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Liver Adenosine Monophosphate-Activated Kinase-α2 Catalytic Subunit Is a Key Target for the Control of Hepatic Glucose Production by Adiponectin and Leptin But Not Insulin

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The AMP-activated kinase (AMPK) is a serine threonine kinase that functions as a fuel sensor to regulate energy balance at both cellular and whole-body levels. Here we studied how hepatic AMPKα2 isoform affects hepatic glucose production and peripheral glucose uptake *in vivo*. We generated mice deleted for the AMPKα2 gene specifically in the liver (liverα2KO). Liverα2KO mice were glucose intolerant and hyperglycemic in the fasted state. Hyperglycemia was associated with a 50% higher endogenous glucose production than in controls as assessed *in vivo*. We then investigated whether this increased glucose production was sensitive to insulin. Insulin, when infused at a rate inducing physiological hyperinsulinemia, totally inhibited endogenous glucose production in liverα2KO mice, showing that they had normal insulin sensitivity. This was confirmed *in vivo* by normal insulin-induced phosphorylation of Akt and transcriptional regulation of the phosphoenolpyruvate carboxykinase, glucose-6 phosphatase, and pyruvate kinase in liver during the fasted/fed transition. Leptin and adiponectin regulate hepatic glucose production, so we then infused these adipokines into liverα2KO mice. Neither of these adipokines regulated hepatic glucose production in mice lacking hepatic AMPKα2, whereas both did so in control mice. In conclusion, we show that the hepatic AMPKα2 isoform is essential for suppressing hepatic glucose production and maintaining fasting blood glucose levels in the physiological range. We also demonstrate that regulation of hepatic glucose production by leptin and adiponectin, but not insulin, requires hepatic AMPKα2 activity. (*Endocrinology* 147: 2432–2441, 2006)

LIVER IS A KEY organ for the control of systemic glucose homeostasis by maintaining a balance between glucose uptake and release. In the fasted state, one of its major function is to produce glucose for all other organs, by either glycogenolysis or gluconeogenesis. Inactivation of glycogen synthase and activation of glycogen phosphorylase by phosphorylation are key regulatory mechanisms of glycogenolysis (1). Similarly, d*e novo* synthesis of glucose in liver from precursors provides the organism with glucose in postabsorptive state (2). Deficient hepatic glucose output may lead to hypoglycemia and cause tissue and organ malfunction. On the other hand, hyperglycemia in both types 1 and 2 diabetes is associated with high hepatic glucose production (3, 4). Suppression of gluconeogenesis, a key metabolic pathways for hepatic glucose output, has been shown to improve overall glycemic control in both human patients and type 2 diabetes animal models (5). The control of gluconeogenesis depends on numerous factors, including the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), concentration of gluconeogenic substrates (lactate, gluconeogenic amino acids, and glycerol), and insulin levels. However, the signaling molecules for both gluconeogenesis and glycogenolysis remain to be identified.

Recently the evolutionarily conserved serine/threonine kinase, AMP-activated protein kinase (AMPK), has been implicated in the control of hepatic glucose output. Mammalian AMPK is a heterotrimeric complex consisting of a catalytic (α) and two regulatory (β and γ) subunits. Two to three isoforms of each subunit (α1, α2, β1, β2, γ1, γ2, and γ3),

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Abbreviations: AICAR, 5-Aminoimidazole-4-carboxamide riboside; AMPK, AMP-activated protein kinase; EGP, endogenous glucose production; FFA, free fatty acid; G6Pase, glucose-6-phosphatase; HDL, high-density lipoprotein; liverα2KO, mice deleted for the AMPKα2 gene specifically in the liver; L-PK, L-pyruvate kinase; PEPCK, phosphoenolpyruvate carboxykinase.

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encoded by different genes, are known. In situations of energy depletion, a decrease in the cellular ATP to AMP ratio leads to AMPK activation and inhibition of energy-consuming biosynthetic pathways, such as fatty acid and sterol synthesis, and activation of ATP-producing catabolic pathways, such as fatty acid oxidation (6). Work with pharmacological compounds and adenovirus-mediated AMPK activation/inactivation strategies suggests that hepatic AMPK has a regulatory role in glucose homeostasis. Bergeron *et al.* (7) first demonstrated that 5-aminoimidazole-4-carboxamide riboside (AICAR), a potent AMPK activator, inhibits endogenous glucose production (EGP) in normal rats and insulin-resistant obese Zucker rats *in vivo*. Zhou *et al.* (8) provided evidence that metformin-mediated inhibition of glucagon-stimulated glucose production in primary cultured rat hepatocytes required AMPK activation. Indeed, an AMPK inhibitor (compound C) was able to blunt the inhibitory effect of metformin on hepatocyte glucose production. Metformin also reduces hepatic lipid synthesis and prevents development of fatty liver by inhibiting sterol regulatory element-binding protein-1 gene expression through a mechanism that requires AMPK activation (8). Administration of full-length adiponectin reduces blood glucose levels in mice (9) and inhibits genes in the liver involved in the gluconeogenic pathway such as PEPCK and G6Pase (10). The concomitant injection of adenoviruses encoding a dominant-negative form of AMPK prevented the hypoglycemic effect of adiponectin, and this led to the suggestion that the glucose-lowering effect of full-length adiponectin was due to increased liver AMPK activity (10).

Most of the known metabolic effects of AMPK are mediated by the α2 catalytic subunit but have been described only in the hypothalamus and skeletal muscle (11–14). We recently demonstrated that acute activation of AMPK specifically in the liver, achieved by adenovirus-mediated gene transfer of a constitutively active form of AMPKα2, is sufficient to decrease blood glucose levels and reduce hepatic gluconeogenic gene expression (15). It has also been reported that the livers of mice lacking the adipocyte hormone resistin produce less glucose due to activation of AMPK in the liver (16). These findings suggest that hepatic glucose production can be modulated by changing the AMPKα2 activity in the liver. Nevertheless, whether insulin, leptin, and adiponectin control glucose homeostasis by acting through the hepatic AMPKα2 isoform is not known.

We studied the contribution of hepatic AMPKα2 to EGP and its general metabolic role in whole-body glucose metabolism. We used a conditional gene knockout strategy to abolish AMPKα2 activity specifically in the liver. Here we report the first description of the metabolic consequences of hepatic deletion of the AMPKα2 catalytic subunit gene *in vivo* in mouse (liverα2KO). EGP in the fasted state was increased in the absence of hepatic AMPKα2, and remaining AMPKα1 in liver was not sufficient to control the EGP. We also showed that *in vivo* insulin-mediated suppression of EGP was preserved but leptin and adiponectin were unable to control EGP in this model. These observations demonstrate that the absence of the AMPKα2 catalytic subunit from the liver is sufficient to increase EGP, a key feature of type 2 diabetes mellitus, and that the AMPKα2 catalytic subunit is essential for control by adipokines of EGP but not for the action of insulin.

