PRLR Regulates Hepatic Insulin Sensitivity in Mice via STAT5

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Insulin resistance is one of the major contributing factors in the development of metabolic diseases. The mechanisms responsible for insulin resistance, however, remain poorly understood. Although numerous functions of the prolactin receptor (PRLR) have been identified, a direct effect on insulin sensitivity has not been previously described. The aim of our current study is to investigate this possibility and elucidate underlying mechanisms. Here we show that insulin sensitivity is improved or impaired in mice injected with adenovirus that overexpress or knock down PRLR expression, respectively. Similar observations were obtained in in vitro studies. In addition, we discovered that the signal transducer and activator of transcription-5 pathway are required for regulating insulin sensitivity by PRLR. Moreover, we observed that PRLR expression is decreased or increased under insulin-resistant (*db/db* mice) or insulin-sensitive (leucine deprivation) conditions, respectively, and found that altering PRLR expression significantly reverses insulin sensitivity under both conditions. Finally, we found that PRLR expression levels are increased under leucine deprivation via a general control nonderepressible 2/mammalian target of rapamycin/ribosomal protein S6 kinase-1–dependent pathway. These results demonstrate a novel function for hepatic PRLR in the regulation of insulin sensitivity and provide important insights concerning the nutritional regulation of PRLR expression. ***Diabetes*** **62:3103–3113, 2013**

In recent years, there has been an increase in the global prevalence of type 2 diabetes (T2D) for which insulin resistance is a common feature (1). Hormones secreted from different tissues, such as adiponectin, greatly contribute to the regulation of insulin sensitivity (2). Because the functions of hormones are mediated via binding to their cell surface receptors (2), hormone receptors are the key factors that ultimately determine specific physiological responses. Although prolactin (PRL), a hormone produced predominantly by the anterior pituitary gland (3), has previously been implicated in T2D and the regulation of glucose metabolism (4,5), the role of the prolactin receptor (PRLR) in the regulation of insulin sensitivity is unknown.

PRLR was first identified as a specific, high-affinity, membrane-anchored receptor in 1975 (6), and the cDNA encoding the rat PRLR was isolated in the late 1980s (7). The biological functions of PRLR are mainly mediated by the activation of signal transducer and activator of transcription (STAT)-5, extracellular signal–related kinases (ERKs), and phosphatidylinositol 3-kinase pathways (8). PRLR is expressed in all organs and/or tissues and functions in the regulation of numerous biological events (9).

In addition to its well-known functions in reproductive processes (3), PRLR has been implicated in glucose homeostasis, as glucose levels are higher in *Prlr* knock-out mice after glucose injection (10). PRLR regulates glucose levels indirectly by modulating the secretion of insulin via changes in islet density, β-cell number, and mass (10,11). Consistent with these results, knocking down PRLR expression decreases glucose-induced insulin secretion in islets isolated from rats (12). It is unknown, however, whether PRLR is involved in the regulation of insulin sensitivity, another critical mechanism for glucose homeostasis (13). Previous in vitro studies have shown that PRLR increases protein kinase B (Akt) phosphorylation (14) and forms a complex with insulin receptor (IR) substrate 1 (IRS1) after prolactin stimulation (15). Because IRS1 and Akt are major components of insulin signaling (16), PRLR may play a role in regulating insulin sensitivity. Furthermore, because PRLR expression and serum prolactin levels are decreased in response to reduced maternal nutrition or low-protein diet (17,18), the availability of amino acids may play an important role in regulating PRLR expression. The aim of our current study is to investigate these possibilities and elucidate the underlying mechanisms.

In our current study, we observed that whole-body and hepatic insulin sensitivity are improved or impaired in mice injected with genetically engineered adenoviruses (Ads) that overexpress or knock down PRLR expression, respectively. In addition, we found that STAT5 is required for regulating insulin sensitivity by PRLR. Furthermore, we observed that the expression of PRLR is altered under conditions of insulin resistance (*db/db* mice) and increased insulin sensitivity (dietary leucine deprivation), and manipulations of PRLR expression have significant impact on insulin sensitivity under both conditions. Moreover, we found that PRLR expression levels are regulated by the availability of amino acids via a general control nonderepressible 2 (GCN2)/ mammalian target of rapamycin (mTOR)/ribosomal protein S6 kinase-1 (S6K1)–dependent pathway. Finally, we confirmed the effects of PRLR on insulin signaling in in vitro studies. Taken together, these results identify a novel function for hepatic PRLR in the regulation of insulin sensitivity and provide important insights into the nutritional regulation of PRLR expression.

RESEARCH DESIGN AND METHODS

Animals and treatment. Male C57BL/6J mice were obtained from Shanghai Laboratory Animal Co., Ltd. (Shanghai, People's Republic of China). GCN2 knock-out ($Gcn2^{-/-}$) and leptin receptor–deficient (*db/db*) mice were provided by Dr. Douglas Cavener (Penn State University, State College, PA) and Dr. Xiang Gao (Nanjing University, Nanjing, People's Republic of China),

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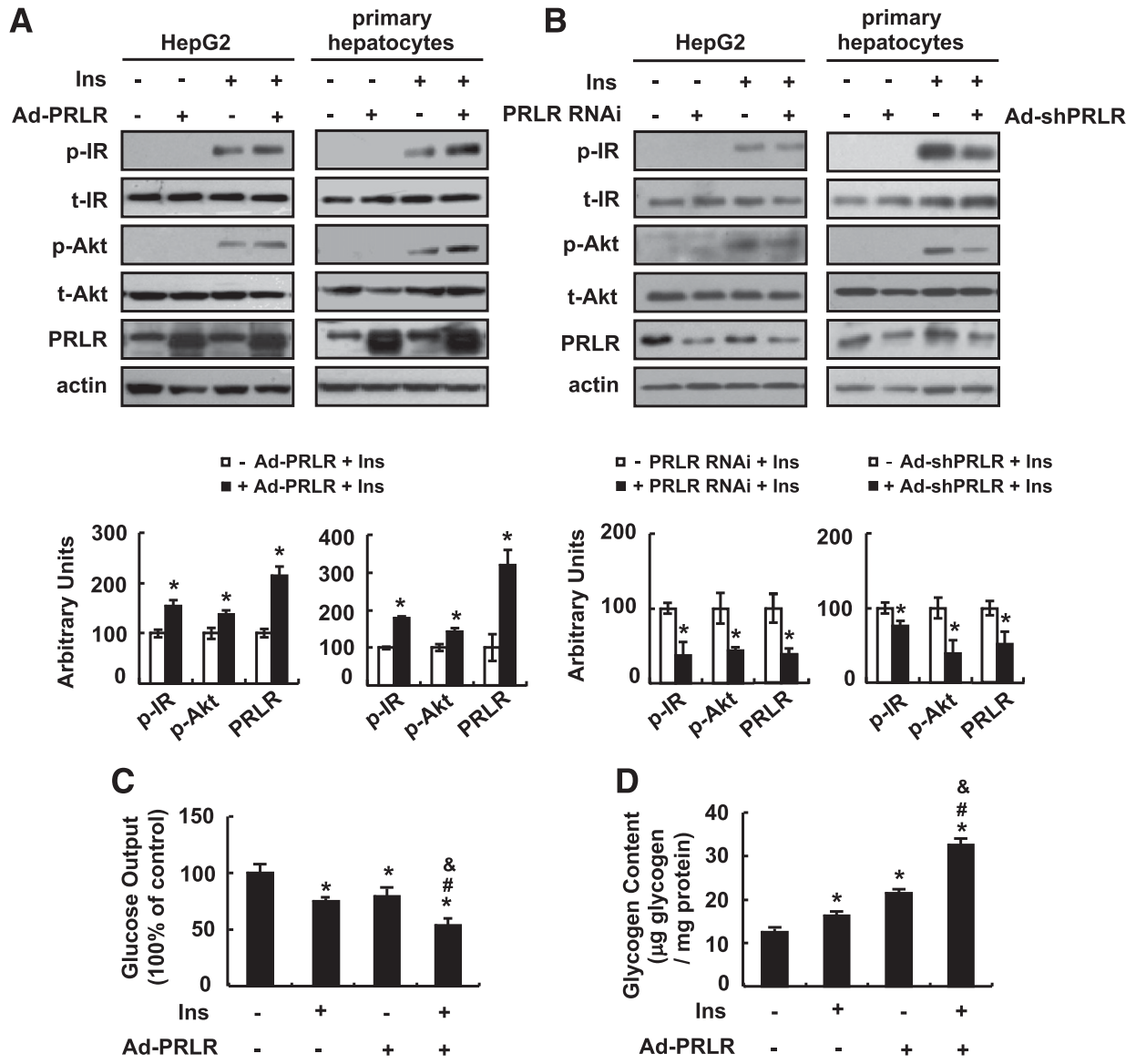

