Leptin ameliorates insulin resistance and hepatic steatosis in $Agpat2^{-/-}$ lipodystrophic mice independent of hepatocyte leptin receptors[s]

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Abstract   Leptin is essential for energy homeostasis and regulation of food intake. Patients with congenital generalized lipodystrophy (CGL) due to mutations in 1-acylglycerol-3-phosphate-*O*-acyltransferase 2 (*AGPAT2*) and the CGL murine model (*Agpat2*$^{-/-}$ mice) both have severe insulin resistance, diabetes mellitus, hepatic steatosis, and low plasma leptin levels. In this study, we show that continuous leptin treatment of *Agpat2*$^{-/-}$ mice for 28 days reduced plasma insulin and glucose levels and normalized hepatic steatosis and hypertriglyceridemia. Leptin also partially, but significantly, reversed the low plasma thyroxine and high corticosterone levels found in *Agpat2*$^{-/-}$ mice. Levels of carbohydrate response element binding protein (ChREBP) were reduced, whereas lipogenic gene expression were increased in the livers of *Agpat2*$^{-/-}$ mice, suggesting that deregulated ChREBP contributed to the development of fatty livers in these mice and that this transcription factor is a target of leptin's beneficial metabolic action. Leptin administration did not change hepatic fatty acid oxidation enzymes mRNA levels in *Agpat2*$^{-/-}$ mice. The selective deletion of leptin receptors only in hepatocytes did not prevent the positive metabolic actions of leptin in *Agpat2*$^{-/-}$ mice, supporting the notion that the majority of metabolic actions of leptin are dependent on its action in nonhepatocyte cells and/or the central nervous system.—Cortés, V. A., K. M. Cautivo, S. Rong, A. Garg, J. D. Horton, and A. K. Agarwal. **Leptin ameliorates insulin resistance and hepatic steatosis in *Agpat2*$^{-/-}$ lipodystrophic mice independent of hepatocyte leptin receptors.** *J. Lipid Res.* 2014. **55:** 276–288.

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The relevance of white adipose tissue (WAT) as an endocrine organ rather than a mere passive fat depot was unveiled by the discovery of leptin, a 167-amino acid peptide hormone predominantly secreted by mature adipocytes (1). This adipokine regulates multiple functions ranging from energy homeostasis to sexual maturation, bone mass accrual, and innate and acquired immune function (2). Leptin plasma levels are directly correlated with total body adiposity (3).

Leptin acts on cell surface receptors located throughout the central nervous system (CNS) (4, 5) and on a variety of peripheral tissues, including lungs, kidneys, skeletal muscle, heart, and liver (6). The relevance of central versus peripheral leptin receptor has been addressed in recent years by several investigators (7, 8). Reduced leptin signaling is observed in leptin-deficient (*ob/ob*) and leptin receptor-deficient (*db/db*) mice. Humans with mutations in the leptin and leptin receptor gene (9–11) have markedly increased food intake (hyperphagia), reduced energy expenditure, early-onset morbid obesity, lack of pubertal development and infertility, insulin resistance, diabetes mellitus, and fatty liver. Notably, peripheral administration of leptin into both the *ob/ob* mice (12–14) and humans with congenital leptin deficiency (15, 16) reverses most of the above-mentioned complications, unequivocally demonstrating the key role of this adipokine in whole-body metabolic regulation (17).

The leptin receptor (LepR) belongs to the interleukin-6 receptor family of class 1 cytokine receptors (6). There are six identified LepR isoforms, named LepRa to LepRf, due

Abbreviations: BAT, brown adipose tissue; CGL, congenital generalized lipodystrophy; ChIP, chromatin immunoprecipitation; ChREBP, carbohydrate response element-binding protein; LepR, leptin receptor; WAT, white adipose tissue.

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[s] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one table.

to the alternative splicing of a single common transcript (18). The four short-forms (LepRa, LepRc, LepRd, and LepRf) and the one long-form (LepRb) share the extracellular and transmembrane domains as well as the first 29 amino acids of the intracellular domain. Only the intracellular domain of LepRb contains a sequence motif for the binding of JAK protein kinases that is crucial for leptin signaling (18, 19). The *db/db* mice, which exclusively lack the LepRb isoform (18, 20), have an identical metabolic phenotype to that observed in db^{3J}/db^{3J} mice that lack all the LepR isoforms (21) and *ob/ob* mice, which primarily lack leptin (1), indicating that LepRb mediates most, if not all, metabolic actions of leptin.

The role of WAT in whole-body metabolic regulation has been further addressed by the observation that severe WAT deficiency associates with the same metabolic complications of obesity. In fact, both the congenital generalized lipodystrophy (CGL) patients (22) and lipodystrophic mouse model, including aP2-tg-*Srebp1c,* A-*ZIP/F-1,* and $Agpat2^{-/-}$ mice (23–25), develop hyperinsulinemia, diabetes mellitus, dyslipidemia, and fatty liver, indicating that both extremes of adiposity might share common pathophysiologic pathways that lead to same final metabolic derangement.

The missing link in CGL patients and lipodystrophy mouse models could be the abnormal levels of some adipokines, such as leptin, due to lack of adipose tissue. This is in contrast to elevated circulating levels of leptin in obese subjects in whom the action of this hormone in the CNS appears to be impaired and is referred to as "leptin-resistance" state (26). CGL is characterized by very low levels of plasma leptin as a consequence of primary WAT deficiency. Seminal studies reported by Brown and Goldstein (27) demonstrated that leptin supplementation results in remarkable amelioration of hyperphagia, plasma insulin, and glucose and liver triglyceride concentrations in their hypoleptinemic aP2-tg-*Srebp1c* lipodystrophy mouse model (27). The clinical relevance of these observations was later corroborated by leptin supplementation in severely hypoleptinemic lipodystrophic patients (28). These subjects responded dramatically and favorably to leptin treatment, significantly improving insulin resistance and liver and plasma triglyceride levels. However, such improvements have failed to be observed in common obese subjects when infused with leptin, most likely due to resistance to the metabolic actions of this hormone (29).

The mechanism(s) underlying leptin's insulin-sensitizing actions in both leptin-deficient humans and rodents and the reason why obese individuals develop leptin resistance remains obscure. We recently generated and characterized a murine model of human CGL type 1 syndrome, the $Agpat2^{-/-}$ mouse (25). The AGPATs are intermediate enzymes in the pathway for the biosynthesis of glycerophospholipids (GPL) and triacylglycerol (TAG) (30). AGPATs esterify the *sn-2* position of 1-acylglycerol-3-phosphate (lysophosphatidic acid or LPA) to phosphatidic acid (PA) (31–33). Mutations in *AGPAT2* cause congenital generalized lipodystrophy in humans (34), and $Agpat2^{-/-}$ mice recapitulate all the features of lipodystrophy observed in humans, including severe hypoleptinemia, thus providing

an animal model system to further explore the role of leptin in insulin sensitivity and energy balance in a medically relevant murine model of lipodystrophy.

As a step forward in understanding the molecular mechanism(s) involved in the insulin sensitizing effect of leptin, we here describe the metabolic and molecular effects of leptin replacement in $Agpat2^{-/-}$ mice and determine the functional relevance of hepatic LepR for metabolic actions of leptin in $Agpat2^{-/-}$ mice by selectively deleting it from hepatocytes.

RESEARCH DESIGN AND METHODS

Animals

Generation of $Agpat2^{-/-}$ mouse and genotyping is described elsewhere (25). Mice were housed in colony cages (four per cage) and maintained on a 12 h light/12 h dark cycle and fed Teklad Mouse/Rat Diet 7002 from Harlan Teklad Premier Laboratory Diets (Madison, WI). Animals were euthanized at the end of the dark cycle by isoflurane overdose; blood and tissues were harvested in less than 1 min. All mouse housing, breeding, surgical procedures, euthanasia, and tissue collection were conducted in conformity with Public Health Service (PHS) policy and performed with the approval of the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. All efforts were made to minimize animal suffering.

Generation of $Agpat2^{-/-}$;$Lepr^{f/f}$;*Alb-cre+* ($Agpat2^{-/-}$;$LepR^{hepato-ko}$) mice

To generate mice that lacked leptin receptor in liver on the *Agpat2* null background, *Lepr* floxed ($Lepr^{f/f}$) mice (7) were first crossed to mice that expressed Cre recombinase under the transcriptional control of albumin promoter (*Alb-Cre*) (obtained from Jackson Laboratory). Resulting $Lepr^{f/f}$;*Alb-cre+* and $Lepr^{f/f}$;*Alb-cre-* mice were bred to $Agpat2^{+/-}$ mice to obtain $Agpat2^{+/-}$;$Lepr^{f/f}$;*Alb-cre+* and $Agpat2^{+/-}$;$Lepr^{f/f}$;*Alb-cre-* mice. These mice were bred to produce the $Agpat2^{+/+}$;$Lepr^{f/f}$;*Alb-cre+*, $Agpat2^{+/-}$;$Lepr^{f/f}$;*Alb-cre-*, $Agpat2^{-/-}$;$Lepr^{f/f}$;*Alb-cre+* (now referred to as $Agpat2^{-/-}$;$LepR^{hepato-ko}$) and $Agpat2^{-/-}$;$Lepr^{f/f}$;*Alb-cre-* (now referred to as $Agpat2^{-/-}$;$LepR^{hepato-wt}$) mice which were used in these reported experiments.