Materials and Methods

Generation of liver-specific AMPKα2 knockout gene mice model

Mice harboring a floxed AMPKα2 gene (AMPKα2flox/flox) in which the sequence encoding the catalytic domain was flanked with loxP sequences (17) were bred with AlfpCre transgenic mice (18) to generate mice with a liver-specific deletion. We used only male mice in these studies. AMPKα2$^{+/+}$; Cre^{+} (control) mice were used as wild-type controls, and AMPKα2flox/flox; Cre^{+} (liverα2KO) mice were used as homozygous mutants. Mice were genotyped using primers specific for the Cre transgene (5′-CAGGGTGTTATAAGCAATCCC-3′ and 5′-CCTGGAAAATGCTTCTGTCCG-3′) and the floxed AMPK2 gene (5′-GCTTAGCACGTTACCCTGGATGG-3′ and 5′-GTTATCAGCCCAACTAATTACAC-3′). Cre recombination efficiency was assessed by Southern blot analysis of *Hin*dIII-digested liver DNA as described previously (17).

Animals

Control and liverα2KO male mice born in our animal facility were studied at 12 wk of age. Animals were housed under controlled temperature (21 C) and lighting [12-h light (0700–1900 h), 12-h dark (1900 h to 0700 h] with free access to water and standard mouse chow diet (rodent diet no. A03, UAR, 60% carbohydrate, 13% fat, and 27% protein on a caloric basis). All procedures were performed in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals.

Blood parameter determinations

Blood was withdrawn from the tail vein for both fed and fasted experiments using EDTA-aprotinin as the anticoagulant. In the fed state, blood was collected at 2300 h. For fasting experiments, food was removed at 1800 h, and the mice were kept in a clean new cage for 5 h before collecting blood. For the oral glucose tolerance test, blood glucose levels were evaluated using a glucometer (Glucotrend II; Roche Laboratories, Indianapolis, IN). Serum insulin concentrations were measured using a rat insulin ELISA kit with a mouse insulin standard (Crystal Chem, Chicago, IL). Serum leptin and adiponectin concentrations were assessed with mouse leptin and adiponectin RIA kits, respectively (Linco Research, St. Charles, MO). Serum concentrations of triglycerides, free fatty acids (FFAs), ketone bodies, and total and high-density lipoprotein (HDL) cholesterol were determined using an automatized Monarch device (CEFI, IFR02, Paris, France) as described previously (17).

Oral glucose tolerance test

Mice fasted overnight were loaded orally with glucose (3 g/kg), and blood was collected from the tail vein 0, 20, 40, and 60 min later for determination of glucose levels. Blood was also collected after 40 min into chilled heparinized tubes for determination of plasma insulin concentration as described previously (17).

Glucose turnover analysis during basal fasting and under hyperinsulinemic clamp conditions

To determine the rate of glucose use, a catheter was indwelled into the femoral vein under anesthesia, sealed under the back skin, and glued on the top of the skull (17). The mice were then housed individually. The mice were allowed to recover for 4–6 d, and after 2 d, they showed normal feeding behavior and motor activity. On the day of the experiment, the mice were fasted for 6 h. The whole-body glucose use rate was determined in basal and in hyperinsulinemic euglycemic conditions. In the basal state, HPLC purified D-(^{3}H)3-glucose (PerkinElmer, Boston, MA) was continuously infused through the femoral vein at a rate of 10 μCi/kg·min for 3 h. Under the physiological hyperinsulinemic condition, insulin was infused at a rate of 4 mU/kg·min for 3 h and D-(^{3}H)3-glucose was infused at a rate of 30 μCi/kg·min, higher than for the basal

condition, to ensure a detectable plasma D-(^{3}H)3-glucose enrichment. Throughout the infusion, the blood glucose concentration was assessed with a glucose meter in blood samples (3.5 μl) collected as appropriate from the tip of the tail vein. Euglycemia was maintained by periodically adjusting a variable infusion of 16.5% (wt/vol) glucose. Plasma glucose concentrations, and D-(^{3}H)3-glucose specific activity were determined in 5 μl of blood sampled from the tip of the tail vein every 10 min during the last hour of the infusion. Mice showing variations in the specific activity larger than 15% were excluded from the study.

Isotope measurements

Tritiated H_2O and D-(^{3}H-3)glucose enrichments were determined in total blood after deproteinization performed as follows and described previously (19). Five microliters of tail venous blood were mixed with 250 μl of 0.3 M $ZnSO_4$. Then 250 μl of 0.3 M $Ba(OH)_2$ were added to precipitate the proteins and blood cells. The $Zn(OH)_2$ precipitate formed was spun down. An aliquot of the supernatant was evaporated to dryness and the radioactivity corresponding to D-(^{3}H-3)glucose determined. Another aliquot was directly mixed with the scintillation buffer to determine the radioactivity corresponding to ^3H_2O and D-(^{3}H-3)glucose. Therefore, the difference between values for the first and the second aliquot corresponds to the ^3H_2O produced. In a third aliquot of the same supernatant, the glucose concentration was assayed by the glucose oxidase method (Trinder; Sigma, St. Louis, MO).

Adiponectin and leptin infusion in control and liverα2KO mice

Murine globular adiponectin (kindly provided by Drs. Tsu-Shuen Tsao and Harvey Lodish, Massachusetts Institute of Technology, Boston, MA) was infused at a rate of 24 ng/kg·min. This infusion rate was determined in the light of preliminary results in which a dose-response curve of the hormone has been performed on the inhibition of hepatic glucose production in normal mice. The infusions tested were 24, 7.2, 2.4, 0.72, and 0.24 ng/kg·min. The data showed that the last dose was fully able to inhibit hepatic glucose production (see Fig. 3C). It was hence used in mutant mice. Murine leptin (Peprotech, Rocky Hill, NJ) was infused at the rate of 0.5 μg/kg·min for 3 h. Saline was infused into liverα2KO mice as a control. In all groups, HPLC-purified D-(^{3}H)3-glucose was continuously infused through the femoral vein at a rate of 10 μCi/kg·min for 3 h to measure EGP, as described (19).

Western blot analysis for AMPK and insulin signaling pathways

For *in vivo* analysis of insulin signaling, mice fasted overnight were anesthetized with xylazine/ ketamine, and 5 U/kg regular human insulin (NovoNordisk, Copenhagen, Denmark) were injected into the inferior venae cavae. Five minutes later, the liver was harvested and frozen in liquid nitrogen until analysis. For Western blot analysis, liver, skeletal muscle, and gonadal white adipose tissue samples were homogenized in buffer A [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 10% (vol/vol) glycerol, 1 mM dithiothreitol, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride] + 1% Triton X-100 and then centrifuged at 7000 × *g* for 10 min at 4 C. The resulting supernatants were used as total extracts. Aliquots of these extracts containing 50 μg protein were subjected to SDS-PAGE on a 7.5% gel and transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) with protein transfer confirmed by Ponceau S staining to control for equal loading. Immunoreactive proteins were detected using the ECL plus Western blotting detection system (Amersham Biosciences, Piscataway, NJ). Blots were probed with the following primary antibodies: anti-AMPKα1 and -α2 (kindly provided by Prof. G. Hardie, Dundee, Scotland, UK), anti-phospho-ACC Ser79, anti-phospho-AMPKα Thr172, anti-phospho-Akt Ser473, and anti-Akt (Cell Signaling, Beverly, MA). Bands were quantified using Image J software (National Institutes of Health, Bethesda, MD).