FIG. 1. PRLR regulates insulin (Ins) sensitivity in vitro. *A* and *B*: Cells were infected with Ad-PRLR (+ Ad-PRLR) or GFP (− Ad-PRLR) for 48 h in *A*. HepG2 cells were treated with PRLR siRNA (+ PRLR RNAi) or control reagent (− PRLR RNAi) for 48 h, and primary hepatocytes were infected with Ad-shPRLR (+ Ad-shPRLR) or scrambled vector (− Ad-shPRLR) for 72 h in *B*; both cases followed with (+ Ins) or without (− Ins) 100 nmol/L insulin stimulation for 20 min. *C* and *D*: Primary hepatocytes were exposed to Ad-PRLR (+ Ad-PRLR) or were without Ad-PRLR (− Ad-PRLR) for 24 h. The cells were then treated with or without 100 nmol/L insulin for another 24 h, followed by the measurement of glucose production or glycogen content. The mean ± SEM values shown are representative of at least three independent in vitro experiments. Statistical significance was calculated using one-way ANOVA followed by the SNK test for the effects of any group vs. the − Ad-PRLR group without insulin stimulation (*$P < 0.05$), with vs. without insulin stimulation in the + Ad-PRLR group (#$P < 0.05$), or Ad-PRLR vs. the control group after insulin stimulation (&$P < 0.05$). *A* and *B*: p-IR (tyr1150/1151), p-Akt (ser473), and PRLR protein (*top*, Western blot; *bottom*, quantitative measurements of p-IR, p-Akt, and PRLR protein relative to their total protein or actin). *C*: Glucose output assay. *D*: Glycogen content. t, total.

respectively. Eight- to 10-week-old mice were maintained on a 12-h light/dark cycle at 25°C, and provided free access to commercial rodent chow and tap water prior to initiation of the experiments. Control diet (a nutritionally complete amino acid) and leucine-deficient [(−) leu] diet were obtained from Research Diets, Inc. (New Brunswick, NJ), and feeding experiments were performed as previously described (19). In rapamycin-treated groups, rapamycin (Tauto Biotech, Shanghai, People's Republic of China) was delivered by intraperitoneal injection at a dose of 1 mg/kg body weight, with control animals receiving vehicle alone (20). These experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences.

Primary hepatocyte isolation, cell culture, and treatments. Hepatocytes were prepared by collagenase perfusion as described previously (21). An insulin-resistance model was induced by incubating HepG2 cells with 18 mmol/L glucosamine for 18 h as previously described (22). Control (complete amino acid) and (−) leu medium were prepared as described previously (19). For small interfering RNA (siRNA) transfection in HepG2 cells, double-stranded siRNA targeting human PRLR was purchased from GenePharma (Shanghai, People's Republic of China). The siRNA sequence is specific for human PRLR: 5′- GAAGCAUUGUUCUAGACAATTUUGUCUAGAACAAUGCUUCTT-3′. Cells were transfected with PRLR siRNA using X-tremeGene siRNA Transfection Reagent (Roche Diagnostics, Mannheim, Germany). Glucose output and glycogen synthesis were measured as previously described (23,24).

Generation and administration of recombinant Ads. Recombinant Ads for expression of mouse long-form PRLR or hemagglutinin (HA)-tagged constitutively active S6K1 (CA-S6K1) were generated using the AdEasy Adenoviral Vector System (Qbiogene, Irvine, CA) according to the manufacturer's instructions. The cDNA of mouse long-form PRLR was a gift from Dr. Akihiko Yoshimura (Kyushu University, Fukuoka, Japan). The cDNA of HA-tagged CA-S6K1 was from the Addgene plasmid 8991, and originally from John Blenis's laboratory (19). Ads expressing either scrambled short hairpin RNA (shRNA) or

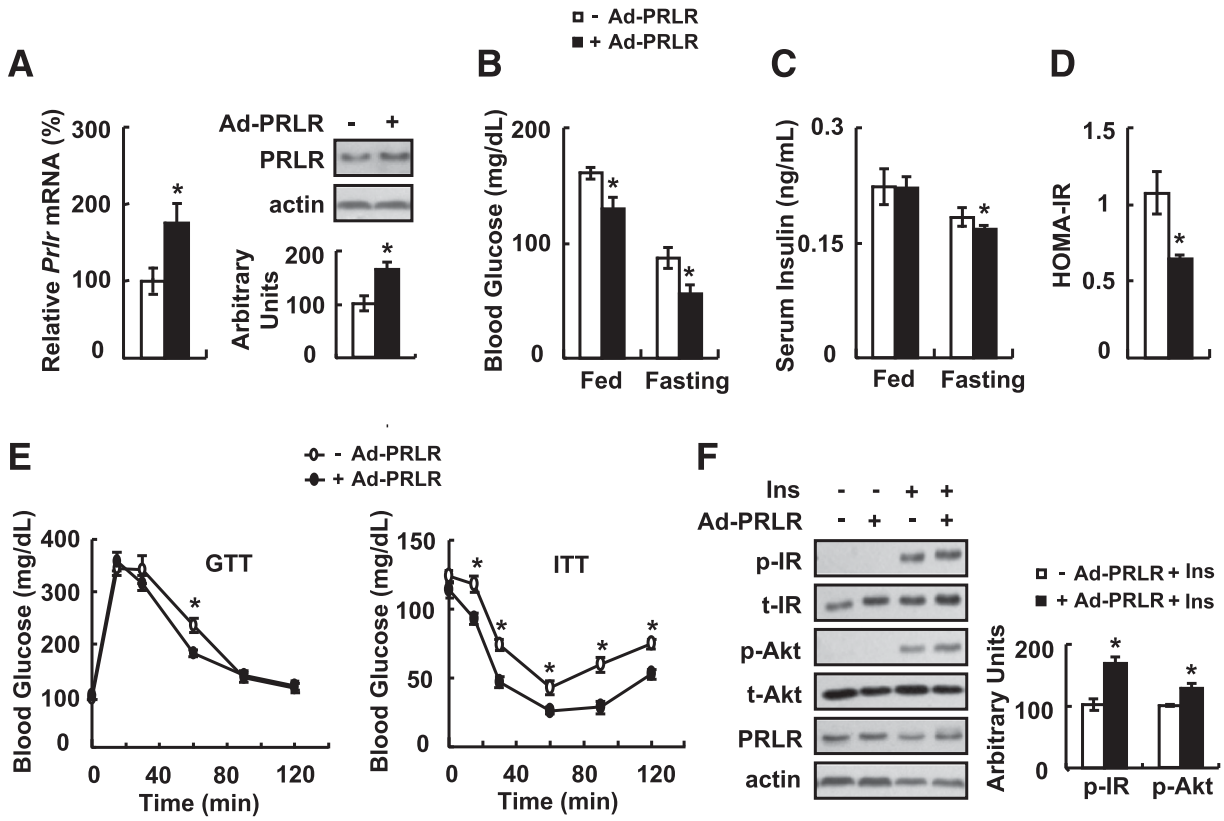

FIG. 2. Overexpression of PRLR using Ad-PRLR improves insulin (Ins) sensitivity in vivo. Male C57BL/6J mice were infected with Ad-PRLR (+ Ad-PRLR) or GFP (− Ad-PRLR) via tail-vein injection, followed by examination of PRLR expression in the liver at day 12 in *A*, measurement of blood glucose and serum insulin levels at day 14 in *B* and *C*, calculating HOMA-IR index in *D*, performance of GTTs and ITTs at day 8 or 10 in *E*, and examination of insulin signaling in liver before (− Ins) and after (+ Ins) 2 units/kg insulin stimulation for 3 min at day 12 in *F*. The mean ± SEM values shown are representative of at least two independent in vivo experiments, with the number of mice included in each group in each experiment indicated (n = 5–6). Statistical significance was calculated using the two-tailed Student t test for the effects of the Ad-PRLR vs. the control group (*$P < 0.05$). *A*: *Prlr* mRNA and PRLR protein (*top*, Western blot; *bottom*, quantitative measurement of PRLR protein relative to actin). *B*: Blood glucose levels. *C*: Serum insulin levels. *D*: HOMA-IR index. *E*: GTT and ITT. *F*: p-IR (tyr1150/1151), p-Akt (ser473), and PRLR protein (*left*, Western blot; *right*, quantitative measurements of p-IR and p-Akt protein relative to their total protein). t, total.

