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# Liver Triglyceride Secretion and Lipid Oxidative Metabolism Are Rapidly Altered by Leptin in *Vivo*

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**Leptin has potent lipid-lowering effects in peripheral tissues and plasma that are proposed to be important for the prevention of cellular lipotoxicity and insulin resistance. The current study addressed *in vivo* the effects of acute leptin delivery on liver triglyceride (TG) metabolism, the consequence of hepatic leptin action on whole-body TG homeostasis, and the mechanisms of leptin action. A 120-min iv leptin infusion (plasma leptin, ~14 ng/ml) decreased liver TG levels (53 ± 3%; $P = 0.001$), but not skeletal muscle TG levels, and increased liver phosphatidylinositol 3-kinase activity (341 ± 95%; $P = 0.01$) in lean rats. Leptin had no effect on liver TG levels or phosphatidylinositol 3-kinase activity in diet-induced obese rats. In lean animals, leptin decreased the plasma TG concentration (20 ± 7%; $P = 0.017$), the rate of TG accumulation in plasma after tyloxapol administration (26 ± 6%; $P = 0.003$), and TG secretion from isolated liver (51 ± 8%; $P = 0.004$). To determine possible metabolic fates of depleted hepatic TG, we assessed leptin effects on liver oxidative metabolism. Leptin increased hepatic acetyl-coenzyme A carboxylase phosphorylation (85 ± 13%; $P = 0.006$), fatty acid oxidation (49 ± 7%; $P = 0.001$) and ketogenesis (69 ± 15%; $P = 0.004$). Finally, intracerebroventricular delivery of leptin for 120 min had no effect on liver TG levels, but did increase signal transducer and activator of transcription 3 phosphorylation (162 ± 40%; $P = 0.02$). These data present *in vivo* evidence for a role for leptin in the acute regulation of hepatic TG metabolism, and whole body TG homeostasis. A likely contributing mechanism for these effects is leptin-induced partitioning of TG into oxidative pathways. (*Endocrinology* 147: 1480–1487, 2006)**

LEPTIN-DEFICIENT states resulting from leptin gene mutations or lipodystrophy in rodents and humans are characterized by hyperlipidemia, excessive storage of lipid in tissues such as liver and skeletal muscle, and insulin resistance. These defects are markedly improved by the administration of leptin in humans and rodents (1–6), establishing leptin as an important regulator of lipid metabolism and insulin sensitivity. In a previous study (7) we demonstrated that a 90-min perfusion of isolated rat liver with leptin was sufficient to decrease triglyceride levels by approximately 50%. These effects were absent in diet-induced obese (DIO) rats, a situation of elevated liver and plasma triglyceride (TG) concentrations, establishing hepatic leptin resistance as a potential mechanism contributing to dyslipidemia in obesity.

A number of questions arose from these observations. First, because our previous observations were made in a perfused liver model, the acute *in vivo* effects of leptin on liver TG levels are unclear. This is an important issue, because the regulation of liver TG metabolism *in vivo* occurs in a neural, nutrient, and hormonal milieu that is disrupted in the isolated liver. Second, the rapid depleting effects of leptin on liver TG levels suggest that leptin may play a role in the acute regulation of hepatic lipoprotein metabolism. TG export to peripheral tissues from the liver as a component of very low-density lipoprotein (VLDL) is important for the maintenance of normal lipid homeostasis. However, dysregulation of lipoprotein metabolism is common in obesity, resulting in a so-called atherogenic lipid profile [large VLDL and small, dense low-density lipoprotein (LDL)]. Interestingly, the plasma TG concentration is markedly decreased by chronic leptin treatment in leptin-deficient humans and in rodents (1, 2, 8–10). However, it is unclear what role, if any, leptin-induced alterations in TG secretion may play in reducing plasma TG. Finally, the mechanisms responsible for the depleting effects of leptin on liver TG levels were not addressed. In skeletal muscle and heart, leptin acutely increases fatty acid oxidation (11–17), an effect that in skeletal muscle is a consequence of decreased acetyl-coenzyme A carboxylase (ACC) activity mediated by increased AMPK activity (16). Leptin-induced increases in oxidative metabolism have also been demonstrated (18, 19) or implied (20) in other tissues, but the effects of leptin in liver are unknown. The current study was undertaken to address each of these issues. Specifically, we determined *in vivo* the effects of acute leptin delivery on liver TG metabolism, the consequence of hepatic leptin action on whole-body triglyceride homeostasis, and the potential mechanisms of leptin action on liver TG levels. The data demonstrate that iv delivery of leptin for 120 min substantially decreases liver TG levels and activates phosphatidylinositol 3-kinase (PI3-kinase) in lean, but not DIO, rats; that leptin decreases liver TG secretion and increases hepatic fatty acid oxidation and ketogenesis (most