PEPCK and G6Pase enzymatic activities

Hepatic PEPCK and G6Pase enzyme activities were measured in 6-h-fasted liverα2KO and control mice. Frozen liver samples were powdered into liquid nitrogen and homogenized by sonication in 20 mM HEPES, 0.25 M sucrose (pH 7.3) (100 μg of wet tissue per milliliter). Homogenates were diluted 10 times before determination of G6Pase at maximal velocity (20 mmol/liter glucose-6 phosphate) at 30 C on complex formation of pi produced from G6P, as described previously (20). Specific G6Pase activity was cleared of the contribution of nonspecific phosphohydrolase activities by subtracting the activity toward β-glycerophosphate (20 mmol/liter) (20). PEPCK was determined in 100,000 × *g* supernatants of the homogenates, using the decarboxylation assay described by Jomain-Baum and Schramm (21). The decarboxylation assay determines the formation of phosphoenolpyruvate by an equilibrium displacement generating oxaloacetate from malate by malate dehydrogenase. The reaction was started by adding 10 mmol/liter malate. The oxaloacetate formation was determined spectrophotometrically by measuring the synthesis of nicotinamide adenine dinucleotide hydroxide, as previously described (22).

Nutritional regulation of hepatic gene expression, RNA isolation, and RT-PCR analysis

Expression of the hepatic PEPCK, G6Pase, and L-pyruvate kinase (L-PK) genes was studied in control and liverα2KO mice after an overnight fast (n = 4 for each group) and after refeeding (n = 4 for each group). Total RNA was isolated from liver with RNA plus (Qbiogene, Cambridge, UK), and single-strand cDNA was synthesized from 5 μg of total RNA with random hexamer primers (Applied Biosystems, Foster City, CA) and Superscript II (Invitrogen, Carlsbad, CA). A LightCycler reaction kit (Eurogenetec, Herstal, Belgium) and specific PCR primers used for real-time PCR were as follows: Ob-R sense, 5′-ACTGGGACATAGAGTGCTGG-3′, antisense 5′-ATACATCAGAAGAGCGTAGTTG-3′; Adipo-R1 sense, 5′-GGTGGTGGCAGCAGCTTTCGTC-3′, antisense 5′-GAGCTGACATGTCACTGGTACTG-3′; Adipo-R2 sense, 5′-GCAGGAGTGTTCGTGGGCTTAGG-3′, antisense 5′-GCAGGCCTGGCTCAGGGTCAGAG-3′; and for PEPCK, G6Pase, and L-PK as described previously (15).

Statistical analysis

Data are expressed as means ± SE. A two-tailed Student's unpaired *t* test was used for statistical analyses, and the null hypothesis was rejected at the 0.05 level.

Results

Animal model: liver-specific AMPKα2 KO mice

We generated a liver-specific knockout mouse for the AMPKα2 catalytic subunit gene by breeding AMPKα2flox/flox mice with Alfp-Cre transgenic mice. Liverα2KO mice were born with the expected Mendelian ratio, were fertile, appeared indistinguishable from their wild-type littermates, and had normal life span. There was no significant difference in body weight, body composition, and food intake between liverα2KO and control groups (data not shown). The AMPKα2 deletion induced by Cre recombinase activity was manifest by the conversion of the 2-kb floxed allele fragment into a 3.8-kb deleted allele fragment (Fig. 1A). Southern blot analysis of genomic DNA from different organs of AMPK-flox/flox animals carrying the AlfpCre transgene showed specific recombination in the liver but not in other organs including adipose tissue, skeletal muscle, pancreas, and hypothalamus (Fig. 1B). The efficiency of AMPKα2 gene deletion was estimated to be greater than 90% in hepatocytes because the Cre recombinase is specifically expressed in hepatocytes that represent 70–80% of liver cells (18). Western blot analysis of protein extracts prepared from liver of liverα2KO mice failed to detect AMPKα2 protein, although hepatic AMPKα1 content was unchanged (Fig. 1C). The pro-

