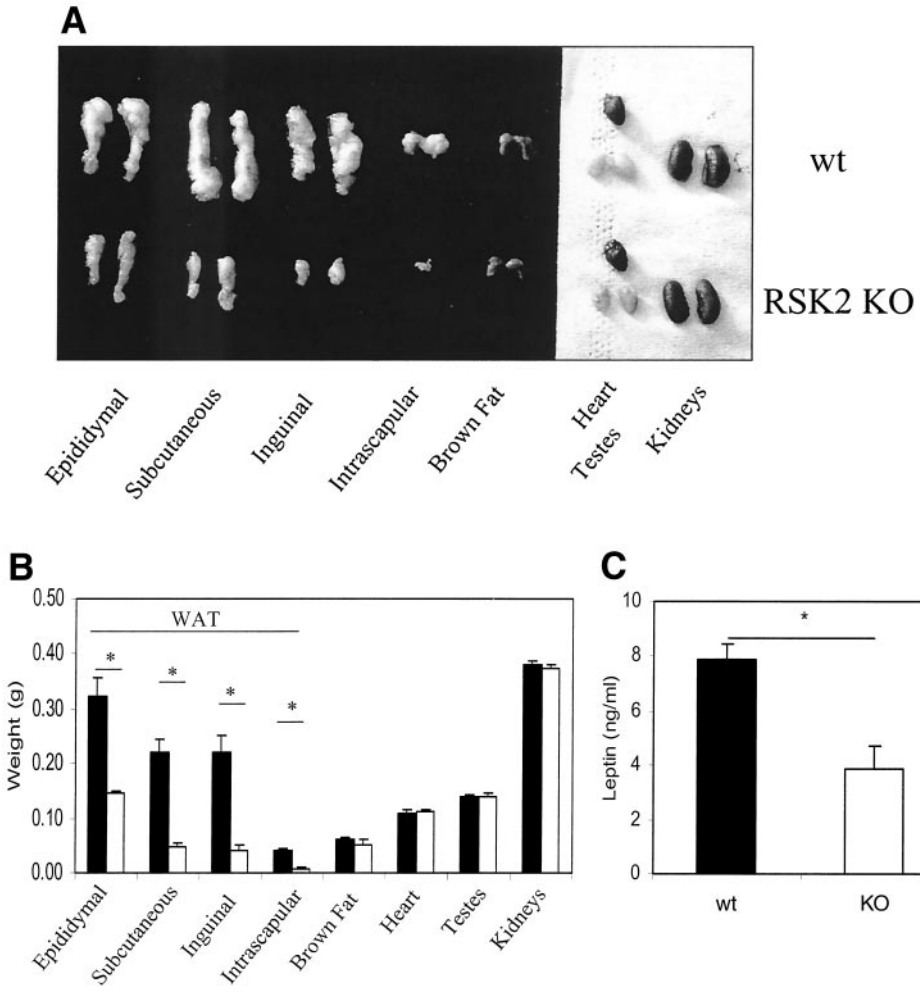

FIG. 2. Reduced adipose tissue size and reduced serum leptin levels in RSK2 KO mice. *A*: Photograph of epididymal, inguinal, subcutaneous, and interscapular white fat pads, as well as other tissues and organs, were dissected from 1-year-old wild-type (wt) and RSK2 KO mice, and those from one representative animal of each genotype are displayed. *B*: Dry weights of the different white fat pads and other tissue from RSK2 KO (n = 3) and wild-type (n = 3) mice. *C*: Serum leptin levels of overnight fasted wild-type (n = 4) and KO (n = 8) mice at the age of 30 weeks. Data are means ± SE. Significance: *P < 0.01. The differences in WAT content were measured three times.

lower fat mass, RSK2 KO mice also had a reduced serum concentration (50%) of the adipocyte-derived hormone leptin at 20 weeks of age (Fig. 2*C*). Other endocrine parameters such as serum corticosterone, thyroid hormones, and testosterone levels were similar in KO and wild-type male mice (not shown). These results show that RSK2 is important for regulation of WAT mass.