shRNA specific for mouse PRLR were generated using the BLOCK-iT Adenoviral RNAi Expression System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The scrambled sequence is 5′-TTCTCCGAACGTGTCACGT-3′. The shRNA sequence for mouse PRLR is 5′-GCCACCTACCATAACTGATGT-3′, which targets both long- and short-form PRLR. In the liver, STAT5b is 20-fold more abundant than STAT5a (25), so we used recombinant Ads expressing shRNA against STAT5b in the current study. The shRNA sequence specific for mouse STAT5b: 5′-GGGACCTGAATTACCTCATAT-3′. High-titer stocks of amplified recombinant Ads were purified as previously described (19). Viruses were diluted in PBS and administered at a dose of 10^7 plaque-forming units/well in 12-well plates or via tail-vein injection using 10^9 plaque-forming units/mouse.

Blood glucose, serum insulin, glucose tolerance test, insulin tolerance test, and homeostasis model assessment of IR index. Levels of blood glucose and serum insulin were measured using a Glucometer Elite monitor or Mercodia Ultrasensitive Rat Insulin ELISA kit (ALPCO Diagnostic, Salem, NH), respectively. Glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) were performed by intraperitoneal injection of 2 g/kg glucose after overnight fasting and 0.75 units/kg insulin after 4-h fasting, respectively. Homeostasis model assessment (HOMA) of IR index was calculated according to the following formula: [fasting glucose levels (mmol/L)] × [fasting serum insulin (μU/mL)]/22.5. The area under the curves was calculated as previously described (26).

In vivo insulin signaling assay. Mice maintained on different diets were fasted for 6 h prior to insulin injection as previously described (21). Sections of liver were excised from anesthetized mice, snap frozen, and kept as untreated controls. Three minutes after injection with 2 units/kg insulin via the portal vein, pieces of liver section were excised and snap frozen for Western blot analysis.

Western blot analysis. Western blot analysis was performed as previously described (19). Primary antibodies [anti-phosphorylated (p)-IR (tyr1150/1151), anti-IR, anti-p-Akt (ser473), anti-Akt, anti-p-STAT5 (Tyr 694), anti-STAT5, anti-p-S6 (ser235/236), and anti-S6 (all from Cell Signaling Technology, Beverly, MA), and anti-PRLR [sc-20992 (only detects the long-form PRLR); Santa Cruz Biotechnology, Santa Cruz, CA] were incubated overnight at 4°C, and specific proteins were visualized by ECL Plus (Amersham Biosciences, Amersham, Buckinghamshire, U.K.). Band intensities were measured using Quantity One (Bio-Rad Laboratories, Hercules, CA) and normalized to total protein or actin.

RNA isolation and relative quantitative RT-PCR. *Prlr* mRNA levels were examined by RT-PCR as previously described (27). The sequences of primers used to specifically detect the mouse long-form PRLR were as follows: sense primer, 5′-TGAGGACGAGCGGCTAATG-3′; and antisense primer, 5′-GGTGTGTGGGTT TAACACCTTGA-3′.

Statistics. All data are expressed as the mean ± SEM. Significant differences were assessed either by two-tailed Student t test or one-way ANOVA followed by the Student-Newman-Keuls (SNK) test. $P < 0.05$ was considered statistically significant.

RESULTS

PRLR regulates insulin sensitivity in vitro. To explore a role for PRLR in the regulation of insulin sensitivity, we examined the effect of PRLR overexpression on phosphorylation of IR (tyr1150/1151) and Akt (ser473), the two major components of insulin signaling (16), by infecting HepG2 cells and primary cultured hepatocytes with Ad expressing PRLR (Ad-PRLR) or control green fluorescent protein (Ad-GFP). Previous studies have shown that multiple functions of PRL are enhanced by the upregulation of PRLR, and the overexpression of PRLR stimulates the PRL-related intracellular signaling (28). We found that insulin-stimulated phosphorylation of IR and Akt was significantly elevated in

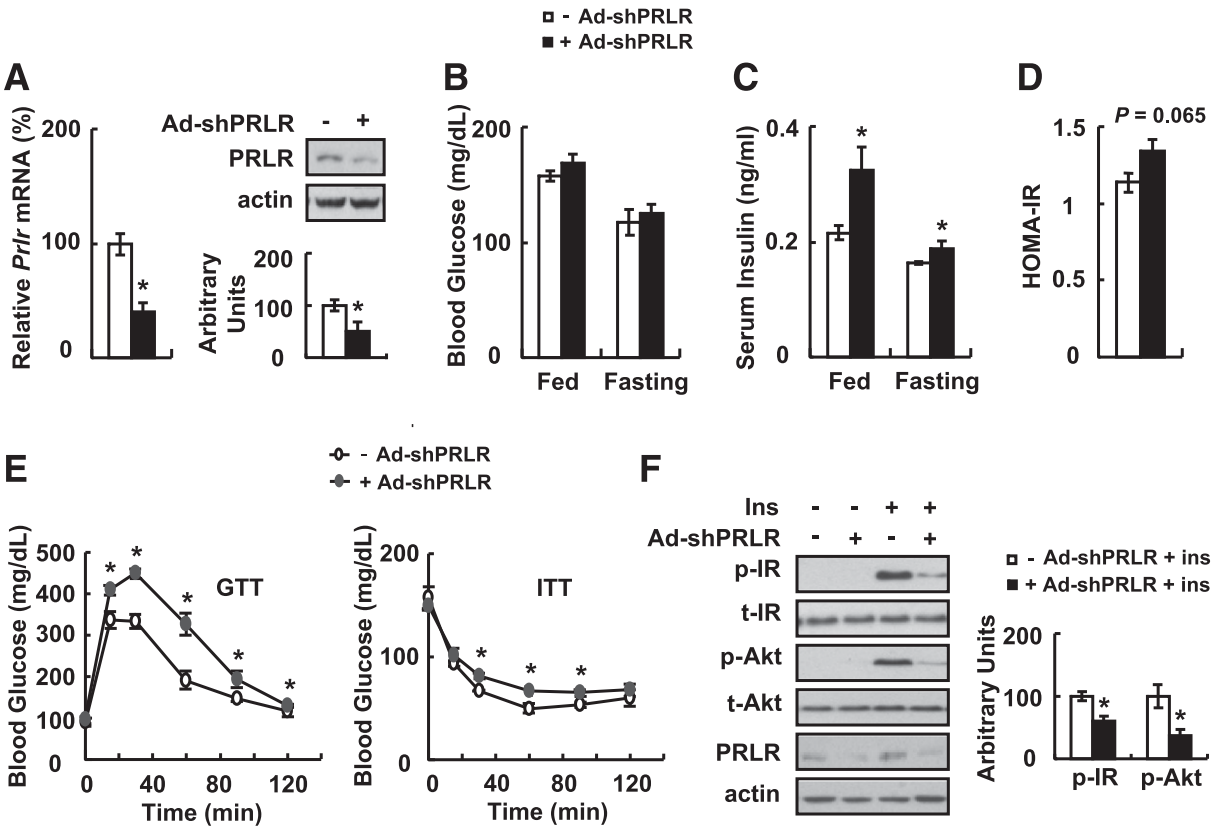

FIG. 3. Knocking down PRLR using Ad-shPRLR decreases insulin (Ins) sensitivity in vivo. Male C57BL/6J mice were infected with Ad-shPRLR (+ Ad-shPRLR) or scrambled vector (− Ad-shPRLR) via tail-vein injection, followed by examination of PRLR expression in the liver at day 7 in *A*, measurement of blood glucose and serum insulin levels at day 14 in *B* and *C*, calculating HOMA-IR index in *D*, performance of GTTs and ITTs at day 3 or 4 in *E*, and examination of insulin signaling in liver before (− Ins) and after (+ Ins) 2 units/kg insulin stimulation for 3 min at day 7 in *F*. The mean ± SEM values shown are representative of at least two independent in vivo experiments, with the number of mice included in each group in each experiments indicated (n = 5–6). Statistical significance was calculated using the two-tailed Student t test for the effects of the Ad-shPRLR vs. the control group (*$P < 0.05$). *A*: *Prlr* mRNA and PRLR protein (*top*, Western blot; *bottom*, quantitative measurement of PRLR protein relative to actin). *B*: Blood glucose levels. *C*: Serum insulin levels. *D*: HOMA-IR index. *E*: GTT and ITT. *F*: p-IR (tyr1150/1151), p-Akt (ser473), and PRLR protein (*left*, Western blot; *right*, quantitative measurements of p-IR and p-Akt protein relative to their total protein). t, total.