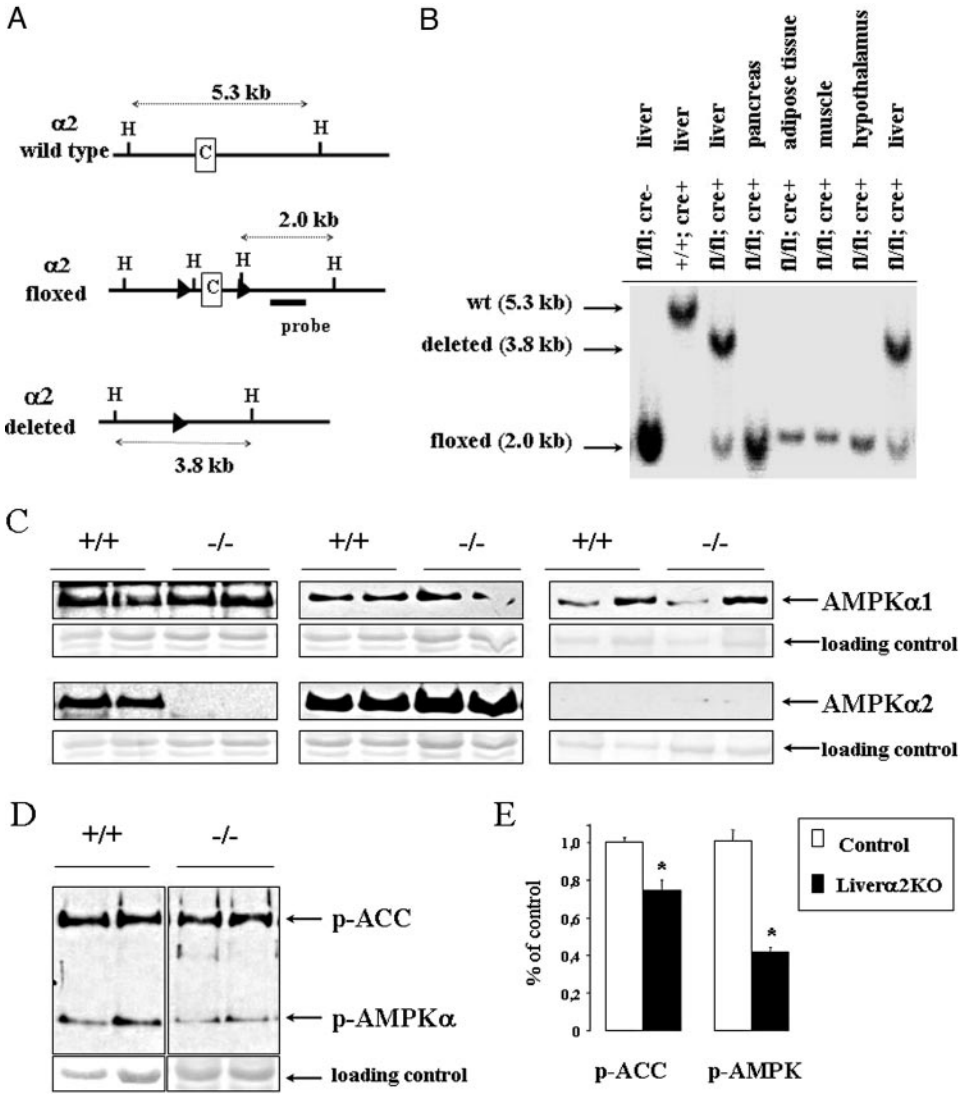

FIG. 1. Characterization of liverα2KO mice. A, Schematic representation (not to scale) of the genomic structure of the *AMPKα2* wild-type allele, *AMPKα2* floxed allele, and *AMPKα2* deleted allele. *Triangles* indicate *loxP* sites, and H indicates *Hin*dIII restriction sites. C, Exon encoding the *AMPKα2* catalytic domain (amino acids 189–260). B, Southern blot analysis after *Hin*dIII digestion of liver, adipose tissue, pancreas, skeletal muscle (vastus lateralis), and hypothalamus DNA from AMPKα2$^{+/+}$; Cre^{-}, AMPKα2flox/flox; Cre^{-}, and AMPKα2flox/flox; Cre^{+} mice. Expected fragment sizes of the *AMPKα2* wild-type (5.3 kb), floxed (2.0 kb), and deleted (3.8 kb) alleles after *Hin*dIII digestion and hybridization with the indicated probe (*solid bar*, A) are shown. Western blot analysis of AMPKα1 subunit (C), AMPKα2 subunit in liver, skeletal muscle (vastus lateralis), and gonadal white adipose tissue. Ponceau-stained band of protein extracts is presented as a loading control. D, Total phosphorylated Thr-172 AMPKα subunit (P-AMPKα), phosphorylated Ser-79 acetyl-CoA carboxylase (P-ACC), and Ponceau-stained band of liver protein extracts from control ($^{+/+}$) and AMPKα2flox/flox; Cre^{+} ($^{-/-}$) mice is presented as a loading control. E, Quantification of hepatic AMPK and ACC phosphorylation in control (*open bars*) and liverα2KO (*closed bars*) mice (n = 4–5 mice per group. *, $P < 0.05$).

tein levels for AMPKα1 and AMPKα2 were not modified in extrahepatic tissue, skeletal muscle, and white adipose tissue (Fig. 1C). Hepatic content of phosphorylated Ser79-ACC was 25% lower in liverα2KO than wild-type mice (Fig. 1D), indicating lack of compensation by the remaining AMPKα1 isoform for full AMPK activity in the liver.

TABLE 1. Metabolic markers in liverα2KO mice during fasted and fed periods

	Fasted		Fed	
	Control (n = 10)	Liverα2KO (n = 10)	Control (n = 10)	Liverα2KO (n = 10)
Glycemia (mg/dl)	130 ± 14	163 ± 8[a]	196 ± 11[b]	221 ± 10[b]
Insulinemia (ng/ml)	0.35 ± 0.11	0.73 ± 0.11[c]	2.09 ± 0.61	2.17 ± 0.41
Leptin (ng/ml)	0.93 ± 0.44	3.31 ± 0.69[a]	1.65 ± 0.25[b]	2.60 ± 0.26
Adiponectin (μg/ml)	10.0 ± 0.5	15.4 ± 1.1[a]	ND	ND
FFAs (mM)	1.98 ± 0.14	2.56 ± 0.17[c]	1.36 ± 0.55[d]	1.64 ± 0.18[b]
Triglycerides (mM)	0.80 ± 0.09	1.29 ± 0.13[c]	1.03 ± 0.29	1.18 ± 0.15
β-Hydroxy butyrate (mM)	0.49 ± 0.05	0.26 ± 0.06[c]	ND	ND
Total cholesterol (mM)	3.27 ± 0.22	3.64 ± 0.23	2.83 ± 0.23	3.38 ± 0.16
HDL cholesterol (mM)	2.42 ± 0.16	2.68 ± 0.15	2.03 ± 0.15	2.38 ± 0.11

Metabolic parameters were measured from liverα2KO and control mice during fasted and fed periods. Blood was collected at 2300 h for both fed and fasted state. For fasting experiments, food was removed at 1800 h. Data are means ± SE. ND, Not determined.

[a] $P < 0.01$.

[b] $P < 0.01$.

[c] $P < 0.05$ liverα2KO *vs.* control group.

[d] $P < 0.05$ fasted *vs.* fed period by two-tailed *t* test.

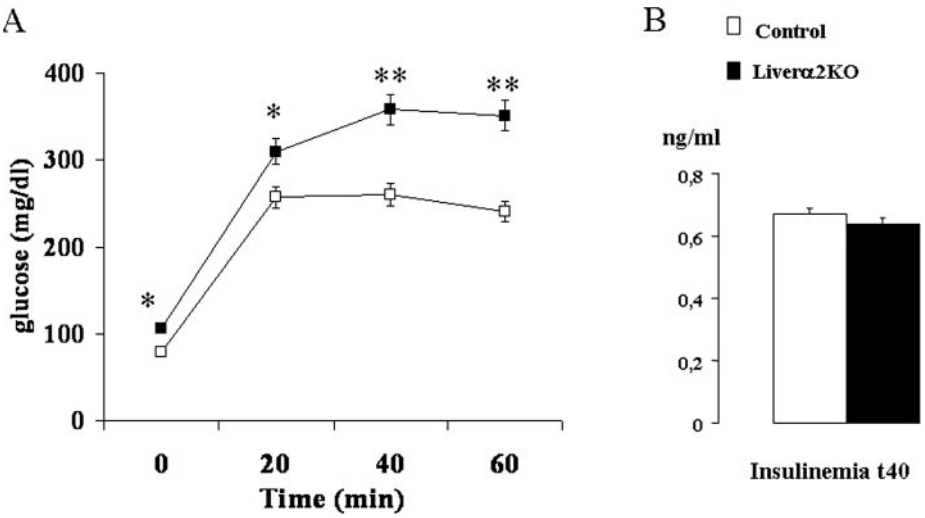

FIG. 2. Specific deletion of the AMPKα2 subunit gene in liver is associated with oral glucose intolerance. A, Glucose tolerance tests were performed after an overnight fast on 2-month-old male control (*open squares*, n = 10) and liverα2KO (*filled squares*, n = 8) mice. Animals were loaded orally with glucose (3 g/kg). Blood glucose was measured immediately before and 20, 40, and 60 min after glucose injection. *, $P < 0.05$; **, $P < 0.01$. B, Plasma insulin concentration (nanograms per milliliter) 40 min after the oral glucose administration is shown for control (*open bars*) and liverα2KO (n = 8–10 per group) mice.

Metabolic variables

In fasted conditions, plasma glucose, insulin, leptin, adiponectin, triglycerides, and FFA levels were statistically higher in liverα2KO mice than controls (Table 1). Hydroxybutyric acid plasma levels were lower in liverα2KO mice in the fasted state and undetectable in both groups in the fed condition (Table 1). In the fed state, there was no difference in plasma glucose, insulin, triglycerides, FFAs, and total and HDL cholesterol between liverα2KO mice and their wild-type controls (Table 1). The expected increase of leptin levels after feeding was not observed in liverα2KO mice (Table 1).