Because RSK2 KO mice have reduced body weight and

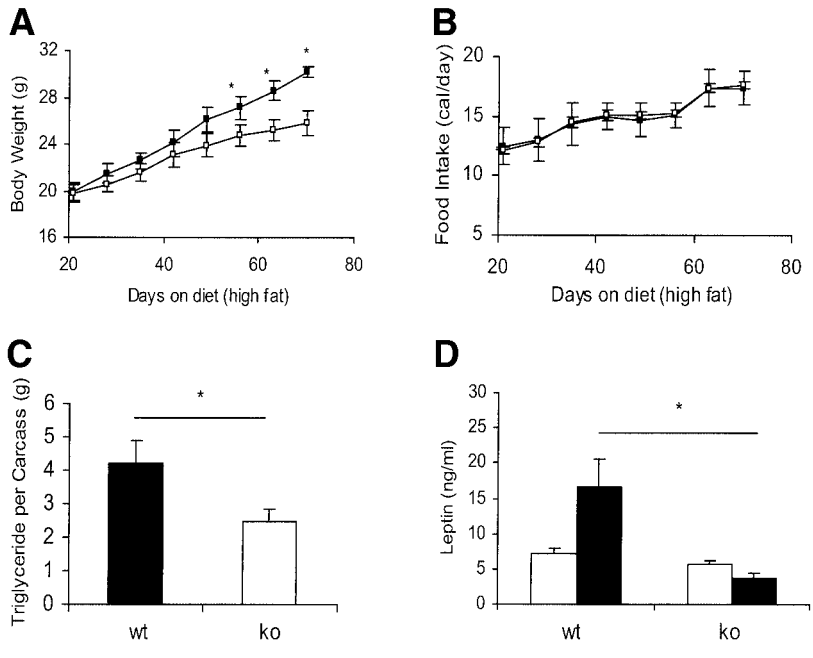

FIG. 3. RSK2 KO mice are resistant to diet-induced obesity. *A*: Effects of a HFD on body weight. ■, wild-type (wt); □, KO. Data are means ± SE. Significance: *P < 0.01. Wild-type (n = 11) and KO mice (n = 13) were given a HFD (45% of the calories as fat) for 10 weeks. Mice were housed individually at the age of 4 weeks and had free access to the diet. Body weight was measured weekly. *B*: Food intake in kilocalories per day measured weekly. *C*: Carcass analysis of triglyceride body content. Significance: *P < 0.05. *D*: Serum leptin levels before (□) and at the end (■) of a HFD. Significance: *P < 0.02. Data are means ± SE

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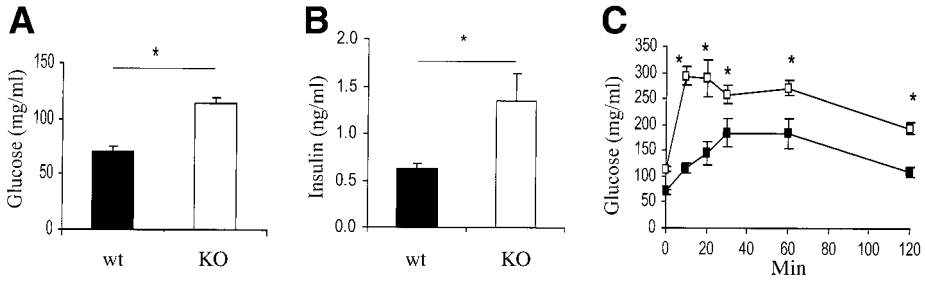

FIG. 4. Insulin resistance and mild diabetes in 10-week-old RSK2 KO mice. *A*: Glucose concentration after overnight fasting of wild-type (wt; $n = 6$) and KO ($n = 6$) mice. Significance: *$P < 0.05$. *B*: Insulin concentrations after overnight fasting. Significance: *$P < 0.001$. *C*: Glucose tolerance test. Significance: *$P < 0.03$. Data are means ± SE. Similar results were obtained from studies of 18-week-old animals.

fat mass when fed a normal chow diet, we next determined whether they would be resistant to weight gain when given a HFD (45% of calories in the form of fat). At 4 weeks of age, wild-type and KO mice were fed the HFD and body weights were measured. The results are shown in Fig. 3*A*. We found that KO mice gained less weight over time than the wild-type littermates. At the end of 10 weeks on the HFD, the KO mice weighed 15% less than their wild-type littermates. Both groups had similar food intake throughout this period, which increased slightly in both groups during the 10 weeks of the study (Fig. 3*B*). At the end of the study, we found a significant difference in the body composition between the groups, with 58% less triglycerides per RSK2 KO carcass (Fig. 3*C*), consistent with a lower fat mass in these animals when fed a chow diet. We also measured fasted serum leptin levels at 4 weeks (before a HFD) and after 10 weeks with the HFD. As expected for the obesity-prone C57BL6 mice (43), leptin levels more than doubled over the course of high-fat feeding. However, serum leptin concentrations were unchanged (or slightly lower) in RSK2 KO animals during the same period, consistent with the relative lower body weight and triglyceride content of these mice (Fig. 3*D*). We conclude that mice lacking RSK2 are resistant to weight gain and fat gain when challenged with a HFD.