cells overexpressing PRLR (as shown by Western blotting using anti-PRLR antibodies) in both HepG2 and primary cultured hepatocytes (Fig. 1*A*). Furthermore, insulin-stimulated phosphorylation of IR and Akt was impaired when endogenous PRLR protein levels were reduced by knocking down PRLR expression via RNA interference (RNAi) or Ad expressing shRNA directed against the coding region of PRLR (Ad-shPRLR) (Fig. 1*B*). We also examined glucose production and glycogen synthesis in primary hepatocytes infected with Ad-PRLR or control Ads. We found that the overexpression of PRLR significantly decreased glucose production and increased glycogen synthesis compared with control cells, in the presence or absence of insulin (Fig. 1*C* and *D*).

Overexpression of PRLR improves insulin sensitivity in vivo. To investigate the effects of PRLR on the regulation of insulin sensitivity in vivo, we infected male mice with Ad-PRLR or Ad-GFP via tail-vein injection and found that *Prlr* mRNA and protein levels were significantly increased in the livers of these mice (Fig. 2*A*). Increased PRLR expression significantly decreased blood glucose levels in mice under both fed and fasting conditions (Fig. 2*B*). Although fed serum insulin levels remained unchanged, fasting serum insulin levels were significantly decreased in Ad-PRLR mice (Fig. 2*C*). Consistently, the HOMA-IR index was also decreased in these mice (Fig. 2*D*). Glucose tolerance and clearance were further examined by GTTs and ITTs, respectively. Although the difference in GTT between Ad-PRLR mice and the control group was small, blood glucose levels decreased much more quickly 15 min after the administration of insulin in Ad-PRLR mice (Fig. 2*E*). In addition, insulin-stimulated phosphorylation of IR and Akt was also increased in the livers of these mice (Fig. 2*F*). Similar results were obtained in female mice (data not shown).

Knocking down PRLR expression impairs insulin sensitivity in vivo. To further investigate the impact of PRLR on insulin sensitivity in vivo, we examined insulin signaling in mice injected with Ad-shPRLR or Ad scrambled. As predicted, *Prlr* mRNA and protein expression levels were significantly reduced in the livers of Ad-shPRLR mice (Fig. 3*A*). Though fed and fasting blood glucose levels remained unchanged (Fig. 3*B*), serum insulin levels were significantly increased in Ad-shPRLR mice under both fed and fasting conditions (Fig. 3*C*). HOMA-IR index values were also increased in these mice (Fig. 3*D*). Consistent with these changes, Ad-shPRLR mice exhibited decreased glucose tolerance and clearance as measured by GTTs and ITTs, respectively (Fig. 3*E*). Insulin-stimulated phosphorylation of IR and Akt was also greatly impaired in the livers of mice infected with Ad-shPRLR (Fig. 3*F*).

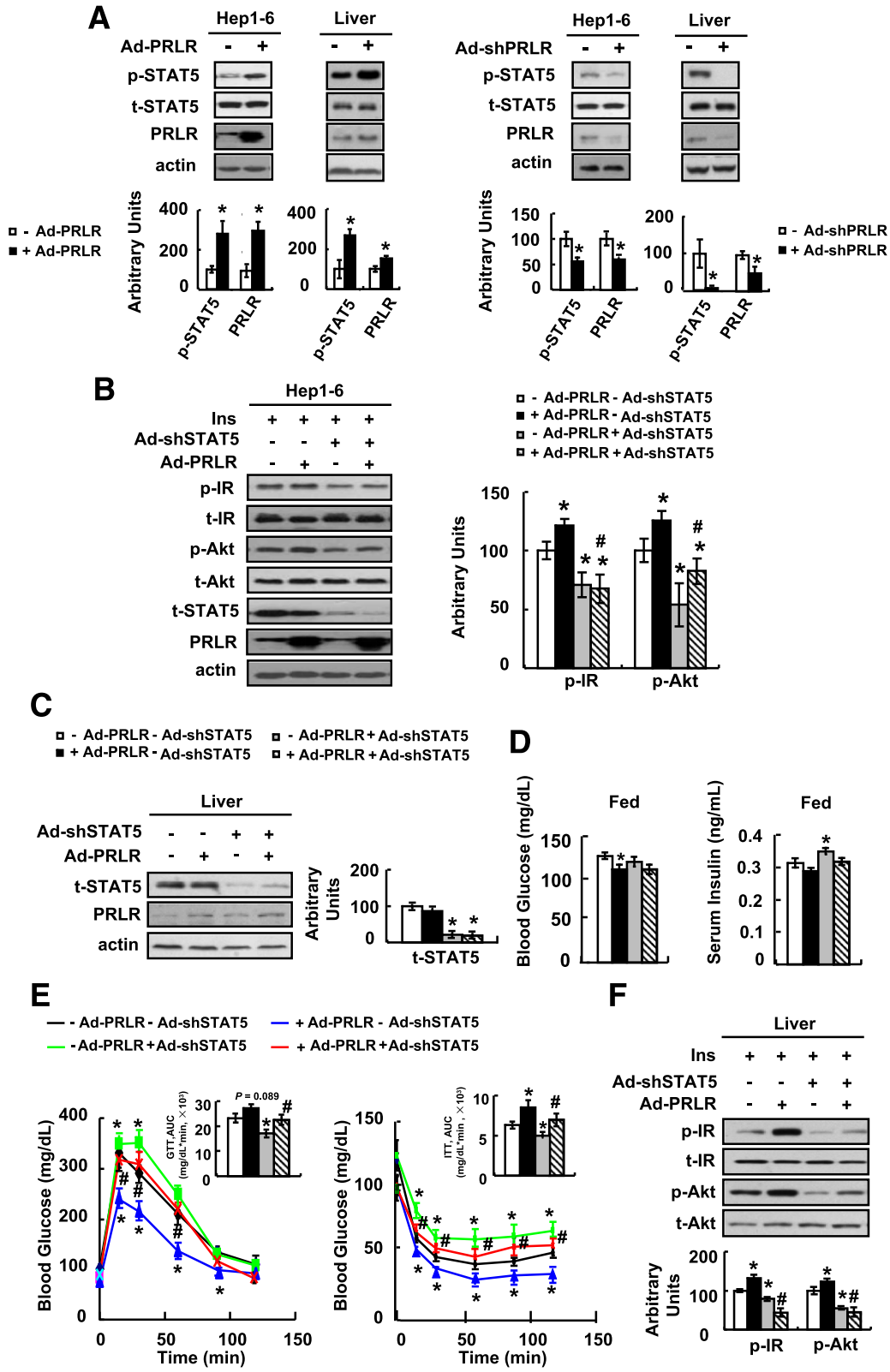

FIG. 4. Ad-PRLR increases insulin sensitivity by activation of STAT5. *A*, *left*: Hep1–6 cells were infected with Ad-PRLR (+ Ad-PRLR) or GFP (− Ad-PRLR) for 48 h, or male C57BL/6J mice were infected with Ad-PRLR (+ Ad-PRLR) or Ad-GFP (− Ad-PRLR) via tail-vein injection, followed by examination of p-STAT5 in the liver at day 12. *A*, *right*: Hep1–6 cells were exposed to Ad-shPRLR (+ Ad-shPRLR) or scrambled vector (− Ad-shPRLR) for 72 h, or male C57BL/6J mice were infected with Ad-shPRLR (+ Ad-shPRLR) or scrambled vector (− Ad-shPRLR) via tail-vein injection, followed by examination of p-STAT5 in the liver at day 7. *B*: Hep1–6 cells were infected with Ad-shRNA against STAT5b (+ Ad-shSTAT5) or scrambled Ad (− Ad-shSTAT5) for 24 h prior to being infected with Ad-PRLR (+ Ad-PRLR) or Ad-GFP (− Ad-PRLR) for 48 h, followed with 10 nmol/L insulin stimulation for 3 min. *C–F*: Male C57BL/6J mice were infected with Ad-PRLR (+ Ad-PRLR) or Ad-GFP (− Ad-PRLR) and with Ad-shSTAT5b (+ Ad-shSTAT5b) or scrambled Ad (− Ad-shSTAT5b) via tail-vein injection, followed by examination of STAT5 expression in the liver at day 8 in *C*, measurement of fed blood glucose and serum insulin levels at day 7 in *D*, performance of GTTs and ITTs at day 4 or 6 in *E*, and examination of insulin signaling in liver before (− Ins) and after (+ Ins) 2 units/kg insulin stimulation for 3 min at day 8 in *F*. The mean ± SEM