Liverα2KO mice are glucose intolerant

To assess glucose homeostasis in liverα2KO mice, we performed an oral glucose challenge. After glucose administration, liverα2KO mice, hyperglycemic in the fasted state, had a markedly higher blood glucose levels than wild type demonstrating glucose intolerance (Fig. 2A). Despite higher plasma glucose concentration, insulinemia at 40 min was similar to that in wild-type controls (Fig. 2B).

Liverα2KO mice have higher basal EGP

Basal EGP was significantly higher in liverα2KO mice than controls (39.0 ± 0.7 *vs.* 27.7 ± 1.3 mg/kg·min in knockout and controls, respectively, $P < 0.01$; Table 2 and Fig. 3A). Basal EGP was inhibited by physiological hyperinsulinemia (the mean for all groups was 66 ± 14 μU/ml) in liverα2KO mice to the same extent as in control mice, suggesting normal hepatic insulin sensitivity (Table 2 and Fig. 3A). We next infused leptin and adiponectin separately into control and liverα2KO mice in the basal fasted state to compare their regulatory effects on hepatic glucose production (Fig. 3C and Tables 3 and 4). We first assessed the efficient dose of adiponectin, which was able to efficiently inhibit hepatic glucose production in control mice. The rate of 24 ng/kg·min was then chosen and used in mutant mice (Fig. 3C). Leptin was infused at a rate previously determined to be sufficient to control hepatic glucose production in wild-type mice (23). As previously described (23), leptin infusion stimulated whole-body glycolysis, with reference to saline-infused controls (Table 3). Similarly, adiponectin infusion increased whole-body glycolysis and the glycogen synthesis rate and consequently whole-body glucose production (Table 3). EGP at the end of leptin and adiponectin infusions in liverα2KO mice was the same as that in saline-infused controls (Table 4). This was not due to the lack of leptin and adiponectin receptors in the liver of liverα2KO mice. As shown in Fig. 3D, expression levels for these receptors were unaltered in the liver of AMPKα2KO mice, compared with control mice. These results suggest that hepatic AMPKα2 deletion is sufficient to abolish regulation of EGP mediated by adipokines completely, but not that by insulin. The hepatic insulin sensitivity of liverα2KO mice is thus not impaired despite the absence of hepatic AMPKα2. In addition, whole-body glucose turnover stimulation by adipokines was impaired in liverα2KO mice despite expression of AMPKα2 in extrahepatic tissues.

TABLE 2. Metabolic markers in liverα2KO mice and control mice during basal and euglycemic hyperinsulinemic clamp studies

	Weight (g)	Glucose turnover (mg/kg·min)	Hepatic glucose production (mg/kg·min)	Glycolysis rate (mg/kg·min)	Glycogen synthesis rate (mg/kg·min)
Basal					
Control (n = 6)	25.3 ± 2.6		27.7 ± 1.3	7.6 ± 0.8	19.0 ± 1.5
Liverα2KO (n = 7)	27.9 ± 0.9		39.0 ± 0.7[a]	15.4 ± 1.8[a]	23.6 ± 1.9
Clamp					
Control	25.8 ± 1.4	62.3 ± 3.1	−0.57 ± 2.0	27.1 ± 0.33	32.1 ± 2.9
Liverα2KO	26.2 ± 0.9	65.4 ± 5.1	0.83 ± 4.3	28.5 ± 2.0	38.6 ± 4.2

For euglycemic hyperinsulinemic clamp studies, control and liverα2KO mice were fasted for 6 h and infused with insulin during 3 h at a rate of 4 mU/kg·min. Data are means ± SE.

[a] $P < 0.01$ *vs.* control group by two-tailed *t* test.