Changes in adipose tissue amounts are often associated with alterations in glucose homeostasis in rodents (44) and humans (45). We therefore measured serum glucose and insulin levels and performed glucose tolerance tests. We found that KO mice have impaired glucose tolerance with elevated fasting glucose levels and elevated fasting insulin levels at the age of 10 weeks (Fig. 4*A*–*C*). Similar results were obtained in 18- to 20-week-old animals (not shown). Combined with the results from above, the data suggest that RSK2 KO mice are characterized by reduced fat mass and by the presence of mild diabetes and insulin resistance. In addition, older KO mice have a tendency toward fatty livers (not shown), phenotypes that are all characteristic of human and rodent forms of lipodystrophy (46,47).

Leptin has been reported to increase whole-body insulin sensitivity and glucose uptake in rodents (48,49). Furthermore, lack of leptin release from adipose tissue has been suggested to play an important role in the severe insulin resistance present in mice and patients with lipodystrophy (50–53). To investigate whether leptin could improve glucose homeostasis in the RSK2 KO mice, we implanted subcutaneous micro-osmotic pumps releasing 5 μg recombinant leptin per day, or vehicle, for 14 days in KO and wild-type mice at the age of 20–22 weeks. As expected, leptin treatment resulted in a modest ($P < 0.05$) increase in serum leptin concentrations in the RSK2 KO mice, but the levels remained lower than those measured in the wild-type animals. The 14 days of treatment produced a striking reduction of fasting insulin and fasting glucose concentrations in KO mice (Fig. 5*A*, *D*, and *E*). This occurred without effects of leptin on food intake (Fig. 5*B*) or body weight (Fig. 5*C*). These results support the notion that leptin can regulate glucose disposal and/or insulin sensitivity, independent of effects on food consumption, and that lack of leptin in RSK2 KO mice negatively affects glucose homeostasis in these animals.

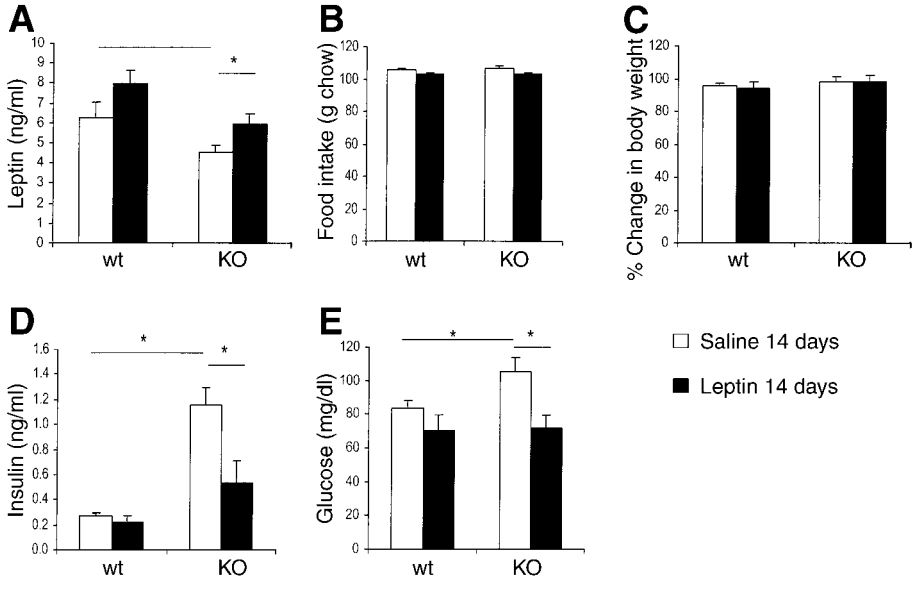

FIG. 5. Leptin normalizes glucose and insulin levels in RSK2 KO mice. Metabolic parameters in wild-type (wt; $n = 9$) and KO ($n = 10$) mice (18–20 weeks of age) after continuous subcutaneous administration via osmotic minipumps of recombinant leptin (5 μg per day for 14 days) (■) or saline (□). *A*: Fasting plasma leptin levels. Significance: *$P < 0.05$. *B*: Cumulative food intake (in grams of chow) during 14 days of the experiment. *C*: Change in body weight after insertion of the pump until death. Mean = 100% values before insertion of subcutaneous pump for wild-type and KO mice. These values were 31.3 and 27.8 g, respectively. *D*: Fasting serum insulin. Significance: *$P < 0.01$. *E*: Fasting serum glucose. Significance: *$P < 0.05$. Data are means ± SE.

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DISCUSSION

Numerous studies have linked RSK proteins to a variety of cellular processes. One member of the family, RSK2, has received particular attention—initially because of its potential role in glycogen metabolism (54,55) and more recently because of its possible role in regulation of cell survival (56,57). In addition, loss of RSK2 activity causes mental retardation and growth defects in humans with CLS (34). Yet the function of individual RSK proteins in vivo, including their substrate(s) and tissue-specific roles, remain elusive. We have recently described the generation of RSK2 KO mice (39). We found that RSK2 is not important for the regulation of glycogen synthesis by insulin in skeletal muscle but that the RSK2 KO mouse does have mild defects in brain function and is therefore a potential useful animal model of CLS.