Ad-PRLR increases insulin sensitivity by activation of STAT5. Previous studies have proposed STAT5 as a downstream target of PRLR signaling (29), and STAT5 has also been implicated in regulating insulin responses (30,31), suggesting the possibility that STAT5 may mediate the effect of PRLR on insulin sensitivity. For these reasons, we examined the effects of PRLR on STAT5 phosphorylation and found that STAT5 phosphorylation was increased by Ad-PRLR and decreased by Ad-shPRLR in Hep1–6 cells and liver (Fig. 4*A*). The possible involvement of STAT5 in PRLR-stimulated insulin signaling was then investigated in Hep1–6 cells infected with Ads expressing shRNA against STAT5b (Ad-shSTAT5b), the most abundant isoform of STAT5 in the liver (25). As predicted, we found that knocking down STAT5b significantly blocked PRLR-enhanced insulin signaling in these cells and Ad-shSTAT5b alone also decreased insulin signaling in the absence of PRLR (Fig. 4*B*). To gain further insights into the importance of STAT5 in regulating insulin sensitivity by PRLR in vivo, we injected mice with Ad-shSTAT5b and examined its effect on PRLR-enhanced insulin sensitivity. Functional validation of Ad-shSTAT5b was demonstrated by its ability to reduce levels of STAT5 in the liver (Fig. 4*C*). Although Ad-shSTAT5b had no effect on PRLR-decreased blood glucose levels (Fig. 4*D*), the injection of Ad-shSTAT5b largely attenuated PRLR-potentiated glucose tolerance, glucose clearance, and insulin signaling in the liver (Fig. 4*E* and *F*). Consistent with previous reports (30), knocking down STAT5b alone also decreased glucose tolerance, glucose clearance, and insulin signaling in the liver (Fig. 4*E* and *F*). Fed serum insulin levels also increased in mice infected with Ad-shSTAT5b alone compared with control mice (Fig. 4*D*).

Overexpression of PRLR improves insulin sensitivity under insulin-resistant conditions in vitro and in vivo. Based on the above results, we speculated that PRLR might also be involved in the regulation of insulin sensitivity under insulin-resistant or insulin-sensitive conditions. To test this possibility, we first examined expression levels of PRLR in an insulin-resistant cell model, in which insulin resistance was induced by pretreatment of HepG2 cells with 18 mmol/L glucosamine for 18 h as described previously (22). PRLR protein levels were found to decrease significantly in these cells (Fig. 5*A*). Overexpression of PRLR significantly reversed the effect of glucosamine on decreasing phosphorylation of IR and Akt (Fig. 5*B*).

We next examined PRLR expression under insulin resistance conditions in vivo using *db/db* mice, a genetic model for insulin resistance (32). PRLR protein levels were decreased in the livers of *db/db* mice compared with wild-type mice (Fig. 5*C*). To investigate whether decreased PRLR was responsible for the impaired insulin signaling in *db/db* mice, we injected these mice with Ad-PRLR or Ad-GFP and examined the effects on insulin sensitivity. Ad-PRLR injection in *db/db* mice significantly decreased fed and fasting blood glucose levels, though serum insulin levels were not affected (Fig. 5*D* and *E*). Impaired glucose tolerance and clearance in *db/db* mice, as demonstrated in GTTs and ITTs, were also significantly improved by Ad-PRLR injection (Fig. 5*F*). Similar to a previous report (33), blood glucose levels were increased 15 min after insulin injection in *db/db* mice in ITT. Possible reasons for this in *db/db* mice include significantly decreased insulin sensitivity, which prolongs the time for insulin to decrease glucose levels, or a greater susceptibility to stress, such as that by insulin injection.

Knocking down PRLR expression decreases leucine deprivation–enhanced insulin sensitivity in vitro and in vivo. We further explored the possible involvement of PRLR in regulating insulin sensitivity under leucine deprivation, which we have previously shown to enhance insulin sensitivity (19). We found that levels of *Prlr* mRNA and protein were significantly increased in HepG2 cells and livers of mice maintained on a (−) leu medium or diet, respectively (Fig. 6*A* and *B*).

We speculated that increased PRLR expression may contribute to the improved insulin sensitivity under leucine deprivation. To test this hypothesis, we knocked down PRLR expression using RNAi and observed blocking effects on leucine deprivation–potentiated phosphorylation of IR and Akt in HepG2 cells (Fig. 6*C*). We then injected mice with Ad-shPRLR or Ad scrambled and maintained these mice on a control or (−) leu diet for 7 days, as previously described (19). Similar to results obtained in vitro, leucine deprivation–increased glucose tolerance and clearance were impaired by Ad-shPRLR (Fig. 6*D* and *E*). Insulin-stimulated phosphorylation of IR and Akt was also decreased in the livers of these mice (Fig. 6*F*).

PRLR expression is increased by leucine deprivation via a GCN2/S6K1–dependent pathway. Although PRLR plays an important role in regulating insulin sensitivity during leucine deprivation, the mechanisms by which leucine deprivation regulates PRLR expression are unclear. GCN2 is a serine protein kinase that functions as a sensor for amino acid deprivation (34), suggesting the possible involvement of GCN2 in the regulation of PRLR expression during leucine deprivation. To test this possibility, we examined PRLR expression in the livers of $Gcn2^{+/+}$ and $Gcn2^{-/-}$ mice maintained on a control or (−) leu diet for 7 days. As predicted, leucine deprivation–increased *Prlr* mRNA and protein levels were largely blocked in the livers of $Gcn2^{-/-}$ mice (Fig. 7*A* and *B*).

Our previous work has shown that the mTOR/S6K1 signaling pathway is decreased in the livers of leucine-deprived mice (19), suggesting that the decreased mTOR/S6K1 activity might be involved in the regulation of PRLR expression. To test this possibility, we examined whether the injection of Ads expressing CA-S6K1 (Ad-CA-S6K1; as confirmed by Western blotting using anti-HA-tagged antibodies) decreases leucine deprivation-induced PRLR expression. As predicted, leucine deprivation–increased *Prlr* mRNA and protein levels were significantly decreased in

values shown are representative of at least three independent in vitro experiments or at least two independent in vivo experiments, with the number of mice included in each group in each experiment indicated (n = 5–7). Statistical significance was calculated using one-way ANOVA followed by the SNK test for the effects of any group vs. without Ad-PRLR and Ad-shSTAT5b (*P < 0.05), or with vs. without Ad-shSTAT5b in the + Ad-PRLR group (#P < 0.05). *A*: p-STAT5 and PRLR protein (*top*, Western blot; *bottom*, quantitative measurements of p-STAT5 protein relative to its total protein). *B*: p-IR, p-Akt, and STAT5 protein (*left*, Western blot; *right*, quantitative measurements of p-IR and p-Akt protein relative to their total protein). *C*: STAT5 and PRLR protein (*left*, Western blot; *right*, quantitative measurement of STAT5 protein relative to actin). *D*: Blood glucose and serum insulin levels. *E*: GTT (*left*) and ITT (*right*). *F*: p-IR and p-Akt protein in liver (*top*, Western blot; *bottom*, quantitative measurements of p-IR and p-Akt protein relative to their total protein). t, total.