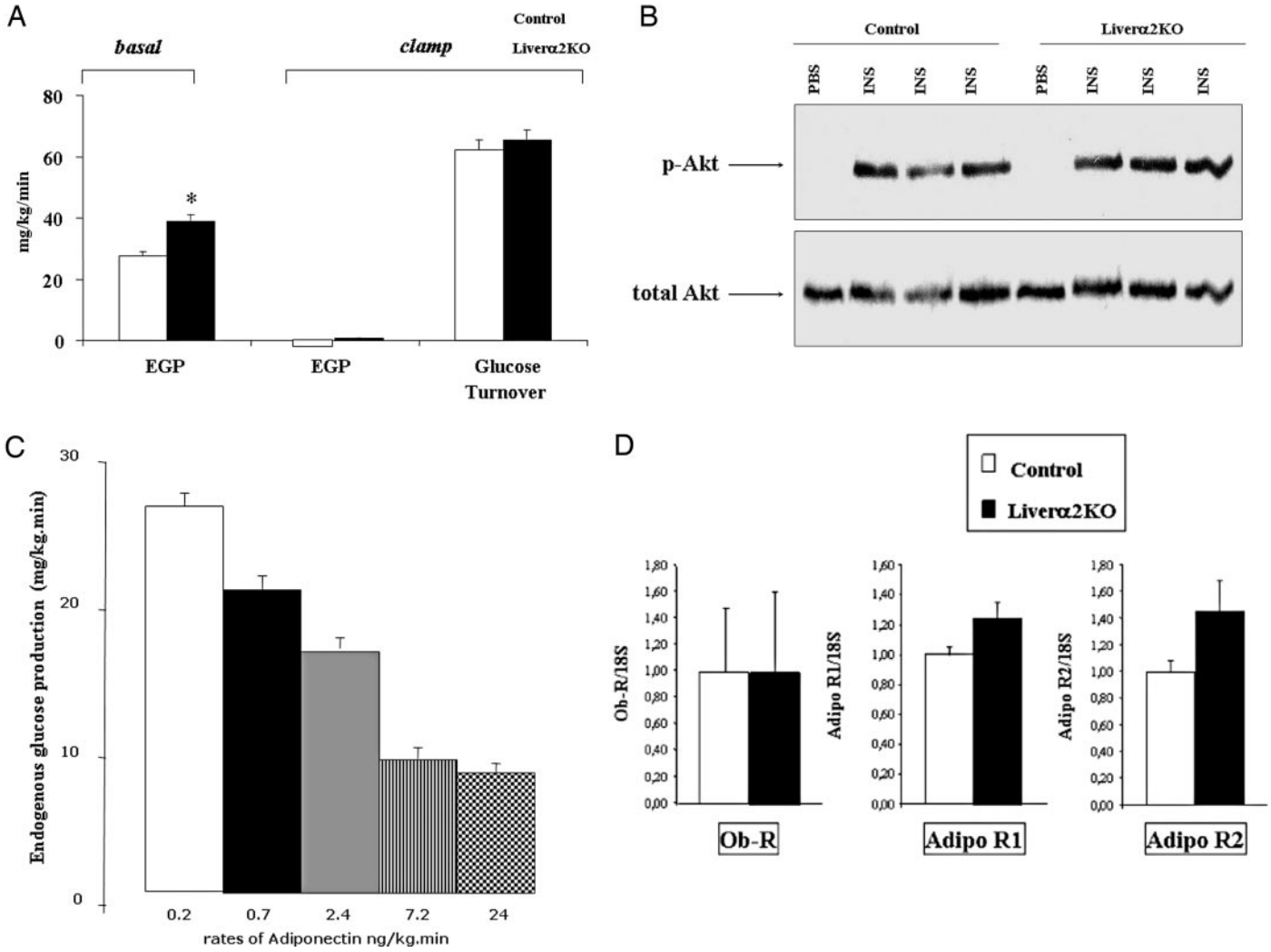

FIG. 3. Glucose fluxes in basal and insulin-stimulated states and hepatic adipokine receptor expression. A, EGP was assessed in wild-type mice (control, n = 6, *open bars*) and liverα2KO mice (n = 7, *closed bars*) in the absence (basal) or presence (clamp) of a physiological insulin infusion (euglycemic hyperinsulinemic clamp). *, $P < 0.05$. B, Representative Western blot of total and phospho-Akt (pAKT) in the liver of control or liverα2KO mice in the presence (INS) or absence (PBS) of insulin. No difference in insulin signaling between control and liverα2KO mice was noted. C, EGP (milligrams per kilogram per minute) was assessed in wild-type mice (n = 6–8/group) in the presence of a continuous iv infusion of adiponectin (at the rates indicated on the figure) to determine the rate that efficiently inhibited EGP. The rate of 24 ng/kg·min was then used in the mutant mice (see Table 3). D, Quantitative gene expression determined by real-time PCR for hepatic leptin (Ob-R) and adiponectin R1 and R2 (Adipo-R1 and Adipo-R2) receptors from 6-h-fasted liverα2KO (*closed bars*) and control mice (*open bars*) (n = 4 for each group).

Whole-body insulin sensitivity is preserved in liverα2KO mice

Insulin-stimulated whole-body glucose turnover was similar in liverα2KO mice and controls (65.4 ± 1.8 *vs.* 62.1 ± 3.1 mg/kg·min in controls, NS) (Table 2 and Fig. 3A). Insulin-stimulated whole-body glycolysis (28.5 ± 2.0 *vs.* 27.1 ± 0.3 mg/kg·min in liverα2KO mice and controls, respectively, NS) and insulin-stimulated whole-body glycogen synthesis rates (38.6 ± 4.2 *vs.* 32.1 ± 2.9 mg/kg·min in liverα2KO mice and controls, respectively, NS) did not differ between the two groups of mice (Table 2). Insulin-induced Akt phosphorylation *in vivo* was similar in liverα2KO mice and their cor-

TABLE 3. Metabolic markers during adiponectin or leptin infusion in control mice

	Weight (g)	Hepatic glucose production (mg/kg·min)	Glycolysis rate (mg/kg·min)	Glycogen synthesis rate (mg/kg·min)
Saline (n = 5)	29.2 ± 2.4	25.6 ± 2.9	14.0 ± 0.9	11.6 ± 2.9
Adiponectin (n = 5)	28.2 ± 1.7	43.9 ± 6.6[a]	22.6 ± 5.0[a]	21.3 ± 5.0[a]
Leptin (n = 4)	32.2 ± 1.8	43.5 ± 6.6[a]	18.8 ± 2.2[a]	24.7 ± 8.4[a]

Control mice were fasted for 6 h and murine globular adiponectin was infused at a rate of 24 ng/kg·min and murine leptin at 0.5 μg/kg·min for 3 h. Data are means ± SE.

[a] $P < 0.05$ *vs.* saline-infused mice by two-tailed *t* test.

TABLE 4. Metabolic markers during adiponectin or leptin infusion in liver AMPKα2KO mice

	Weight (g)	Hepatic glucose production (mg/kg·min)	Glycolysis rate (mg/kg·min)	Glycogen synthesis rate (mg/kg·min)
Saline (n = 5)	26.8 ± 1.3	39.0 ± 0.7	15.4 ± 1.8	23.6 ± 1.9
Adiponectin (n = 6)	26.1 ± 0.7	34.1 ± 2.4	11.4 ± 1.2	22.7 ± 2.9
Leptin (n = 6)	28.2 ± 1.1	34.1 ± 2.6	20.2 ± 1.7[a]	13.9 ± 2.4[a]

Liverα2KO mice were fasted for 6 h, and murine globular adiponectin was infused at a rate of 24 ng/kg·min and murine leptin at 0.5 μg/kg·min for 3 h. Data are means ± SE.

[a] $P < 0.05$ *vs.* saline infused mice by two-tailed *t* test.

responding controls (Fig. 3B). Therefore, whole-body insulin sensitivity was not affected by the deletion of the AMPKα2 subunit gene in the liver.

Liver gene expression in fasted and fed state

To elucidate the mechanisms by which basal hepatic glucose production was increased in liver AMPKα2$^{-/-}$ mice, we evaluated PEPCK, G6Pase, and L-PK mRNA levels by RT-PCR in the liver in both fasted and fed conditions. In fasted conditions, mRNA levels for PEPCK and G6Pase were similar in livers of control and liverα2KO mice (Fig. 4). In the fed state, both these genes were down-regulated in both groups as expected (Fig. 4), but L-PK mRNA was up-regulated.

PEPCK and G6Pase enzymatic activities

Contrasting with unaffected hepatic PEPCK and G6Pase gene expression, the enzymatic activity levels for these two enzymes were much higher in 6-h-fasted liverα2KO than control mice (Fig. 5). These data strongly suggested that liver AMPKα2 is involved in the control of enzymatic activity of key players of the gluconeogenic pathway.

Discussion

We here describe the metabolic consequences of specific hepatic deletion of the AMPKα2 catalytic subunit gene *in vivo* in the mouse. We demonstrated that the absence of hepatic AMPKα2 increased fasted EGP, resulting in hyperglycemia