Leptin infusion

Mouse recombinant leptin (Sigma, St. Louis, MO) was dissolved in phosphate saline buffer (1 mg/ml, release rate of ~0.25/μg/h), and 100 μl was loaded into osmotic minipumps (model 1002, Alzet, Cupertino, CA). Leptin was delivered at a rate of 0.25 ± 0.05 μg/h. The pumps were subcutaneously inserted in the interscapular space under isoflurane general anesthesia. The surgical wound was closed with metal clips and the animals were allowed to recover on a heating pad. Control mice were implanted with minipumps filled with PBS. The minipumps were removed and replaced with new ones after two weeks. At the end of the fourth week, the animals were euthanized and blood and liver were harvested for analysis.

Pair feeding and indirect calorimetry

For pair-feeding studies in $Agpat2^{-/-}$ mice, food intake was restricted in the saline-infused $Agpat2^{-/-}$ mice to the same level as that of leptin-infused ones. For this, individual animals were housed in calorimetric cages (CLAMS, Columbus Instruments, Columbus, OH) and allowed to acclimate for one week. Basal food and water intake, VO_2, VCO_2, and locomotor activity were

determined during this period. Osmotic minipumps, filled either with leptin (1 mg/ml, n = 6) or a saline solution (PBS, n = 6), were implanted in $Agpat2^{-/-}$ mice at the beginning of the second week. Animals were replaced in their original calorimetric cages with unrestricted food access. In the next three weeks, food access was unlimited for the leptin-treated mice, whereas it was limited for the saline-infused ones. For the food restriction experiment, the feeders of the food-restricted group were refilled at the beginning of the dark phase, and the amount of food consumed was calculated on the basis of individual mice body weight to that of the leptin-treated mice.

Gene expression analysis by RT-PCR

Total RNA was prepared from the mouse livers using RNA STAT-60 kit (Tel-Test Inc., Friendswood, TX). Genomic DNA in the RNA samples was eliminated by RNase free DNase digestion (Ambion, Austin, TX). cDNA was generated using TaqMan® reverse transcription kit (Applied Biosystems-Roche, Branchburg, NJ). Real-time PCR was carried out in 384-well plates, read, and analyzed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). PCR conditions and primers used were previously reported (25). Primers sequences are presented in supplementary Table I. Q-PCR expression levels were normalized to the expression level of cyclophilin and the fold change was estimated by the $\Delta\Delta C_T$ method.

Antibodies and immunoblot analysis

For ACC-1, FAS, IR, PI3K, AKT (total and phosphorylated), GSK3β (total and phosphorylated), ERK1/2 (total and phosphorylated), AMPKα (total and phosphorylated), and RAP immunoblot analysis, whole-liver protein extracts were prepared from individual animals as described (35). Equal aliquots of total protein from each liver were pooled, and 30 µg was separated in 8% SDS-PAGE, transferred onto nitrocellulose membranes, blocked, and incubated with primary and secondary antibodies. For SREBP-1, total membranes and nuclear extracts were prepared from individual mice as described (25). Equal aliquots from each animal were pooled and separated in 8% SDS-PAGE, transferred onto nitrocellulose membranes, blocked, and incubated with primary and secondary antibodies as described (25). The primary antibodies used were insulin receptor (IR) subunit β (BD Transduction Laboratories, San Jose, CA), PI3K subunit p85 (Upstate, Lake Placid, NY), SREBP-1 (36), ChREBP (Novus Biologicals, Saint Louis, MI), and FAS and ACC1 (Genepia, Seoul, South Korea). The primary antibodies against AKT, phospho-AKT (Ser473), GSK3β, phospho-GSK3β (Ser9), αAMPK, phospho-αAMPK (Thr172), ERK1/2 and phospho-ERK1/2 (Thr202, Tyr204) were all obtained from Cell Signaling Technology. Secondary peroxidase-conjugated donkey anti-mouse and anti-rabbit IgG were obtained from Jackson Immunoresearch (West Grove, PA) and GE Healthcare (Little Chalfont, UK), respectively. All immunoblots were visualized by SuperSignal® West Pico Chemiluminiscent Substrate (Pierce, Rockford, IL).

ChIP assay for mouse liver pyruvate kinase (*L-pk*) promoter occupancy by ChREBP

Chromatin immunoprecipitation (ChIP) assays were performed as described before (37). Proteins were crosslinked to DNA by addition of 1% formaldehyde to ∼70 mg of mouse whole-liver tissue by incubating for 10 min at room temperature. Sonication was performed by eight pulses of 10 s each. Immunoprecipitation was performed with a ChREBP antibody (NB400–135, Novus Biologicals). Immune complexes were pulled down with 60 µl of Protein A/G PLUS-agarose (Santa Cruz Biotechnology). DNA fragments were amplified by PCR and separated in agarose gels.

The primers used for amplification were: *L-pk* promoter: forward, 5′-GAAGGATGCCCACTACAGCC-3′ and reverse, 5′-TGCCTT-TGTCAGTGGGATGG-3. As a negative control, albumin promoter was amplified with the primers: forward, 5′-CAAGGCCCACAC-TGAAATGC-3′ and reverse, 5′-TGTGTGCAGAAAGACTCGCTC-3′.

Detection of glycosylated ChREBP in the liver by immunoblotting

To measure posttranslation modification for ChREBP, this transcription factor was immunoprecipitated and immunoblotted with an antibody specific for *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAcylation). Whole-liver protein extracts were prepared from 100 mg of individual livers (n = 4–6 per group) by homogenization in RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate) supplemented with phosphatase inhibitors (2.5 mM Na_3VO_4 and 2.5 mM $Na_4P_2O_7$) and protease inhibitor cocktail (Roche). One milligram of pooled proteins was immunoprecipitated with a ChREBP antibody (NB400–135, Novus Biologicals). Immune complexes were recovered after addition of 50 ml of protein A/G agarose-PLUS (Santa Cruz Biotechnology) and gentle centrifugation. Bound proteins were visualized by immunoblot analysis using a monoclonal antibody directed against *O*-linked β-N-acetylglucosamine (MMS-248R, Covance).

Biochemical and hormone determinations

Liver triglycerides and cholesterol as well as plasma triglycerides, cholesterol, glucose, insulin, and leptin were measured as previously described (25) at UT Southwestern Medical Center Mouse Metabolic Phenotyping Core. Liver glycogen was quantified by colorimetric detection of free glucose (Glucose Autokit from Wako, Richmond, VA) after amyloglucosidase (Sigma, Saint Louis, MO) digestion of liver homogenates, as described by Grefhorst et al. (38). Total thyroxine (T4) was determined with Mouse/Rat Thyroxine ELISA Kit (Calbiotech, Spring Valley, CA). Corticosterone was measured with Corticosterone EIA Kit (Enzo Life Sciences, Plymouth Meeting, PA) following manufacturer instructions.

Statistical analysis

Plasma leptin, insulin, glucose and liver tissue triglycerides, cholesterol, and glycogen were compared by one-way ANOVA followed by Bonferroni's multiple comparison test using GraphPad Prism version 5.0 (San Diego, CA). Gene expression analysis was initially performed in pooled samples. For this, equal amounts of RNA for each experimental group were pooled and the fold change relative to control groups was determined. If the resulting fold change was greater than 1.5 or less than 0.7, individual RT-PCR was performed to determine the statistical significance of these differences. Individual sample results are presented as mean ± SEM. The number of animals used for experimentation was limited because more than 80% of $Agpat2^{-/-}$ and $Agpat2^{-/-};LepR^{\text{hepato-ko}}$ mice die before the time of weaning, possibly because of severe hyperglycemia and dehydration (25). For this reason, some experimental groups had only three animals. However, both the physiological determinations and the gene expression analysis show that the inter-individual variability is minimal and the differences among experimental groups are statistically significant.