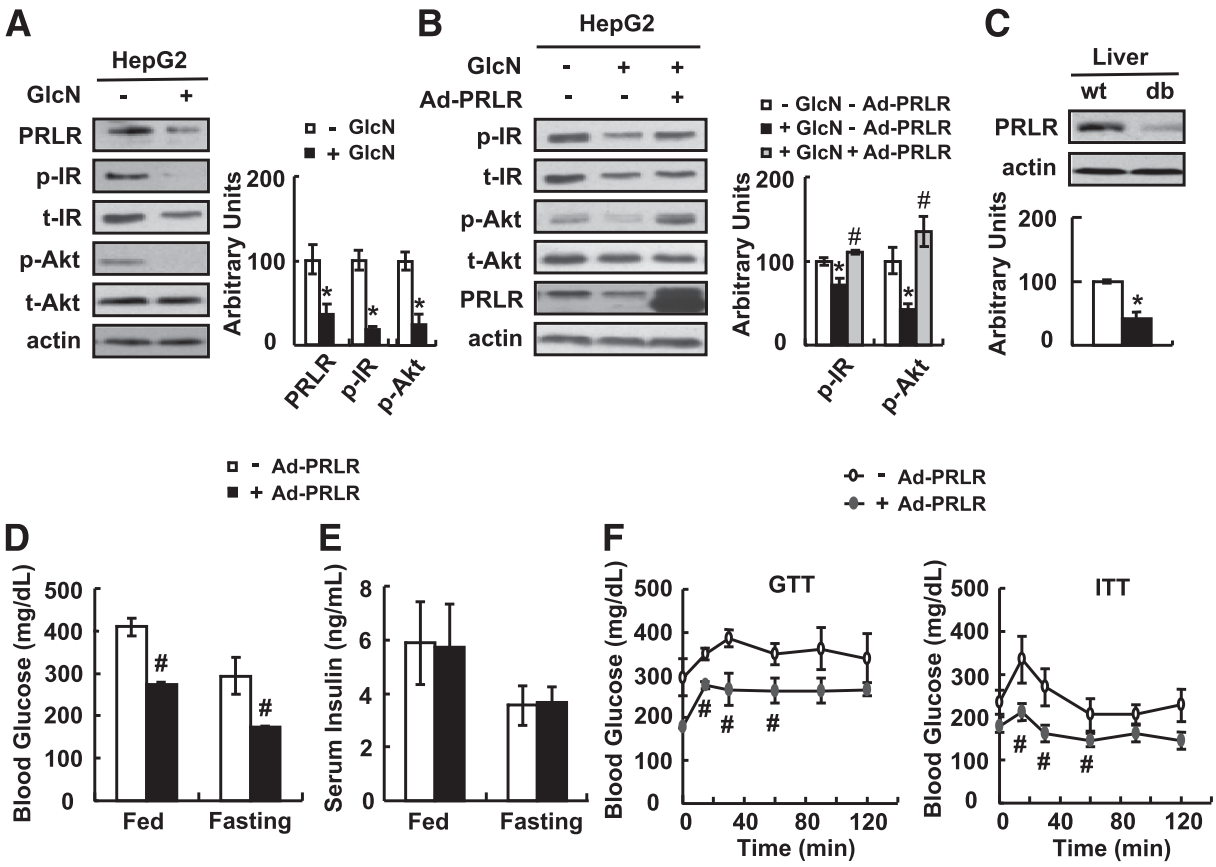

FIG. 5. Overexpression of PRLR by Ad-PRLR improves insulin sensitivity under insulin-resistant conditions. *A* and *B*: HepG2 cells were exposed to Ad-PRLR (+ Ad-PRLR) or GFP (− Ad-PRLR) for 48 h prior to being incubated with (+ GlcN) or without (− GlcN) 18 mmol/L glucosamine for 18 h in the presence of Ad, followed by stimulation with 100 nmol/L insulin for 20 min. *C*: PRLR protein was analyzed by Western blot in the livers of wild-type (wt) and *db/db* (db) mice. *D–F*: Male C57BL/6J *db/db* mice were infected with Ad-PRLR (+ Ad-PRLR) or GFP (− Ad-PRLR) via tail-vein injection, followed by measurement of blood glucose and serum insulin levels at days 11 and 7 in *D* and *E*, and performance of GTTs and ITTs at days 7 and 9 in *F*, respectively. The mean ± SEM values shown are representative of at least three independent in vitro experiments or at least two independent in vivo experiments, with the number of mice included in each group in each experiment indicated (n = 5–7). Statistical significance was calculated using the two-tailed Student t test or one-way ANOVA followed by the SNK test for the effects of insulin-resistant vs. control group (*$P < 0.05$) in *A–C*, Ad-PRLR vs. the control group under insulin resistance (#$P < 0.05$) in *B* and *D–F*. *A* and *B*: PRLR, p-IR (tyr1150/1151), and p-Akt (ser473) protein (*left*, Western blot; *right*, quantitative measurements of PRLR, p-IR, and p-Akt protein relative to actin or their total protein). *C*: PRLR protein (*top*, Western blot; *bottom*, quantitative measurements of PRLR protein relative to actin). *D*: Blood glucose levels. *E*: Serum insulin levels. *F*: GTT and ITT. t, total.

the livers of mice injected with Ad-CA-S6K1 (Fig. 7*C* and *D*). Because decreased S6K1 activity observed under leucine deprivation is found to be GCN2 dependent (19), S6K1 may function in the GCN2 downstream signaling pathway to regulate PRLR expression. This possibility was further investigated in *Gcn2*$^{-/-}$ mice fed a (−) leu diet and injected with the mTOR inhibitor rapamycin (35). Levels of PRLR protein were increased in the livers of rapamycin-treated *Gcn2*$^{-/-}$ mice compared with the control group (Fig. 7*E*).

DISCUSSION

PRLRs are present in nearly all organs and tissues (9). Although numerous biological functions of PRLR have been identified (3,9,10), a direct effect of PRLR on hepatic insulin sensitivity has not previously been described. Several lines of evidence provided in our current study demonstrate a role for PRLR in the regulation of insulin sensitivity. These include the observations that *1*) overexpression of PRLR in vitro increases insulin-stimulated phosphorylation of IR and Akt, and *2*) Ad-PRLR mice exhibit increased glucose clearance and tolerance, whereas knocking down PRLR expression has the opposite effect. Although PRLR has previously been implicated in glucose homeostasis, most of those studies have focused on its effects on insulin secretion by β-cells (10–12). Our study is the first to demonstrate a novel function of PRLR in the regulation of insulin sensitivity. Moreover, these results also suggest that PRLR deficiency in the liver contributes to impaired glucose tolerance in *Prlr*$^{-/-}$ mice, in addition to decreased β-cell function, as shown previously (10). Alternative splicing of the single PRLR gene produces five known isoforms in humans and two isoforms (short- and long-form) in rodents. In rodents, these two isoforms are present in many organs, but are variably expressed in a tissue-specific manner, and different isoforms have independent biological activities (8). Because the Ad-PRLRs used in our experiments overexpress the long-form PRLR, we speculate that the effect of PRLR on insulin sensitivity is most likely mediated by this PRLR isoform, although the shRNA expressing Ads and siRNA used in our study would be expected to knock down both long and short forms of PRLR.