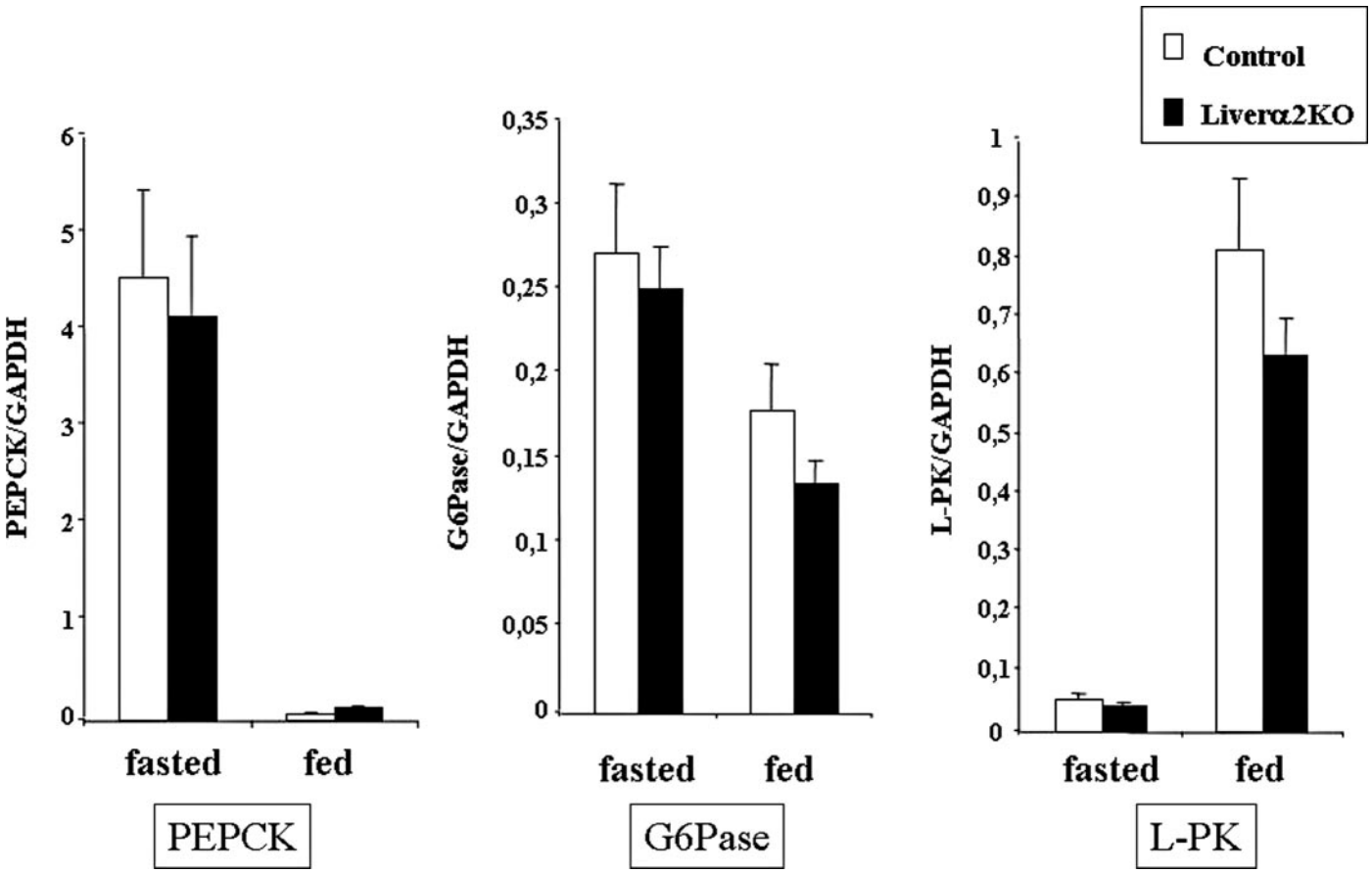

FIG. 4. Hepatic PEPCK, G6Pase, and L-PK gene expression. Regulation of hepatic PEPCK, G6Pase, and L-PK gene expression analyzed by real-time PCR after an overnight fast (n = 4 for each group) and after refeeding (n = 4 for each group) in liverα2KO mice (*closed bars*) and their controls (*open bars*). GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

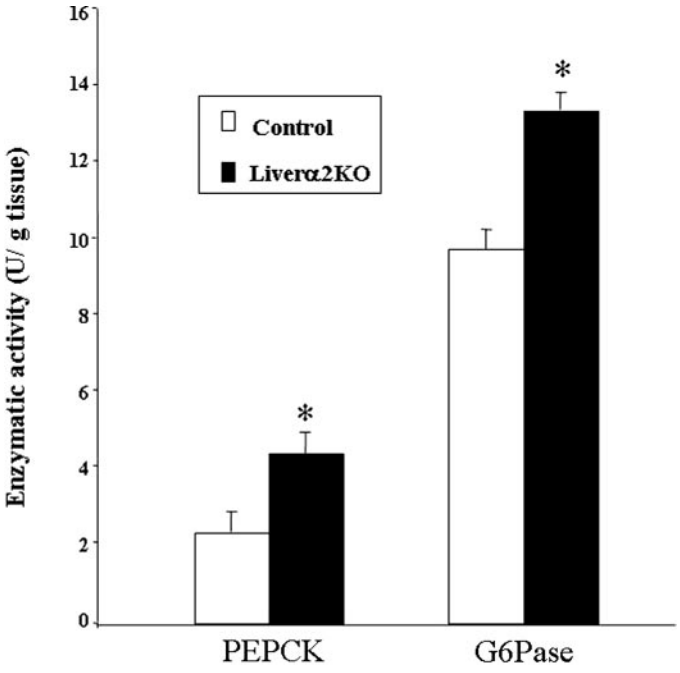

FIG. 5. Hepatic PEPCK and G6Pase enzymatic activities. Enzymatic activities for PEPCK and G6Pase in the liver of 6-h-fasted control (*open bars*) and liverα2KO (*closed bars*) mice (n = 4 for each group. *, $P < 0.05$).

and glucose intolerance *in vivo*. The absence of AMPKα2 did not interfere with hepatic insulin sensitivity: glucose production was totally inhibited by a physiological dose of insulin. In contrast, the absence of AMPKα2 strongly impaired EGP regulation by leptin and adiponectin.

In our model, the AMPKα2 subunit gene was deleted only in liver and was unaffected in other tissues, including skeletal muscle and white adipose tissue. Deletion of AMPKα2 subunit gene in the liver did not affect the AMPKα1 protein content, but the phospho-Ser79 ACC content was reduced by 25% in random fed animals. Ser79-ACC phosphorylation is proportional to the total AMPK activity, and therefore, it appears that the α2-deletion was not compensated by the remaining α1-subunit resulting in a lowered overall AMPK activity. Furthermore, it has been established that AMPKα1 and -α2 isoforms contribute to about 50% of total AMPK activity in the liver (24). The excessive hepatic glucose production in liverα2KO mice, as assessed by the state-of-the-art method using glucose tracers in awake and free-moving mouse, could have been due to either gluconeogenesis or glycogenolysis. However, after 6–8 h of fasting, liver glycogen stores are mostly depleted and hepatic gluconeogenesis accounts for most of the hepatic glucose production (25). Hence, the increased hepatic glucose production by AMPKα2 knockout mice is probably due to increased gluconeogenesis rather than glycogenolysis.

Hepatic AMPK may reduce gluconeogenesis: studies by Bergeron and colleagues (7, 26) suggested that activation of AMPK by systemic AICAR administration suppresses glucose production in overnight-fasted rats. These findings were supported by the observation that injecting mice with adenovirus expressing dominant-negative AMPK (10) increased expression of the gluconeogenic enzyme PEPCK. In contrast, treating hepatocytes in primary culture with the AMPK activator AICAR (26, 27) or adenovirus expressing a constitutively active form of AMPKα2 (15) reduced PEPCK expression and hepatic glucose production. In our study, liver AMPKα2KO mice have higher EGP without any increase in PEPCK or G6Pase gene expression. This is surprising but could be attributed, at least in part, to fasted hyperinsulinemia, which inhibits the expression of the genes for both enzymes (as suggested by the absence of hepatic insulin resistance in our model). Nevertheless, although the expression of these genes was not modified, we clearly demonstrated that hepatic PEPCK and G6Pase enzymatic activities are higher in liverα2KO mice than in controls, suggesting an increase in the gluconeogenic pathway after deletion of AMPKα2 subunit in the liver. Thus, our results suggest that AMPKα2 subunit in the liver probably regulates the gluconeogenic pathway at a posttranscriptional level. In line with this proposal, the existence of posttranscriptional regulation mechanisms of G6Pase activity has been previously documented (28–30).