RESULTS

Leptin normalizes glucose, insulin, and liver triglycerides and glycogen in $Agpat2^{-/-}$ mice

$Agpat2^{-/-}$ mice lack both WAT and brown adipose tissue (BAT) and develop insulin resistance, diabetes mellitus,

hepatic steatosis, and hypertriglyceridemia (25). As a consequence of adipose tissue deficiency, these mice have extremely low levels of circulating leptin (25). To study the metabolic effects as well as the molecular mediators of leptin restitution in $Agpat2^{-/-}$ mice, 16-week-old male mice were infused with leptin for four weeks. Leptin did not reverse the lack of macroscopic white and brown adipose tissue in the $Agpat2^{-/-}$ mice, but it resulted in significant reduction of food intake in the $Agpat2^{-/-}$ mice (compare **Fig. 1A**, before leptin infusion, with Fig. 1D, after leptin infusion). Since metabolic changes in the $Agpat2^{-/-}$ mice could entirely be dependent on reductions in energy intake, a group of $Agpat2^{-/-}$ mice was infused with saline and their food intake was restricted to the level of $Agpat2^{-/-}$ leptin-infused mice (Fig. 1G). Leptin infusion did not affect oxygen consumption rate (VO_2, data not shown) or calculated heat production (Fig. 1B, E, H), but it normalized locomotor activity in the $Agpat2^{-/-}$ mice (Fig. 1F, I).

As shown in the **Fig. 2A**, leptin infusion significantly elevated plasma leptin levels in both wild-type and $Agpat2^{-/-}$ mice, resulting in decreased insulin and glucose levels in $Agpat2^{-/-}$ mice (Fig. 2B, C). Food restriction did not modify any of these parameters in $Agpat2^{-/-}$ mice, and leptin supplementation did not change insulin or glucose in wild-type mice (Fig. 2B, C). Leptin administration to $Agpat2^{-/-}$ mice was also associated with significant decreases in liver triglycerides and cholesterol concentrations (Fig. 2D, E) and significant increases in hepatic glycogen content (Fig. 2F). Food restriction did not change liver triglycerides or cholesterol, but it did significantly elevate glycogen levels in $Agpat2^{-/-}$ mice (Fig. 2F).

Leptin partially normalizes corticosterone and thyroxine plasma levels in $Agpat2^{-/-}$ mice

Murine models of leptin deficiency have altered levels of plasma corticosterone and thyroid hormone levels. Leptin deficient *ob/ob* mouse has adrenal hypertrophy (39), increased levels of circulating corticosterone (40), low thyroidal rates of radioactive iodine uptake (41) and reduced thyroptropin and thyroid hormones circulating levels (42). Lipodystrophic A-ZIP/F-1 mice also have elevated corticosterone levels (43); however, their
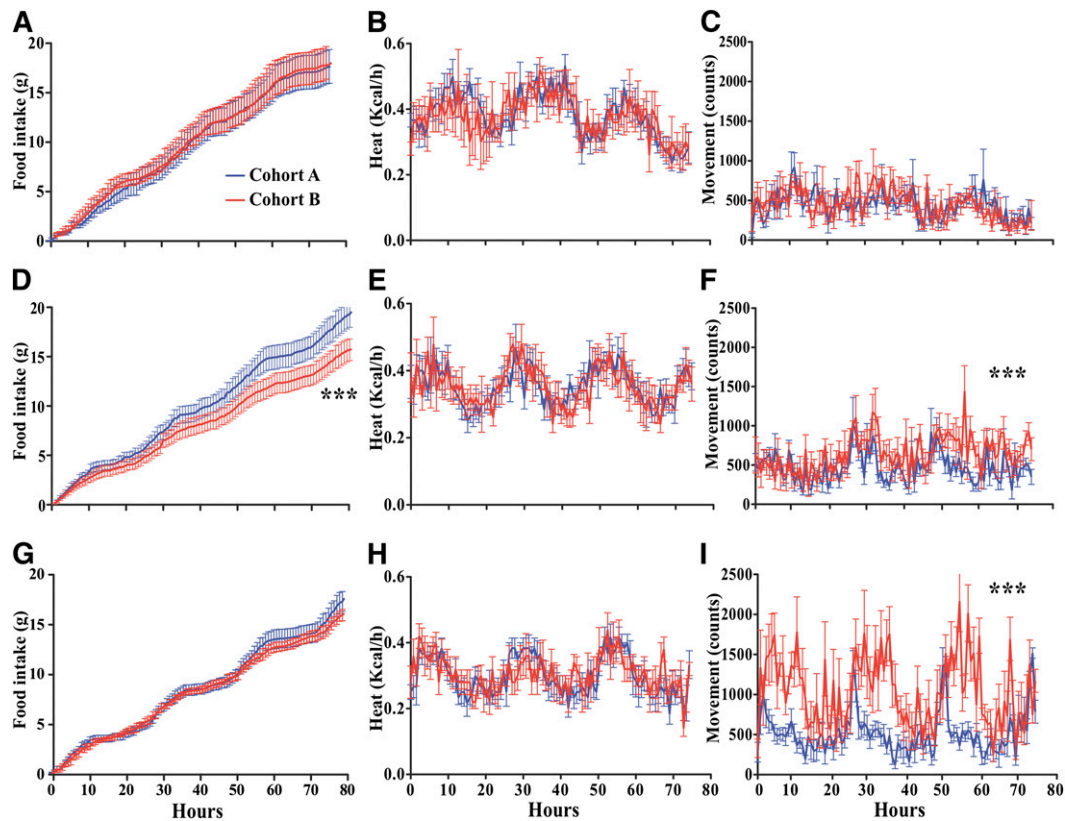

Fig. 1. Individual $Agpat2^{-/-}$ mice were housed in metabolic cages, and food intake, VO_2, VCO_2, and locomotor activity were recorded as detailed in Research Design and Methods. Animals were divided in two cohorts: A (shown in blue) and B (shown in red). In panels A, B. and C. both cohorts remained untreated. In panels D, E, and F, animals in cohort A were implanted with osmotic mini-pumps filled with a saline buffer (PBS), whereas animals in cohort B were implanted with mini-pumps filled with a leptin solution (1 mg/ml) with six mice in each group. In panels G, H, and I, both cohorts remained with the same infusions as in panels D, E, and F, but animals in cohort A were additionally subjected to food restriction, whereas animals in cohort B were fed ad libitum. Panels A, D, and G correspond to accumulated food intake. Panels B, E, and H show the calculated heat production based on body weight corrected for VO_2 and respiratory quotient (data not shown). Panels C, F, and I correspond to total locomotor activity on the X-axis of the metabolic cages. The data were compared by two-way ANOVA. ***$P < 0.05$.
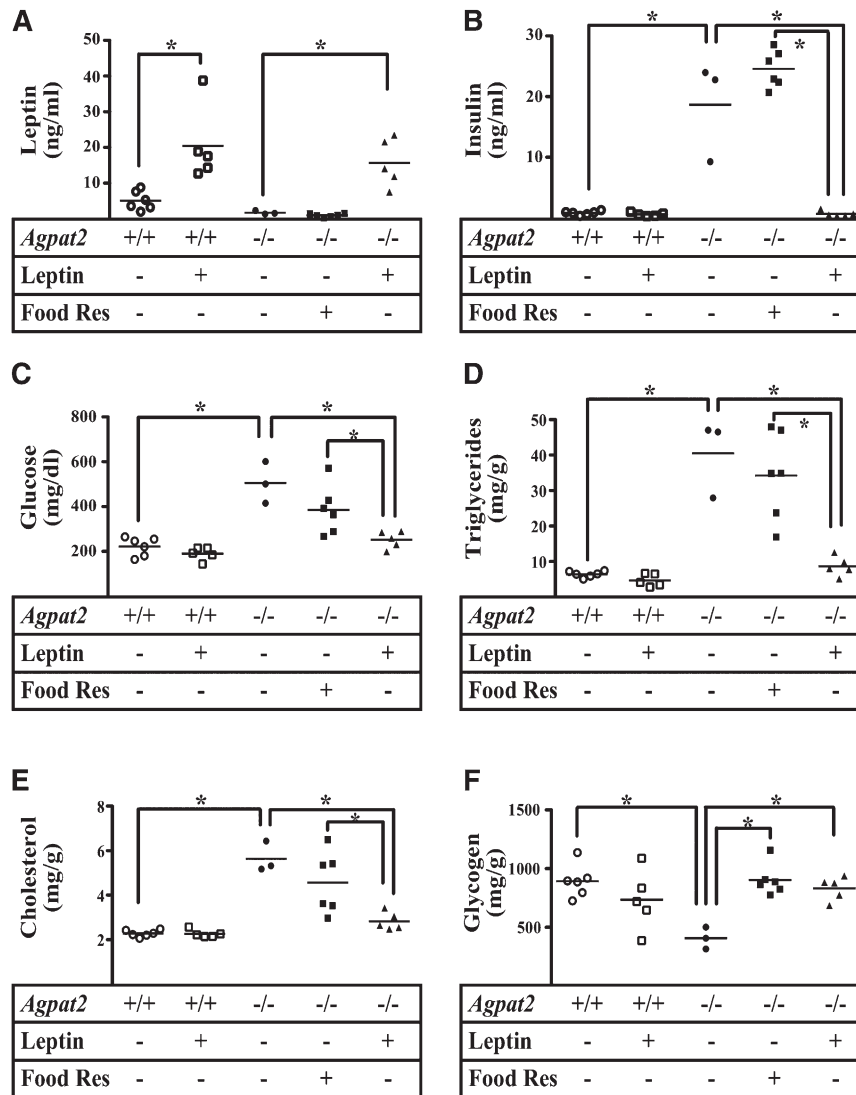
Fig. 2. Plasma and liver metabolites in wild-type ($Agpat2^{+/+}$) and $Agpat2^{-/-}$ mice treated with and without leptin. In panels A-C, plasma leptin, insulin, and glucose levels, respectively, are shown. In panels D–F, liver triglyceride, cholesterol, and glycogen concentrations, respectively, are shown. The horizontal lines correspond to the mean value. The data were compared by one-way ANOVA with Bonferroni's correction for multiple comparisons. *$P < 0.05$.