PRLR belongs to the class 1 cytokine receptor superfamily and therefore lacks an intrinsic kinase domain,

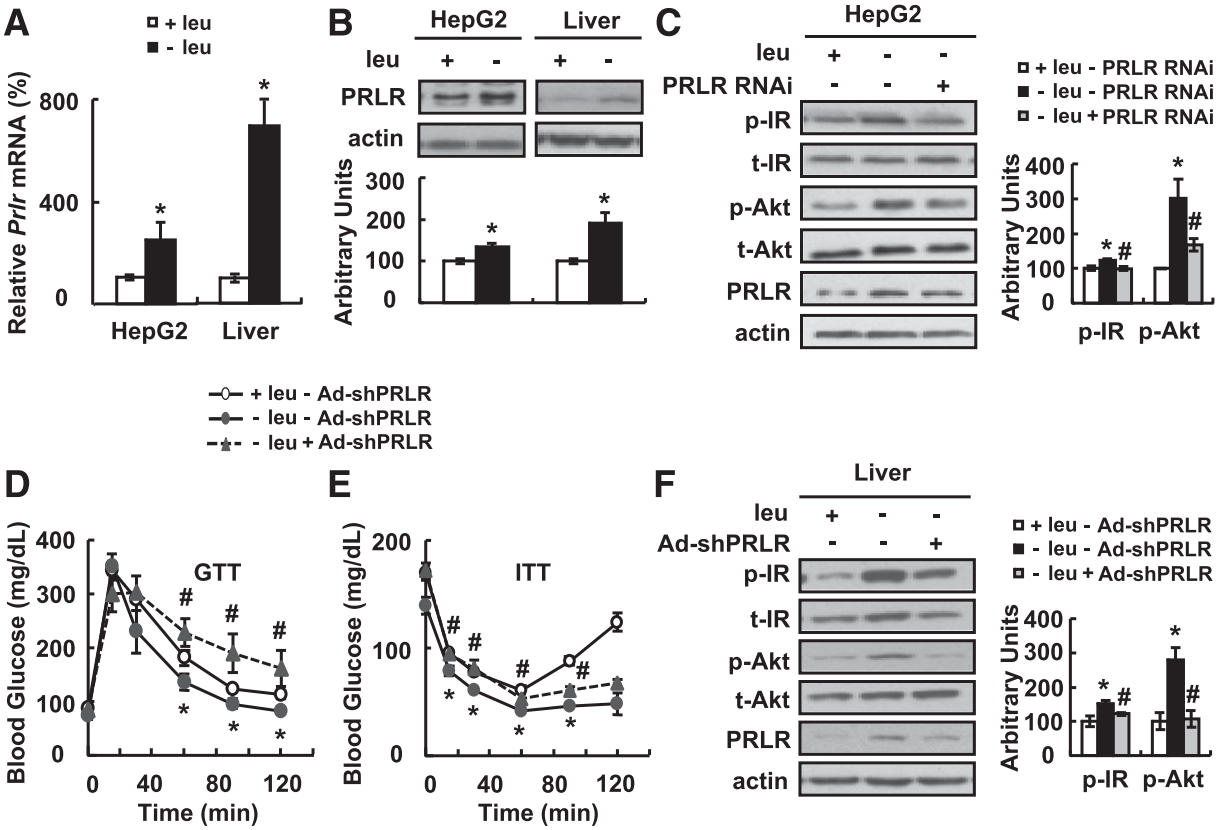

FIG. 6. Knocking down PRLR expression decreases leucine deprivation–enhanced insulin sensitivity in vitro and in vivo. *A* and *B*: Male C57BL/6J mice were fed a control [(+) leu] or (−) leu diet for 7 days, and HepG2 cells were incubated in (+) leu or (−) leu medium for 12 h. *C*: HepG2 cells were treated with PRLR siRNA (+ PRLR RNAi) or control reagent (− PRLR RNAi) for 36 h prior to being cultured in (−) leu or (+) leu media for 12 h, followed by stimulation with 100 nmol/L insulin for 20 min. *D–F*: Mice were infected with scrambled vector (− Ad-shPRLR) and fed a (+) leu diet (indicated by white circles and solid line), infected with scrambled vector (− Ad-shPRLR) and fed a (−) leu diet (indicated by black circles and solid line), or infected with Ad-shRNA against PRLR (+ Ad-shPRLR) and fed a (−) leu diet (indicated by black triangles and dashed line). GTTs and ITTs were performed at day 3 in *D* and *E*, and insulin signaling in liver was examined before (− Ins) and after (+ Ins) 2 units/kg insulin stimulation for 3 min in these mice on day 5 in *F*. The mean ± SEM values shown are representative of at least three independent in vitro experiments or at least two independent in vivo experiments, with the number of mice included in each group in each experiments indicated (n = 5–7). Statistical significance was calculated using the two-tailed Student t test or one-way ANOVA followed by the SNK test for the effects of (−) leu vs. (+) leu treatment (*$P < 0.05$) in *A–F*, Ad-shPRLR vs. the control group under (−) leu treatment (#$P < 0.05$) in *C–F*. *A*: *Prlr* mRNA. *B*: PRLR protein (*top*, Western blot; *bottom*, quantitative measurements of PRLR protein relative to actin). *C* and *F*: p-IR (tyr1150/1151), p-Akt (ser473), and PRLR protein (*left*, Western blot; *right*, quantitative measurements of p-IR and p-Akt protein relative to total IR and Akt, respectively). *D*: GTT. *E*: ITT. t, total.

relying instead on cytoplasmic kinases to transduce signals (36). One of the known downstream targets of PRLR is STAT5 (29), including STAT5a and STAT5b, transcription factors that play roles in diverse biological processes, including mammary gland development, lymphocyte function, and promoting adipogenesis (37,38). Recently, STAT5 has been implicated in the modulation of insulin responsiveness in various models (30,31). For example, liver-specific STAT5-deficient mice displayed significant insulin resistance (30). Consistent with these results, we also observed an inhibitory effect of Ad-shSTAT5b on insulin signaling in vitro and insulin sensitivity in vivo. Moreover, Ad-shSTAT5b also blocks PRLR-enhanced insulin sensitivity. Our results confirm that STAT5 is required for regulating PRLR-mediated insulin sensitivity. STAT5 may regulate insulin sensitivity by modulating the phosphorylation of STAT1 and STAT3, increasing IGF1 expression and/or activating phosphatidylinositol 3-kinase activity, as shown previously (30,39). These possibilities will be studied in the future.

In the current study, we also explored the possible involvement of PRLR in the regulation of insulin sensitivity under insulin-resistant and insulin-sensitive conditions. The insulin resistance models we used included glucosamine-treated cells (22) and *db/db* mice (32). Leptin is a hormone secreted by adipocytes that exerts broad physiological effects on lipid and glucose metabolism. Genetic deletion of its long-form receptor causes T2D (32). Surprisingly, we found that PRLR expression is decreased in these two models and overexpression of PRLR significantly improved insulin sensitivity. The important role of PRLR in regulating insulin sensitivity was further confirmed under insulin-sensitive conditions, which were obtained by the deprivation of leucine in medium or diet, as shown previously (19). In contrast to insulin-resistant conditions, PRLR expression was increased by leucine deprivation, and leucine deprivation–increased insulin sensitivity was blocked by knocking down the expression of PRLR in vitro and in vivo. Taken together, these results imply that PRLR functions in the regulation of insulin sensitivity under both insulin-resistant and insulin-sensitive conditions.

Compared with the well-known regulation of PRLR expression by hormones (40), nutritional regulation of PRLR expression is poorly understood. In contrast to reduced PRLR abundance during maternal nutrition restriction in