Intralipid infusion increases hepatic glucose production and gluconeogenesis in the dog (31). This is in part explained by an increase in lipid oxidation to cover the energetic cost of stimulated gluconeogenesis. This type of mechanism may apply to our model because plasma levels of FFAs and triglycerides were high in liverα2KO mice. However, it seems unlikely that lipid availability can explain the observed rise in EGP in liverα2KO mice for the following reasons. First, FFA and triglyceride plasma levels were higher than control values only in the fasted state and not in the fed state, indicating a preserved regulation of the lipid flux by nutrient and insulin. Second, there was no hepatic steatosis in liverα2KO mice (data not shown), although it is usually associated with insulin resistance and increased EGP. Third, whole-body insulin sensitivity of liverα2KO mice and controls were similar, suggesting that there was no mechanism of lipid-glucose competition affecting both hepatic and peripheral tissues. Lastly, contrasting with rodent models with increased gluconeogenesis (32–34), we showed that fasting/feeding regulation of PEPCK and G6Pase genes was preserved in liverα2KO mice, suggesting that hepatic nutrient sensing as well as the action of the regulatory hormones insulin and glucagon do not be depend on the AMPKα2 activity in the liver. In summary, increased gluconeogenesis contrasted with normal hepatic insulin sensitivity in liverα2KO mice, indicating that inhibition of gluconeogenesis by insulin does not require AMPKα2. Furthermore, we found no evidence of any contribution by lipids to the difference in EGP between liverα2KO mice and controls.

In a second set of experiments, we studied whether regulation of hepatic glucose production by insulin, leptin, and adiponectin required AMPKα2 activity. During hyperinsulinemic clamping by infusing insulin at a physiological rate, whole-body insulin sensitivity was preserved in the absence of hepatic AMPKα2. Furthermore, the absence of the AMPKα2 subunit did not affect insulin signaling in hepatocytes because *in vivo* Akt stimulation by insulin was similar in liverα2KO mice and control mice. Consequently, although we cannot exclude the possibility that remaining AMPKα1

catalytic subunit could account for the inhibitory effect of insulin, it is more likely that the AMPKα2 catalytic subunit is not required for EGP suppression by insulin. It is possible that AMPK and insulin could activate distinct pathways or converge at a point downstream from AMPK to affect gluconeogenic gene expression.

In contrast to the effects of insulin, the regulation of EGP by leptin and adiponectin was impaired in the liverα2KO mice. Rossetti *et al.* (35) demonstrated in the rat that in the presence of a low rate of insulin infusion, leptin had a major metabolic effect on the intrahepatic partitioning of glucose fluxes. The inhibitory effect of insulin on hepatic glucose production is substantially enhanced by leptin treatment (23) due to leptin strongly stimulating gluconeogenic activity. This explains why the PEPCK mRNA concentration was 3-fold higher after the 6-h leptin infusion than before, suggesting that the hormone has gluconeogenic activity. Similarly, we demonstrated here that in the fasted state, the rate of whole-body glucose use was increased during leptin infusion in control mice. Because glycemia remained constant throughout the leptin infusion, these observations show that hepatic glucose production was enhanced to balance the peripheral demand for glucose. Thus, leptin increases peripheral glucose uptake, which in turn enhances hepatic glucose production (via stimulated gluconeogenesis) in a coordinate way. In addition, in both rodent animal models, the glycogen stores are depleted by leptin treatment (23, 35). By affecting hepatic gluconeogenesis and/or glycogen stores, stimulation of hepatic glucose production in C57BL/6J ob/ob mice after an iv leptin infusion was associated with increased G6Pase activity (36).

Our data demonstrate that hepatic glucose production is not regulated by leptin in the absence of AMPKα2, as it is in control mice. Interestingly, leptin plasma levels were increased in liverα2KO mice in the fasted state, and the expected regulation of leptin levels by feeding was lost. These up-regulated and unregulated leptin levels contrast with the absence of obesity or infertility that affects db/db mice. This may be due to the modest increase of leptin levels in liverα2KO mice, compared with that in db/db mice. In genetically engineered or diet-induced rodent models with high plasma levels of leptin, whole-body insulin sensitivity was reduced in a context of leptin resistance (37). Here this is not the case because liverα2KO mice did not display insulin resistance. Consequently, it is not clear how higher leptinemia in liverα2KO mice could indicate an adaptation of endogenous leptin secretion to the lack of hepatic effect of leptin rather than a generalized leptin resistance. Interestingly, although plasma leptin concentrations were higher during infusion than those observed spontaneously, this did not restore control of EGP by leptin. Presumably the hepatic leptin effect depends on active AMPKα2 subunit in the liver rather than on the leptin concentration itself.

The adipocyte-derived hormone adiponectin inhibits PEPCK and G6Pase gene expression through the activation of AMPK (10), suggesting that it can suppress hepatic glucose production in the fasted state. Little is known about the *in vivo* effect of adipokine on glucose fluxes, and therefore, we infused the hormone into fasted mice and studied glucose fluxes. Adiponectin infusion increased EGP and whole-body glucose use in control mice. However, it failed to control EGP and glucose fluxes generally in mutant mice, suggesting that, as observed with leptin, the regulatory effects of adiponectin on EGP are strictly dependent on a functional AMPKα2 isoform in liver. Interestingly, adiponectin plasma levels in liverα2KO mice were higher than in controls. Spiegelman's group reported that there was less adiponectin mRNA in obese diabetic murine model db/db mice than controls (38). Plasma levels of adiponectin have also been reported to be significantly reduced in obese/diabetic mice and humans (39, 40). Thus, reductions in plasma adiponectin levels are commonly observed in a variety of states frequently associated with insulin resistance. In contrast, adiponectin upregulates insulin sensitivity (41). Thus, increased adiponectin levels in liverα2KO mice could be an adaptive phenomenon to compensate for the glucose intolerance and increased EGP. This probably explains, in part, the preserved whole-body insulin sensitivity observed in this model. Furthermore, if adiponectin increases peripheral glucose uptake, in turn enhancing EGP as observed in control mice, the absence of hepatic AMPKα2 subunit might cause resistance to the effects of adiponectin in peripheral tissues in which a functional α2-subunit is present.

This study demonstrates that the absence of the hepatic AMPKα2 catalytic subunit is sufficient to increase basal EGP, a key feature of type 2 diabetes mellitus. In addition, deletion of the hepatic AMPKα2 catalytic subunit gene did not affect hepatic insulin sensitivity but did affect the action of leptin and adiponectin on EGP. Importantly, we also showed that the AMPKα1 isoform could not substitute for the α2-subunit in mediating the hepatic effects of adipokines.

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