thyroid hormones and thyroid functional tests remain normal (43).

In contrast, $Agpat2^{-/-}$ mice have markedly decreased plasma thyroxine (T4) level compared with their wild-type littermates (0.5 ± 0.2 μg/dl versus 3.8 ± 0.3 μg/dl, respectively, **Fig. 3A**). $Agpat2^{-/-}$ mice also have markedly increased plasma corticosterone (9.3 ± 2.1 ng/ml versus 0.3 ± 0.2 ng/ml in wild-type mice, Fig. 3B). Leptin infusion, but not food restriction, significantly reversed both hormone abnormalities in these animals (Fig. 3). However, the corrective effect of leptin was more pronounced for T4 than for corticosterone, which remained ~13-fold higher in the leptin-treated $Agpat2^{-/-}$ mice compared with the wild-type mice (3.8 ± 0.5 ng/ml versus 0.3 ± 0.2 ng/ml).

Considered together, our findings indicate that leptin positively influences insulin sensitivity in the lipodystrophic AGPAT2 deficient mice, restoring liver triglycerides, cholesterol and glycogen to normal levels, and that these metabolic effects do not depend on food intake. Leptin significantly reverses abnormal plasma glucocorticoid and thyroxine levels in the $Agpat2^{-/-}$ mice; however, the complete normalization was not achieved.

Leptin ameliorates the exaggerated hepatic expression of lipogenic genes in the $Agpat2^{-/-}$ mice

To study the molecular mechanisms by which leptin improves insulin sensitivity and hepatic steatosis in $Agpat2^{-/-}$ mice, hepatic mRNA expression levels of lipogenic and oxidative enzymes as well as key transcriptional regulators of de novo lipogenesis were quantified in leptin-treated $Agpat2^{-/-}$ mice. As shown in **Table 1**, leptin supplementation, but not food restriction, was associated with a significant decrease of *Acc1*, *Fas*, *Scd1* and *Elovl6* mRNA levels in the livers of $Agpat2^{-/-}$ mice. These reductions at the mRNA level were consistent with lower ACC-1 and FAS protein concentrations in the livers of leptin-treated $Agpat2^{-/-}$ mice (**Fig. 4A**).
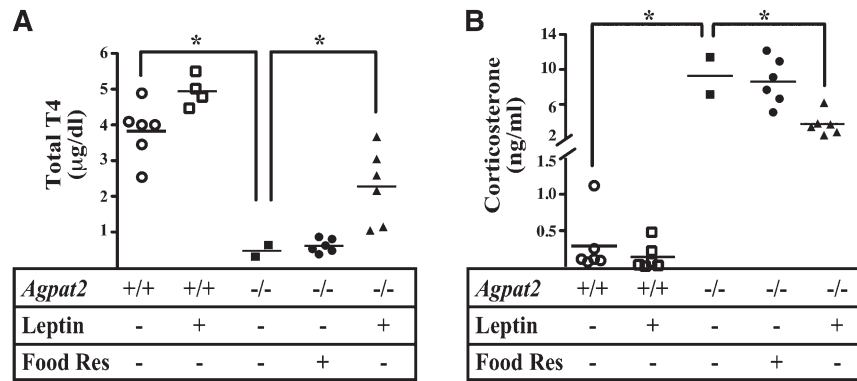
Fig. 3. Plasma total thyroxine and corticosterone levels in wild-type ($Agpat2^{+/+}$) and $Agpat2^{-/-}$ mice. Plasma total thyroxine (T4) (panel A) and corticosterone (panel B) in wild-type and $Agpat2^{-/-}$ mice treated with and without leptin or food restriction. The horizontal lines correspond to the mean value. The data were compared by one-way ANOVA with Bonferroni's correction for multiple comparisons. *$P < 0.05$.

It has been reported that leptin activates fatty acid oxidation, thus decreasing hepatic triglyceride concentration (44, 45). To evaluate whether enhanced fatty acid oxidation rates explained the diminished liver triglyceride levels of leptin-treated $Agpat2^{-/-}$ mice, mRNA levels of several genes involved in mitochondrial and peroxisomal fatty acid oxidation pathways were quantified. No differences were detected in $Cpt1a$, $Lcad$, $Mcad$, or $Acox1$ mRNA levels between leptin and saline-infused $Agpat2^{-/-}$ mice (Table 1). Furthermore, leptin supplementation resulted in lower mRNA levels of hepatic $Ucp2$ in $Agpat2^{-/-}$ mice compared with saline-infused $Agpat2^{-/-}$ mice, suggesting that liver fatty acid oxidation rates could actually be diminished by leptin. Expression of several transcription factors associated with liver lipid metabolism remained unchanged (Table 1). Liver mRNA levels of $Fgf21$, a recently identified hormone associated with starvation-feeding cycle, was increased 70.4-fold in the saline-treated $Agpat2^{-/-}$ relative to saline-infused wild-type mice; leptin supplementation was associated with a 23-fold decrease of $Fgf21$ mRNA levels in $Agpat2^{-/-}$ mouse livers (Table 1).

In skeletal muscle, leptin promotes fatty acid oxidation by stimulating phosphorylation and activation of AMP-activated protein kinase (AMPK). AMPK activation results in decreased ACC-1 activity, lower malonyl-CoA levels and enhanced mitochondrial fatty acid β-oxidation (46). To assess the effects of leptin on hepatic AMPK levels in $Agpat2^{-/-}$ mice, phosphorylation of α catalytic subunit of AMPK (αAMPK) was examined by immunoblot analysis. As shown in Fig. 4B, infusion of leptin did not change hepatic levels of phospho-αAMPK, suggesting that in AGPAT2 deficient mice leptin did not repress ACC-1 activity by AMPK-dependent mechanisms and that it did not stimulate fatty acid oxidation. Others have reported similar lack of AMPK-effects of leptin in cardiac muscle (47) and liver (45).

$Mgat1$ (also known as $Mogat1$), which catalyzes sn-1 (3)- and sn-2-monoacylglycerol acylation is not expressed in the normal adult mouse liver but is detectable in the livers of $Agpat2^{-/-}$ mice, providing an alternative pathway for hepatic diacylglycerol (DAG) and, ultimately, triacylglycerol synthesis (25). In these mice, leptin significantly reduced

$Mgat1$ mRNA levels (Table 1), suggesting that this hormone could also reduce liver triglycerides by decreasing DAG availability.

The above results indicate that leptin may decrease liver triglycerides in AGPAT2 deficient mice by ameliorating both exaggerated de novo lipogenesis and DAG formation. Our results do not support the hypothesis that leptin increases liver fatty acid oxidation rates in absence of AGPAT2.

Transcriptional activity of ChREBP is increased in livers of $Agpat2^{-/-}$ mice and is normalized by leptin infusion

The molecular basis of the exaggerated hepatic de novo lipogenesis in the livers of $Agpat2^{-/-}$ mice remains unclear. $Srebp-1c$, an endoplasmic reticulum membrane bound transcription factor that transcriptionally activates hepatic de novo lipogenesis in response to insulin stimulation, is elevated in the liver of both ob/ob and lipodystrophic Tg-aP2–$Srebp-1c$ hyperinsulinemic mice (48). Paradoxically, despite massive plasma insulin elevations, $Agpat2^{-/-}$ mice have no increase in hepatic nuclear $Srebp-1c$ protein levels (25). In the current study, we corroborated that $Srebp-1c$ remained unchanged in the livers of $Agpat2^{-/-}$ saline-infused mice, at both mRNA and protein levels and leptin treatment did not modify hepatic $Srebp-1c$, in either $Agpat2^{-/-}$ or wild-type mice (Table 1, Fig. 4C), supporting the lack of role for $Srebp-1c$ in the exaggerated hepatic de novo lipogenesis of $Agpat2^{-/-}$ mice.