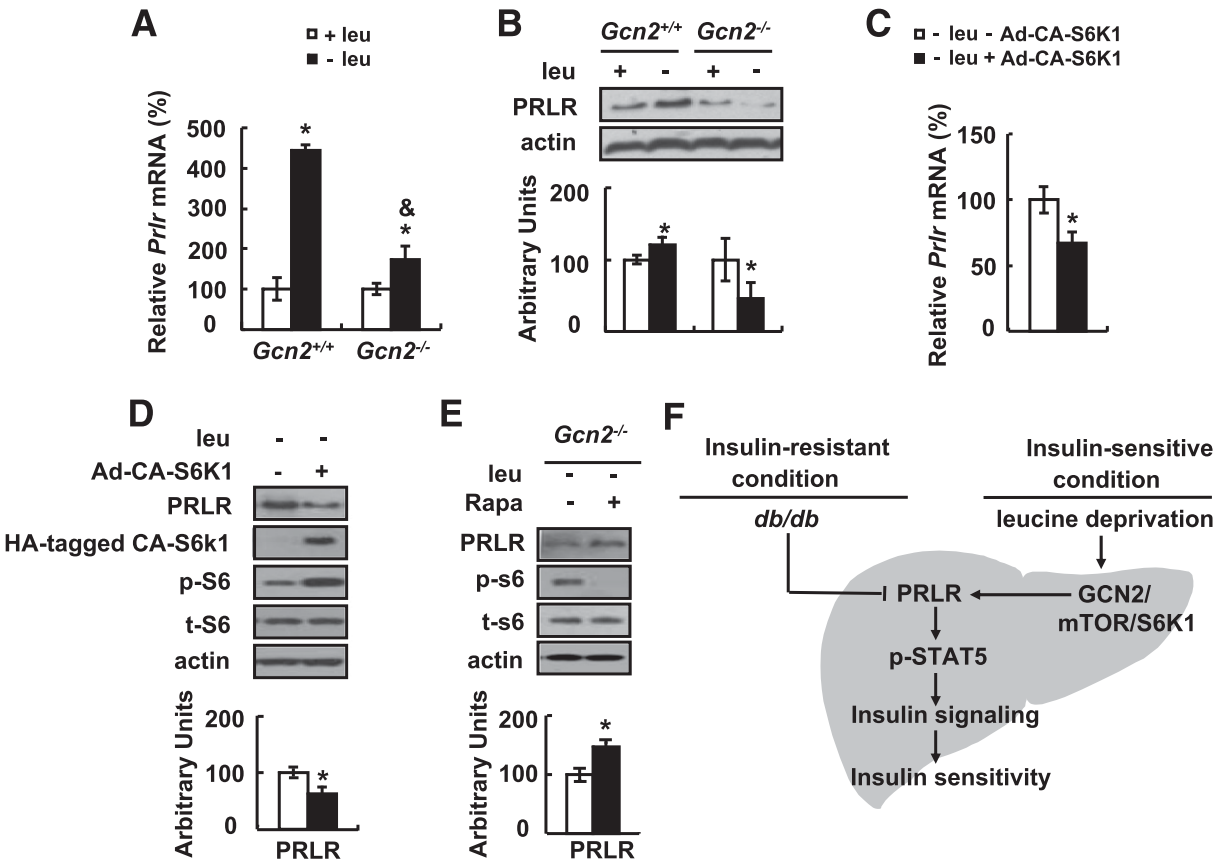

FIG. 7. Leucine deprivation stimulates PRLR expression by activating GCN2 and decreasing mTOR/S6K1 signaling. *A* and *B*: *Gcn2*$^{+/+}$ or *Gcn2*$^{-/-}$ mice were fed a control (+) leu or (−) leu diet for 7 days. *C* and *D*: Mice were infected with Ad-CA-S6K1 (+ Ad-CA-S6K1) or control GFP Ad (− Ad-CA-S6K1) via tail-vein injection and were fed a (−) leu diet for 7 days. *E*: *Gcn2*$^{-/-}$ mice were intraperitoneally injected with rapamycin (Rapa) at a dose of 1 mg/kg body weight (+ Rapa) or vehicle alone (− Rapa) once a day while fed (−) leu diet for 7 days. Data are the mean ± SEM and are representative of at least two independent in vivo experiments with the number of mice as indicated (n = 5–7 per group each time in *A–D*; n = 5 per group each time in *E*). Statistical significance was calculated using a two-tailed Student t test for the effects of (−) leu vs. control diet (*$P < 0.05$) and the effects of *Gcn2*$^{+/+}$ mice fed (−) leu diet vs. *Gcn2*$^{-/-}$ mice fed (−) leu diet (&$P < 0.05$) in *A* and *B*, with vs. without Ad-CA-S6K1 under (−) leu treatment (*$P < 0.05$) in *C* and *D*, with vs. without rapamycin under (−) leu treatment in *E* (*$P < 0.05$). *A* and *C*: *Prlr* mRNA. *B*, *D*, and *E*: PRLR and p-S6 (ser235/236) protein (*top*, Western blot; *bottom*, quantitative measurements of PRLR relative to actin). *F*: Working model. t, total.

lambs (17) and decreased prolactin levels in mice fed a low-protein diet (18), we found that PRLR expression is upregulated by leucine deprivation. Consistent with our findings, recent work has reported that estrogen receptors are also affected by amino acid levels (35), suggesting that dietary amino acids might be particularly important for the expression of these receptors as well as their functions.

While exploring the mechanisms underlying leucine deprivation regulation of PRLR expression, our attention was drawn to the amino acid sensor GCN2, a kinase that is activated by uncharged tRNAs in yeast and mammals in response to deprivation of essential amino acids, including leucine (34). We recently demonstrated that GCN2 also regulates lipid metabolism and insulin sensitivity during leucine deprivation (19,27), suggesting a broad role for GCN2 in regulating metabolism. The involvement of GCN2 in regulating PRLR expression under leucine deprivation was confirmed by the observation that leucine deprivation–induced PRLR expression is significantly blocked in the livers of *Gcn2*$^{-/-}$ mice.

We have previously shown that mTOR/S6K1 functions as a downstream regulator for GCN2 under leucine deprivation (19). S6K1 is a downstream target of mTOR and a serine-threonine protein kinase that has been shown to be essential for protein synthesis, growth, development, and proliferation (41,42). Studies have shown that S6K1 activity is regulated by branched-chain amino acid availability, especially leucine (43). The activities of both mTOR and S6K1 are decreased under leucine deprivation, suggesting that the decreased S6K1 activity might be responsible for the increase of PRLR expression during leucine deprivation. This claim was confirmed by the inhibitory effect of Ad-CA-S6K1 on leucine deprivation–induced PRLR expression. Furthermore, we provided evidence showing that S6K1 functions downstream of GCN2 in regulating PRLR expression under leucine deprivation. Our findings thus identify an important role for the GCN2/mTOR/S6K1 pathway in the regulation of PRLR expression under leucine deprivation. Although it has been shown that mTOR/S6K1 is part of signaling downstream of IR via Akt, mTOR/S6K1 can also have negative feedback effects on insulin signaling (44), which is consistent with our observations demonstrating the effects of mTOR/S6K1 on insulin sensitivity regulation. Furthermore, mTOR/S6K1 may regulate PRLR expression by modulating the expression of CCAAT/enhancer-binding protein-β, a transcription factor that has been shown to regulate PRLR expression (45) and to be upregulated under leucine deprivation (46). In addition to the essential amino acid leucine, PRLR expression regulation by other nutritional components, including fatty acids, glucose, and cholesterol, and its physiological functions under these conditions will require additional studies.

Our current study cannot exclude the possible involvement of additional signaling pathways in the regulation of insulin sensitivity by PRLR. Signaling via ERK, a well-known target of PRLR, is a distinct possibility, since we have observed that phosphorylation of ERK is regulated by Ad-PRLR or Ad-shPRLR in vitro and in vivo (data not shown). Furthermore, PRLR may regulate insulin sensitivity by binding to IRS1 directly, as previously reported in in vitro studies (14,15), or may form a complex with IR after insulin stimulation (our unpublished data). These possibilities will be investigated in the future.

PRLR was thought to be activated by its ligand PRL, which binds to and induces dimerization of the receptor. Recently, increasing evidence has suggested, however, that PRLR is in a predimerized conformation (47). Dimerization of PRLR was observed in the absence of prolactin in HepG2 and other cell lines (48), and Ma et al. showed that the overexpression of PRLR per se is sufficient to activate its downstream signaling (49). These results suggest that PRLR can function independently of PRL. Consistent with this, our results have shown that PRLR can regulate insulin sensitivity in the absence of PRL. These results also suggest that PRLR may function in the regulation of insulin sensitivity independently of changes in levels of serum prolactin. In support of this interpretation, hyperprolactinemia has been reported to be associated with insulin resistance (50).

In conclusion, as described in our working model (Fig. 7*F*), we observed that overexpressing or knocking down PRLR expression improved or impaired insulin sensitivity in vitro and in vivo, respectively, and that STAT5 is required for PRLR-enhanced insulin sensitivity. Furthermore, we demonstrated an important role for PRLR in regulating insulin sensitivity under insulin-resistant and insulin-sensitive conditions. We also showed that PRLR expression levels are regulated under leucine deprivation via a GCN2/mTOR/S6K1 pathway. Taken together, these results identify a novel function for hepatic PRLR in the regulation of insulin sensitivity and provide novel insights into the nutritional regulation of PRLR expression. Because PRLR is expressed in all tissues, the potential role of PRLR in the regulation of insulin sensitivity in other tissues will be investigated in the future.

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J.Y. and F.X. researched data and wrote, reviewed, and edited the manuscript. Q.Z., Y.G., T.X., and K.L. researched data. B.L., Z.L., S.C., and Y.D. provided research material. F.G. directed the project, contributed to discussion, and wrote, reviewed, and edited the manuscript. F.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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