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Abbreviations: ACC, Acetyl-coenzyme A carboxylase; apoB, apolipoprotein B; AMPK, 5′-AMP-activated protein kinase; DIO, diet-induced obesity; ICV, intracerebroventricular; IRS-1, insulin receptor substrate-1; LDL, low-density lipoprotein; PI3-kinase, phosphatidylinositol 3-kinase; STAT, signal transducer and activator of transcription; TG, triglyceride; VLDL, very low-density lipoprotein.

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likely by inhibition of ACC activity), and that 120 min of intracerebroventricular (ICV) leptin administration does not decrease liver TG levels.

## Materials and Methods

### *Animal care and maintenance*

Male Wister rats were purchased from Charles River Laboratories (Madison, WI) at a weight of 175–200 g. After arrival, rats were maintained on a constant 12-h light, 12-h dark cycle with free access to water and were fed *ad libitum* a standard rat chow diet (11% of calories from fat) or a high-fat diet (TD 96001, Harlan Teklad, Madison WI; 45% of calories from fat) for 5–6 wk. All materials and methods were approved by the institutional animal care and use committee of University of Pittsburgh and were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

### *Implantation of chronic indwelling catheters*

Animals were anesthetized with a ketamine:xylazine:acepromazine mix (60 mg/kg ketamine, 5 mg/kg, xylazine, and 1 mg/kg acepromazine). Catheters (PE-50, Intramedic, BD Biosciences, Sparks, MD) were introduced into the left carotid artery (advanced to the aortic arch) and the right jugular vein (advanced to the right atrium) as previously described (9, 21). The catheters were exteriorized at the back of the neck, filled with a sterile glycerol:heparin mix (3:1), and flame-sealed. The wounds were closed with sutures and treated with betadine. The animals were treated with ketoprofen (2 mg/kg, sc), and their recovery was monitored with special attention given to food intake, weight gain, and healing of wounds. Animals were allowed at least 4 d to recover, and only those that had achieved more than 90% of presurgery weights were used in the studies.

### *Implantation of third cerebral ventricle cannula*

Anesthetized animals were placed on a stereotactic apparatus. The incisor bar was set at −3.3 mm. The skull was exposed so that cranial sutures could be viewed. A steel guide cannula was inserted at coordinates from bregma of −2.8 mm anterior/posterior, 0.0 mm medial/lateral from the midsagittal suture, and 8.4 mm ventral to dura according to the brain atlas (22, 23). Three screws placed approximately 5 mm from the cannula insertion site and reinforced with dental cement secured the cannula in place. After checking for cerebrospinal fluid return, a dummy cannula was inserted to prevent clogging of the guide cannula. The skin was closed and sutured over the mound of the dental cement. Antibiotic treatments and recovery procedures were the same as described above.

### *Liver perfusions*

The perfusion equipment and procedures were described previously (7) with modifications. Briefly, livers isolated from lean postprandial rats were perfused with Krebs-Henseleit buffer, which contained 15 mM D-(+)-glucose, 1.7 mM fructose, 2 mM L-lactate, 0.2% BSA, and 0.2 mM palmitate in a recirculating system at a rate of approximately 2.7 ml/min/g liver. Additions of recombinant rat leptin (R&D Systems, Inc., Minneapolis, MN) or vehicle together with human insulin (Eli Lilly & Co., Indianapolis, IN) were accomplished by admixing into the portal vein perfusate using a syringe pump as described previously (7). This administration regimen gave constant perfusate concentrations of leptin (~14 ng/ml) and insulin (~1 mU/ml).