$Chrebp$ is a basic helix-loop-helix transcription factor that also regulates hepatic lipogenesis (49). In contrast to $Srebp-1c$, $Chrebp$ is not regulated by insulin but rather it responds to changes in pentose phosphate pathway metabolites levels (49) and glucose level. Importantly, although the transcriptional program regulated by ChREBP extensively overlaps with that of SREBP-1c (49), liver pyruvate kinase ($L-pk$) is exclusively regulated by ChREBP and can be used to discriminate the actions of one transcription factor over the other.

In $Agpat2^{-/-}$ mice, hepatic ChREBP is decreased at both mRNA and total nuclear protein levels and leptin administration is associated with higher ChREBP protein levels in these animals, with a more pronounced normalizing effect on

TABLE 1. Relative expression of liver mRNAs in *Agpat2*^{+/+} and *Agpat2*^{−/−} mice upon leptin treatment

Genotype		*Agpat2*^{+/+}	*Agpat2*^{−/−}	*Agpat2*^{−/−}	*Agpat2*^{−/−}
Infusion		Leptin	Saline	Saline	Leptin
Food restriction?		No	No	Yes	No
		N = 4	*N = 3*	*N = 6*	*N = 5*
		Fold Change	*Fold Change*	*Fold Change*	*Fold Change*
Fatty acid and TG synthesis	ACC1	0.7 ± 0.07	3.5 ± 0.6[a]	5.4 ± 0.8[a]	1.4 ± 0.2
	FAS	0.5 ± 0.09	3.7 ± 0.2[a]	5.7 ± 0.6[a]	1.3 ± 0.2
	ELOVL6	0.9 ± 0.2	3.6 ± 0.2[a]	5.0 ± 0.5[a]	1.0 ± 0.1
	SCD1	0.7 ± 0.2	2.4 ± 0.2[a]	2.4 ± 0.2[a]	0.9 ± 0.2
	MOGAT1	1.1 ± 0.2	7.9 ± 1.0[a]	17.7 ± 2.4[a]	1.8 ± 0.4
Fatty acid oxidation	CPT1α	1.1	1.5	1.2	1.1
	LCAD	0.8	1.3	1.6	0.9
	MCAD	0.7	1.3	1.7	0.7
	ACOX1	0.7	1.1	1.1	0.8
	UCP2	1.2 ± 0.08	15.8 ± 2.9[a]	7.5 ± 0.6[a]	2.4 ± 0.5
	FGF21	0.7 ± 0.2	70.4 ± 8.0[a]	42.8 ± 7.6[a]	3.0 ± 0.5
Transcriptional regulators	ChREBP	1.0 ± 0.1	0.7 ± 0.3	0.7 ± 0.04	0.9 ± 0.2
	SREBP1a	0.8	1.6	1.4	1.1
	SREBP1c	0.6	0.8	0.9	0.7
	SREBP2	0.8	1.2	1.1	0.9
	PPARα	0.7	0.7	0.9	0.7
	PPARβ	0.6	1.2	2.5	1.9
	PPARγ	0.8	2.3	2.5	1.3
	PGC1α	1.1	0.8	0.9	1.3
	PGC1β	1.1	0.8	0.8	0.6
	FOXO1	0.9	0.8	0.9	0.8
	LXRα	0.9	1.1	1.0	0.8
	LXRβ	0.9	1.2	1.2	0.9
Glucose metabolism	L-PK	0.7 ± 0.1	4.0 ± 0.4[a]	3.6 ± 0.2[a]	1.3 ± 0.1
	G6P	0.8 ± 0.2	0.8 ± 0.1	1.2 ± 0.2	1.1 ± 0.2
	PEPCK	1.6	0.6	0.3	1.4
Leptin signaling pathway	JAK2	0.9	1.4	1.1	1.3
	STAT3	1.0	1.1	0.7	1.2
	TIMP2	0.9	1.9	1.5	1.5
	LEPRb	2.7 ± 0.5[a]	1.0 ± 0.2	0.6 ± 0.1	2.0 ± 0.1[a]

Each value represents the mean fold change (± SEM) of individual mice or the fold change of pooled samples compared with saline-infused nonfood restricted *Agpat2*^{+/+} mice. (See Research Design and Methods for the criteria by which some gene products were selected for individual analysis.) ACC, acyl CoA carboxylase; ACOX1, acyl-CoA oxidase 1; CPT, carnitine palmitoyl transferase; ELOVL6, elongation of long chain fatty acids 6; FAS, fatty acid synthase; FGF21, fibroblast growth factor 21; FOXA2, forkhead box A2; FOXO1, Forkhead box O1; G6P, glucose-6-phosphatase; JAK, janus kinase 3; L-PK, L-pyruvate kinase; LCAD, long chain acyl-CoA dehydrognase; LepRb, leptin receptor isoform b; LXR, liver X receptor; MCAD, medium chain acyl-CoA dehydrogenase; MOGAT, monoacylglycerol acyltransferase; PEPCK, phosphoenolpyruvate carboxykinase; PPAR, peroxisome proliferator-activated receptor; PGC1, PPAR γ coactivator 1; SCD, stearoyl-CoA desaturase; SREBP, sterol responsive element binding protein; STAT3, signal transducer and activator of transcription 3; TIMP2, tissue inhibitor of metalloproteinase 2; UCP2, uncoupling protein 2.

[a] $P < 0.05$ (one-way ANOVA and Bonferroni's multiple comparison test).

ChREBP nuclear protein than on its mRNA concentration (Table 1, **Fig. 5A**). Paradoxically, the opposite happens to *L-pk*: it is elevated in saline-infused *Agpat2*^{−/−} mice, indicating increased ChREBP transcriptional activity, but it is reduced in leptin-infused ones, indicating decreased ChREBP activation (Table 1).

Leptin does not affect ChREBP association with *L-pk* promoter but increases ChREBP *O*-GlcNAcylation

To address the paradoxical effect of leptin on ChREBP nuclear abundance and its transcriptional activity in the livers of *Agpat2*^{−/−} mice, the binding of ChREBP to *L-pk* promoter was assessed by ChIP. As shown in Fig. 5B, we observed no differences in the recruitment of ChREBP to the *L-pk* promoter between *Agpat2*^{+/+} and *Agpat2*^{−/−} mice, and neither food restriction or leptin treatment could modify this association.

O-linked β-N-acetylglucosamine (*O*-GlcNAcylation) has been recently recognized as a modification that increases both nuclear localization and transcriptional activity of ChREBP in the mouse liver (37). While we did not observe increased *L-pk* promoter occupancy by ChREBP (Fig. 5B), immunoblot analysis revealed that the livers of *Agpat2*^{−/−} mice do have increased abundance of *O*-GlcNAcylated ChREBP and that leptin treatment further elevate this modification (Fig. 5C).