We show here that RSK2 deficiency in mice results in reduced body weight, largely because of a specific loss of WAT. Furthermore, the mice are resistant to development of obesity and hyperleptinemia when fed a HFD. For the first time, this study links RSK2 function to the regulation of adipose fat mass. Finally, RSK2 KO mice are mildly diabetic and insulin resistant and have fatty livers. We speculate that the dysregulation of glucose homeostasis is secondary to loss of fat tissue because administration of recombinant leptin, a fat-derived hormone, normalizes glucose and insulin parameters. This conclusion is consistent with other reports of the insulin-sensitizing effects of leptin in both normal mice (48,49) and mice with partial or total lipodystrophy that are hypoleptinemic and severely insulin resistant (53). It is also possible that altered expression of other adipocyte-derived factors involved in the regulation of insulin sensitivity, including adiponectin (58,59) and tumor necrosis factor-α (60,61), may play a role in the decreased insulin sensitivity in the RSK2-deficient mice. Although we did find several phenotypic similarities to the rare cases of humans lacking functional RSK2 proteins, the lean and diabetic phenotypes of the RSK2 KO mice have not been reported in CLS. These differences therefore appear species dependent, and possible explanations include differential tissue distribution of RSK2 and/or differential compensatory mechanisms between humans and mice, including upregulation of other RSK family members.

Although RSK2 KO mice have lower body weights, we did not find any evidence for decreased energy consumption. We therefore speculate that the mice are likely to have increased energy expenditure, with either increased resting metabolic rate and/or increased physical activity. Pointing toward the latter possibility, we did not find any evidence for differences in body temperature or in mRNA expression of uncoupling proteins (UCP1, UCP2, and UCP3) in skeletal muscle, brown adipose tissue, or WAT (data not shown), but further studies are clearly needed to examine the energy expenditure in these mice.

The molecular mechanism underlying the loss of WAT in RSK2 KO mice is currently unknown. Because RSK2 is expressed at its highest levels in WAT (39), we speculated that lack of RSK2 enzyme activity in this tissue could mediate this effect. Consistent with this possibility are recent reports of an anti-apoptotic role of RSK2 involving phosphorylation of BAD (56,57), suggesting that RSK2 mice may exhibit increased cell death in WAT, thus possibly contributing the lean phenotype of the mice. Other RSK isoforms with similar cellular functions as RSK2 could compensate for the lack of RSK2 in other tissues that normally also express RSK2, thus preventing cell death at those sites in RSK2 KO animals. Further studies are required to answer these questions. Specifically, WAT-specific KO or reexpression of RSK2 in adipose tissue in the RSK2 KO mice would give more conclusive evidence for the role of RSK2 in this tissue, both in the regulation of fat mass and in glucose homeostasis.

The reduction of fat tissue in adult RSK2 KO mice is relatively modest (~50%) compared with two recently reported mouse models of lipodystrophy (46,53,62,63). These were generated by transgenic overexpression of versions of two transcription factors (A-ZIP/F-1 and SREBP-1c) both under the control of the adipose-specific AP2 enhancer/promoter. The fat losses were 99% and ~70%, respectively. The severity of disease (i.e., diabetes and insulin resistance) is much more profound in the A-ZIP/F-1 mice, significantly less in the SREBP mice, and mildest in the RSK2 KO animals, thus associated with the relative amounts of fat mass among the strains. Furthermore, the effect of leptin to improve glucose homeostasis toward the normal state was only partial in the A-ZIP/F-1 mice, which almost entirely lack WAT, suggesting that other factors in addition to leptin are critical in this regard. However, our data are consistent with the notion that leptin can positively affect insulin resistance and diabetes in a mouse model of mild lipoatrophic diabetes.

In conclusion, our results demonstrate for the first time a role of RSK2 in the regulation of body weight, adipose mass, and glucose homeostasis in mice. The results also support findings that leptin stimulates insulin sensitivity and glucose disposal, independent of its effect on food intake. Furthermore, we speculate that the reduction of fat mass in RSK2-deficient mice may induce insulin resistance, in part because of leptin deficiency. The RSK2 KO mouse may thus be a new valuable animal model of partial lipodystrophy and insulin resistance in humans, and the data support the idea that leptin may be therapeutically useful in the treatment of humans with lipoatrophic diabetes (64). Finally, further studies of RSK2 signaling pathways and RSK2 action in vivo are likely to yield new important insights in the regulation of glucose metabolism and adipose mass, which could ultimately lead to identification of novel antidiabetic drug targets.

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