Considered together, these findings suggest that leptin reduces the expression of lipogenic enzymes and hepatic triglyceride accumulation by decreasing ChREBP transcriptional activity. There is clearly a striking lack of concordance between total ChREBP nuclear levels, *L-pk* promoter association and *O*-GlcNAcylation and its transcriptional activity that will require further investigation.
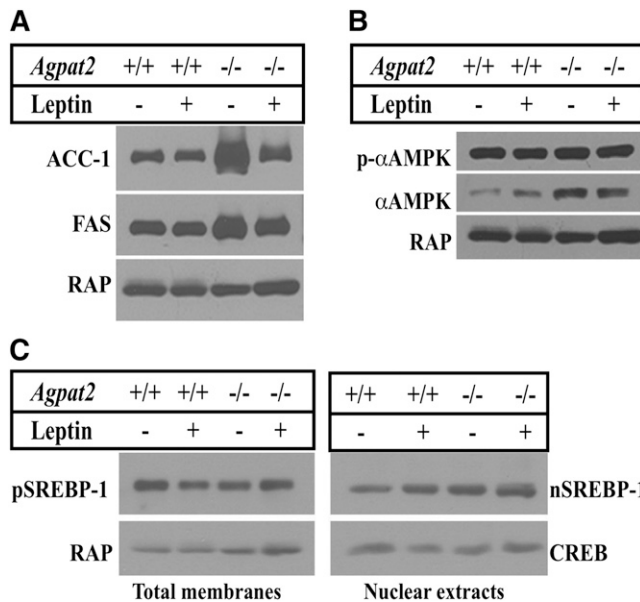
Fig. 4. Immunoblot analysis of proteins involved in fatty acid synthesis (A), αAMPK (B), and SREBP-1 (C) in wild-type (Agpat2$^{+/+}$) and Agpat2$^{-/-}$ mouse livers with and without leptin administration. A: ACC-1 and FAS protein levels in livers of Agpat2$^{+/+}$ and Agpat2$^{-/-}$ mice treated with or without leptin. B: Total and phosphorylated αAMPK (Thr172). C: Hepatic precursor (p) SREBP-1c or mature nuclear (n) SREBP-1c levels in Agpat2$^{+/+}$ and Agpat2$^{-/-}$ mice treated with or without leptin. Receptor-associated protein (RAP) and cAMP response element binding proteins (CREB) were used as loading controls for whole cell and total membrane and nuclear fraction, respectively. For immunoblots in panels A and B, whole liver extracts (30 μg) of pooled livers (n = 4 per pool) were separated in 8% SDS-PAGE. For immunoblots in panel C, total membrane protein (30 μg) or nuclear extract protein (20 μg) were pooled from individual livers (n = 4 per pool) and resolved by SDS-PAGE as indicated in Research Design and Methods.
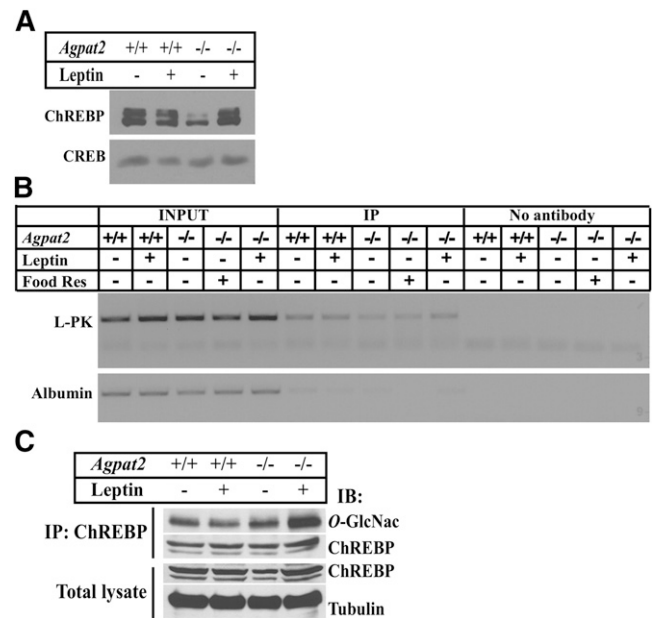

Fig. 5. ChREBP protein total nuclear levels (A), occupancy of L-pk promoter (B), and O-GlcNAcylation (C) in livers of wild-type (Agpat2$^{+/+}$) and Agpat2$^{-/-}$ mice treated with or without leptin or food restriction. For immunoblots in panel A, nuclear extract protein (20 μg) was pooled from individual livers (n = 4–6 per pool) and resolved by SDS-PAGE as indicated in Research Design and Methods. cAMP response element binding proteins (CREB) was used as loading control for nuclear fraction. For ChIP of hepatic ChREBP (B), chromatin/protein complexes were pooled from individual livers (n = 4–6), processed, and analyzed as indicated in Research Design and Methods. L-PK, liver pyruvate kinase. For O-GlcNAcylation immunoblot (IB), analysis of immunoprecipitated (IP) ChREBP (C), whole liver protein extracts of wild-type and Agpat2$^{-/-}$ mice treated with and without leptin or food restriction were pooled from individual livers (n = 4–6) and processed as indicated in Research Design and Methods. Tubulin was used as loading control in total lysate immunoblots.

Leptin reduces hepatic levels of phosphorylated-active AKT in Agpat2$^{-/-}$ mice

Upon leptin treatment, insulin receptor (IR) was increased in the liver of Agpat2$^{-/-}$ mice whereas the phosphorylated active form of AKT (phosphorylated at serine 473) was reduced (**Fig. 6**). Concordantly, leptin also reduced phosphorylated GSK-3β (phosphorylated at serine-9) hepatic levels, indicating lower AKT activation compared with the saline-infused Agpat2$^{-/-}$ mice. Leptin supplementation, therefore, improves insulin sensitivity of Agpat2$^{-/-}$ mice, resulting in decreased circulating insulin levels and lower AKT activation. No changes were noted in PI3K, total AKT, total GSK3 β, or total and phosphorylated ERK1/2 after leptin infusion in the Agpat2$^{-/-}$ mice (Fig. 6).

Leptin-mediated changes in the expression of genes involved in fatty acid synthesis, oxidation, and glucose metabolism in the skeletal muscle of Agpat2$^{-/-}$ mice

Since skeletal muscle is the major target tissue for insulin action, we determined whether leptin influences the expression of genes involved in lipid and glucose metabolism in the skeletal muscle of Agpat2$^{-/-}$ mice. In contrast to our findings in liver, saline-infused Agpat2$^{-/-}$ mice had significantly lower Fas and Scd1 but similar Acc1 and Elovl6

mRNA levels in the skeletal muscle compared with the wild-type mice (**Table 2**). Leptin, as well as food restriction, significantly lowered Acc1 mRNA expression in Agpat2$^{-/-}$ mice compared with the saline-infused Agpat2$^{-/-}$ mice (Table 2). Skeletal muscle mRNA levels for Mcad and Ucp2 were unchanged in saline-treated Agpat2$^{-/-}$ mice and Ucp2 transcript was significantly decreased by leptin treatment. In contrast, the Lcad mRNA was elevated in the Agpat2$^{-/-}$ mice. Unlike the liver, treatment with leptin did not alter Pk and Chrebp mRNA levels in the skeletal muscle of the Agpat2$^{-/-}$ mice. Pepck mRNA levels were significantly lower in saline-infused Agpat2$^{-/-}$ mice compared with saline-infused wild-type mice and leptin did not correct this abnormality (Table 2). Taken together these findings suggest that Agpat2$^{-/-}$ mice have decreased basal skeletal muscle fatty acid synthesis rate and that leptin further reduces it.

Hepatocyte specific gene deletion of LepR does not affect metabolic actions of leptin in Agpat2$^{-/-}$ mice

Leptin receptors are present at the CNS and several peripheral tissues, including the liver (6). To explore whether the action of leptin was mediated by direct signaling in the
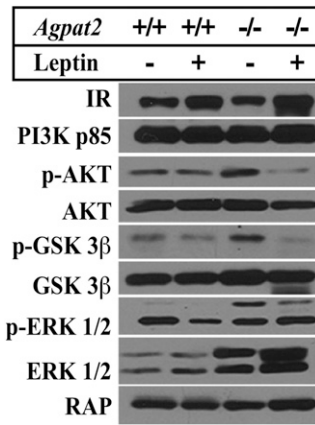
Fig. 6. Immunoblot analysis of proteins associated with insulin signaling in the livers of wild-type ($Agpat2^{+/+}$) and $Agpat2^{-/-}$ mice treated with or without leptin. Equal aliquots of whole liver protein extracts from individual livers (n = 4 per pool) were pooled (total, 30 μg), and proteins were resolved on SDS-PAGE as indicated in Research Design and Methods.

hepatocyte, we generated hepatocyte specific leptin receptor deficient $Agpat2^{-/-}$ mice by breeding $Agpat2^{+/-}$ mice with mice that harbor floxed leptin receptor allele ($LepR^{f/f}$) and Cre recombinase mice driven by albumin promoter (Alb-Cre) (7). The resulting $Agpat2^{-/-}$;$LepR^{f/f}$;Alb-CRE^+ ($Agpat2^{-/-}$;LepR$^{hepato-ko}$) mice were infused with leptin or saline for two weeks, and the effects on insulin activity and hepatic lipogenesis were determined. The deletion of the hepatic leptin receptor did not change plasma leptin levels in the $Agpat2^{-/-}$ mice (**Fig. 7A**, compare with Fig. 2A) and did not reverse their lack of white and brown adipose tissue (data not shown). Leptin infusion increased plasma leptin concentrations in both $Agpat2^{-/-}$;$LepR^{hepato-ko}$ and $Agpat2^{-/-}$;$LepR^{hepato-wt}$ mice, significantly reducing circulating insulin and glucose (Fig. 7B, C), as well as liver

triglycerides and cholesterol (Fig. 7D, E) in both lipodystrophic mouse groups.

The actions of leptin on mRNA levels of selected genes studied in $Agpat2^{-/-}$;$LepR^{hepato-ko}$ and $Agpat2^{-/-}$;$LepR^{hepato-wt}$ mice are shown in **Table 3**. Overall, compared with saline–infused $Agpat2^{-/-}$;$LepR^{heptao-ko}$, leptin supplementation induced equivalent changes in $Agpat2^{-/-}$;$LepR^{hepato-ko}$ and $Agpat2^{-/-}$;$LepR^{hepato-wt}$, indicating that the expression of leptin receptors in hepatocytes is not essential for the metabolic and transcriptional actions of leptin in the $Agpat2^{-/-}$ mice.

DISCUSSION

The worldwide epidemic of obesity and related metabolic diseases has fueled new adipose tissue biology research. Among the most important advances in the field has been the discovery of the hormonal actions of WAT (2). In CGL patients, the lack of WAT determines insulin resistance, diabetes mellitus, hepatic steatosis, and hyperlipidemia. In these patients (28) as well as in all tested murine models of CGL (27, 50, 51), leptin infusion ameliorates all metabolic complications of lipodystrophy, in spite of persistent WAT deficiency, indicating that leptin secretion by adipocytes is necessary for normal metabolic regulation. Importantly, the understanding of the mechanism by which leptin ameliorates the metabolic disturbances CGL is still incomplete.

One hypothesis is that leptin partitions body lipids, preventing excessive lipid buildup in nonadipose tissue (52). This action seems crucial, since independent evidence suggests that ectopic lipid accumulation directly causes insulin resistance and cellular dysfunction (i.e., lipotoxicity) (52).

Then the question is, how does leptin prevent lipotoxicity? It has been suggested that leptin increases fatty acid

TABLE 2. Relative expression of skeletal muscle mRNAs in $Agpat2^{-/-}$ and $Agpat2$ mice upon leptin treatment

Genotype		$Agpat2^{+/+}$	$Agpat2^{-/-}$	$Agpat2^{-/-}$	$Agpat2^{-/-}$
Infusion		Leptin	Saline	Saline	Leptin
Food restriction		No	No	Yes	No
		N = 4	N = 3	N = 6	N = 5
		Fold Change	Fold Change	Fold Change	Fold Change
Fatty acid synthesis	ACC1	0.7 ± 0.1	1.1 ± 0.1	0.4 ± 0.06[ab]	0.4 ± 0.1[ab]
	FAS	0.6 ± 0.1[a]	0.5 ± 0.03[a]	0.2 ± 0.04[a]	0.2 ± 0.05[a]
	ELOVL6	0.9 ± 0.06	0.8 ± 0.06	0.4 ± 0.1[a]	1.0 ± 0.3
	SCD1	0.5 ± 0.5[a]	0.4 ± 0.1[a]	0.3 ± 0.04[a]	0.3 ± 0.07[a]
Fatty acid oxidation	LCAD	2.5 ± 0.3[a]	2.5 ± 0.1[a]	3.9 ± 0.3[ab]	1.8 ± 0.1
	MCAD	1.7 ± 0.03[a]	1.6 ± 0.03	2.1 ± 0.3[a]	1.2 ± 0.1
	UCP2	1.0 ± 0.1	1.8 ± 0.3	0.7 ± 0.2[b]	0.7 ± 0.1[b]
Transcriptional regulators	ChREBP	1.0 ± 0.1	1.1 ± 0.1	0.8 ± 0.2	0.7 ± 0.1
	SREBP1c	2.2 ± 0.4[a]	0.8 ± 0.3	1.9 ± 0.1[b]	0.9 ± 0.1
Glucose metabolism	PKM	1.8 ± 0.4	1.3 ± 0.2	2.0 ± 0.4	1.5 ± 0.1
	PEPCK	0.3 ± 0.03[a]	0.2 ± 0.1[a]	0.1 ± 0.0[a]	0.1 ± 0.0[a]

Each value represents the mean fold change (± SEM) of individual mice compared with saline-infused, nonfood-restricted $Agpat2^{+/+}$ mice. PKM, pyruvate kinase, muscle isoform. For other abbreviations, see Table 1.

[a] $P < 0.05$ compared with saline-infused, nonfood-restricted $Agpat2^{+/+}$ mice (one-way ANOVA and Bonferroni's multiple comparison test).

[b] $P < 0.05$ compared with saline-infused, nonfood-restricted $Agpat2^{-/-}$ mice (one-way ANOVA and Bonferroni's multiple comparison test).
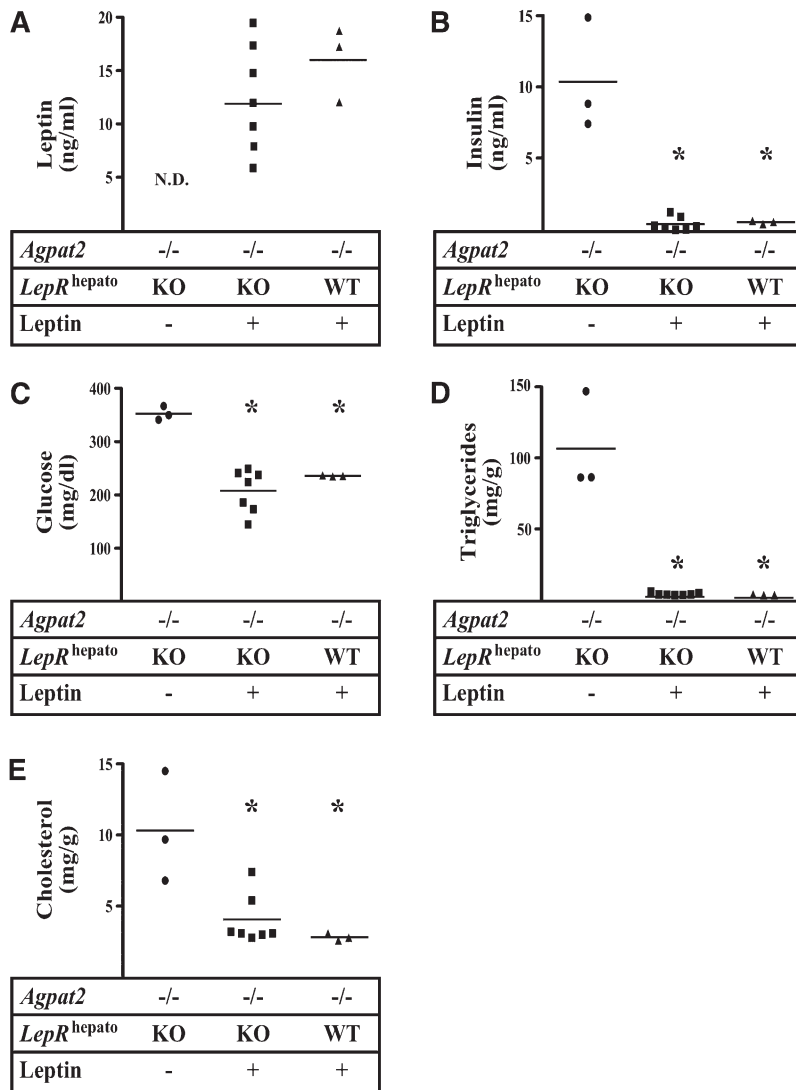
A

Leptin (ng/ml)

N.D.

Agpat2	-/-	-/-	-/-
LepR hepato	KO	KO	WT
Leptin	-	+	+

B

Insulin (ng/ml)

* *

Agpat2	-/-	-/-	-/-
LepR hepato	KO	KO	WT
Leptin	-	+	+

C

Glucose (mg/dl)

* *

Agpat2	-/-	-/-	-/-
LepR hepato	KO	KO	WT
Leptin	-	+	+

D

Triglycerides (mg/g)

* *

Agpat2	-/-	-/-	-/-
LepR hepato	KO	KO	WT
Leptin	-	+	+

E

Cholesterol (mg/g)

* *

Agpat2	-/-	-/-	-/-
LepR hepato	KO	KO	WT
Leptin	-	+	+

Fig. 7. Plasma and liver metabolites in $Agpat2^{-/-}$;$LepR^{hepato-ko}$ and $Agpat2^{-/-}$;$LepR^{hepato-wt}$ mice treated with or without leptin. In panels A–C, plasma leptin, insulin, and glucose levels, respectively, are shown. In panels D and E, liver triglyceride and cholesterol levels, respectively, are shown. The horizontal lines correspond to the mean value. The data were compared by one-way ANOVA with Bonferroni's correction for multiple comparisons. $*P < 0.05$.

oxidation in critical insulin-responsive tissues (44, 45). In our studies, leptin supplementation did not increase mRNA levels of genes involved in mitochondrial or peroxisomal fatty acid oxidation in the $Agpat2^{-/-}$ mice (Table 1). Furthermore, leptin infusion significantly reduced mRNA levels of *Fgf21*, a cognate *Pparα* target gene (Table 1), with potent insulin-sensitizing actions in rodents and nonhuman primates as well as humans (53) Since *Pparα* is the master regulator of hepatic fatty oxidation, it is plausible that leptin supplementation results in diminished *Pparα* activity and lower liver β-oxidation in the $Agpat2^{-/-}$ mice. Concordantly, hepatic mRNA levels of *Ucp2* were reduced upon leptin treatment in these lipodystrophic animals (Table 1).

Alternatively, leptin may prevent lipotoxicity by reducing the exaggerated de novo lipogenesis characteristic of insulin-resistant organisms. Here we report that leptin dramatically decreases the levels of all major lipogenic enzymes in livers of $Agpat2^{-/-}$ mice (Table 1, Fig. 4A) and that this correlates with lower transcriptional activity of ChREBP as estimated by decreased mRNA levels of *L-pk*, a bona fide ChREBP target gene (Table 1). Clearly, leptin does not modify occupancy of *L-pk* promoter by ChREBP (Fig. 5B),

but it increases its *O*-GlcNAcylation (Fig. 5C), a reported modification that results in higher ChREBP nuclear translocation and transcriptional activity (37). Detailed studies of the posttranslational modification and transcriptional co-regulators associated with ChREBP will be necessary to completely understand the actions of leptin on the transcriptional control of hepatic lipogenesis (54, 55).

Unlike the lipodystrophic A-ZIP/F-1 mice (43), $Agpat2^{-/-}$ mice have significantly decreased plasma T4, and this abnormality is significantly corrected by leptin replacement (Fig. 3A). The reported relationship between plasma leptin and thyroid levels is inconsistent (56). Interestingly, a new class of selective thyromimetic drugs potently ameliorates liver triglycerides concentration in murine models of hepatic steatosis (57, 58), suggesting that normalization of hypothyroxiemic state in $Agpat2^{-/-}$ mice could be a relevant mechanism for leptin's beneficial actions in the liver of these animals. The precise mechanisms of hypothyroxinemia in $Agpat2^{-/-}$ mice remain unclear and may require further investigation.

Similarly, leptin decreases hypercorticosteronemia in $Agpat2^{-/-}$ mice (Fig. 3B) and this could, at least partially,

TABLE 3. Relative expression of liver mRNAs in $Agpat2^{-/-};LepR^{+/+};Alb\text{-}CRE$ and $Agpat2^{-/-};LepR^{f/f};Alb\text{-}CRE^+$ mice upon leptin treatment

Genotype		$Agpat2^{-/-};LepR^{hepato\text{-}ko}$	$Agpat2^{-/-};LepR^{hepato\text{-}ko}$	$Agpat2^{-/-};LepR^{hepato\text{-}wt}$
Infusion		None	Leptin	Leptin
		$N = 3$	$N = 6$	$N = 3$
		Fold Change	Fold Change	Fold Change
Fatty acid and TG synthesis	ACC1	0.9 ± 0.06	0.3 ± 0.01^a	0.2 ± 0.02^a
	FAS	1.2 ± 0.03^a	0.3 ± 0.02^a	0.3 ± 0.01^a
	ELOVL6	1.2 ± 0.1	0.3 ± 0.02^a	0.3 ± 0.003^a
	SCD1	0.9 ± 0.01^a	0.1 ± 0.01^a	0.1 ± 0.01^a
	MOGAT1	1.5 ± 0.09^a	0.05 ± 0.01^a	0.1 ± 0.01^a
Fatty acid oxidation	CPT1α	1.2	0.9	0.8
	LCAD	1.4	0.7	0.7
	MCAD	1.2	0.6	0.5
	ACOX1	1.1	0.5	0.5
	UCP2	1.3 ± 0.03^a	0.4 ± 0.02^a	0.3 ± 0.03^a
	FGF21	1.3 ± 0.07	0.04 ± 0.01^a	0.09 ± 0.005^a
Transcriptional regulators	ChREBP	1.0 ± 0.04	1.8 ± 0.06^a	1.5 ± 0.07^a
	SREBP1a	1.2	1.0	0.9
	SREBP1c	1.2	1.1	1.0
	SREBP2	1.5	1.4	1.5
	PPARα	1.3	1.1	1.2
	PPARβ	1.1	1.0	0.8
	PPARγ	1.2	0.4	0.4
	PGC1α	1.2	3.0	2.2
	FOXO1	1.2	1.5	1.1
	LXRα	1.4	1.1	1.2
Glucose metabolism	L-PK	1.0 ± 0.06	0.4 ± 0.01^a	0.3 ± 0.05^a
	G6P	1.2	0.9	0.2
	PEPCK	1.0	3.2	2.7
Leptin signaling pathway	JAK2	1.1	0.8	0.8
	STAT3	1.2	1.0	0.8
	TIMP2	1.0	0.9	0.7
	LEPRb	1.3 ± 0.2	12.3 ± 0.2^a	10.7 ± 2.7^a

Each value represents the mean fold change ($\pm$ SEM) of individual mice or the fold change of pooled samples compared with saline-infused, nonfood-restricted $Agpat2^{-/-};LepR^{hepato\text{-}wt}$ mice. (See Research Design and Methods for the criteria by which some gene products were selected for individual analysis.) For abbreviations, see Table 1.
[a] $P < 0.05$ (one-way ANOVA and Bonferroni's multiple comparison test).

explain insulin-sensitizing effects of leptin in these mice. Elevated corticosterone appears to be a common finding in murine models of leptin deficiency (59), possibly owing to lack of a direct suppressive effect of leptin on glucocorticoid secretion by corticoadrenal cells (60). Interestingly, whereas adrenalectomy dramatically improves both hyperglycemia and hyperinsulinemia in A-ZIP/F-1 lipodystrophic mice, it fails to reduce liver triglyceride concentrations in these animals (61), suggesting that hypercorticosteronemia does not play an essential role in the hepatic steatosis associated with severe lipodystrophy.

Leptin receptor has been detected in all major liver cell types, including hepatocytes, sinusoidal endothelial, Kupffer, and stellate cells (62–64). In contrast, albumin is exclusively expressed in hepatocytes (65). Thus, as expected, leptin receptor will still be present in nonparenchymal liver cells of $Agpat2^{-/-};LepR^{hepato\text{-}ko}$ mice (Table 3). Upon leptin administration liver LepRb mRNA levels were increased in $Agpat2^{-/-};LepR^{hepato\text{-}ko}$ mice (Table 3), strongly suggesting that LepRb is upregulated in nonhepatocyte cell types. This response was also present in wild-type and $Agpat2^{-/-}$ mice (Table 1). The original $LepR^{f/f};Alb\text{-}CRE^+$ mice used to create our $Agpat2^{-/-};LepR^{hepato\text{-}ko}$ mice reportedly have remnant LepR mRNA in their livers, reflecting the contribution of nonhepatocyte cells to the total

liver LepR pool (7). It is unclear, however, in which nonparenchymal liver cells LepR expression is occurring. The clarification of these questions will require investigation of the different cell types of the liver in their physiological tissue context. In agreement with our observations, the hepatocyte-specific deletion of LepR in ob/ob mice did not preclude the ability of infused leptin to increase the circulating levels of insulin-like growth factor binding protein-2 (IGFBP2), a leptin-regulated liver product possibly implicated in leptin metabolic actions (66). Thus, a more complete understanding of the metabolic role of leptin in AGPAT2-deficient mice will require the generation of a total liver-specific leptin receptor knockout mouse. This strategy will be also essential to address the nonmetabolic actions of leptin in the liver, such as fibrogenesis and immunomodulation, which seems to be directly dependent on LepR activation in nonparenchymal liver cells (62–64). Similarly, in vivo euglycemic hyperinsulinemic clamps will be required to corroborate the insulin-sensitizing effect of leptin in the $Agpat2^{-/-}$ mice and to determine the major site of leptin action in these mice.

In conclusion, the results reported here suggest that 1) virtually all the metabolic derangements developed by the $Agpat2^{-/-}$ lipodystrophy mice are derived from leptin deficiency; 2) leptin restitution in $Agpat2^{-/-}$ mice may correct

fatty liver by decreasing de novo hepatic lipogenesis and by elevating thyroxine levels; *3*) the main transcriptional regulator mediating fatty liver in *Agpat2*$^{-/-}$ mice is likely ChREBP, and leptin normalizes its nuclear levels, reduces its transcriptional activity, and increases its glycosylation level but does not change its abundance in the *L-pk* promoter of these mice; and *4*) the beneficial metabolic actions of leptin in *Agpat2*$^{-/-}$ mice do not depend on LepR signaling in hepatocytes.▉▉

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