### *Experimental design*

*Effects of iv leptin delivery on liver TG levels.* Conscious 18-h fasted rats received a continuous iv infusion of either vehicle or leptin using a syringe pump (model 11, Harvard Apparatus, Natick, MA) at a rate of 0.2 μg/kg·min for 120 min (preceded by a 2-min priming dose of 2 μg/kg·min) or for 15 min in the experiments designed for measuring liver PI3-kinase activity and Akt phosphorylation. Volume delivery was 5 ml/kg·h for both vehicle and leptin infusions. Blood samples were taken before infusions and at 120 min, the rats were then anesthetized, and liver and skeletal muscle (soleus) were promptly removed, snap-frozen in liquid nitrogen, and stored at −80 C until analysis.

*Effects of leptin delivery on liver TG secretion.* Three experiments were preformed to address this question. In the first experiment, the plasma TG concentration was measured at the end of a 120-min leptin infusion. A second experiment assessed the rate of TG accumulation in blood after the administration of tyloxapol (Triton WR1339, Sigma-Aldrich Corp., St. Louis, MO), an inhibitor of TG clearance (24, 25). Specifically, leptin or vehicle was infused into lean rats for 120 min, then all animals received a bolus injection of tyloxapol (300 mg/kg). Immediately before tyloxapol administration and 45 and 90 min after Tyloxapol administration and a continuing leptin infusion, blood samples were taken and apolipoprotein B (apoB)-containing lipoproteins were precipitated as described below. Subsequently, the TG content was measured. Because the vast majority of TG is associated with the apoB-containing VLDL particle, we refer to these TG measurements as VLDL-TG. Preliminary experiments determined that the rate of TG appearance in blood was linear for at least 90 min after tyloxapol administration (data not shown). VLDL-TG secretion rates were calculated from the slope of the line after tyloxapol injection, based on the assumption of a plasma volume of 3.5% of body weight. In a third experiment, the secretion of TG from isolated livers was assessed directly. Livers were perfused as described above for 90 min in the absence or presence of leptin. Subsequently, aliquots of perfusate were taken, and VLDL-TG was measured after precipitation of apoB-containing lipoproteins as described below.

*Effects of leptin on hepatic fatty acid oxidation and ketogenesis.* Leptin or vehicle was infused iv for 120 min into 18-h fasted animals. Subsequently, four pieces of liver weighing approximately 200 mg each were isolated; each piece of tissue was then further divided into four pieces of approximately 50 mg and immediately incubated with 9,10-[$^3$H]palmitate for the measurement of fatty acid oxidation, as described below. To assess ketogenesis, isolated livers were perfused for 90 min in the absence or presence of leptin and 0.2 mM palmitate. Subsequently, aliquots of the perfusate were taken, and ketone bodies were measured.

*Effects of acute ICV delivery of leptin on liver TG levels.* Conscious 18-h fasted rats received a continuous ICV infusion of leptin (1 μg/kg·h) or artificial cerebrospinal fluid at a rate of 2 μl/h. At the end of the 120-min infusion, the rats were anesthetized, and the hypothalamus and liver were promptly removed, snap-frozen in liquid nitrogen, and stored at −80 C until analysis.

### *Tissue, plasma, and perfusate measurements*

Tissue TG levels were determined as described previously (7, 9). Briefly, approximately 50 mg frozen liver tissue was extracted in 1 ml of a chloroform-methanol mix (2:1). After redissolving the lipid pellet in 60 μl *tert*-butanol and 40 μl of a Triton X-114-methanol (2:1) mix, TG were measured spectrophotometrically (DU 530, Beckman Coulter, Fullerton, CA) using the glycerol phosphate oxidase-TG kit and Lintrol lipids as standard (Sigma-Aldrich Corp.). Fatty acid oxidation was assessed in liver sections previously exposed to leptin *in vivo* for 120 min as described above. Liver sections (200 mg) were incubated in 2 ml of a culture medium containing 10 mM HEPES, 5 mM glucose, and 0.2 mM 9,10-[$^3$H]palmitate in a shaking water bath at 37 C for 2 h. Palmitate oxidation was assessed by measuring the quantity of tritiated water released into the medium, as previously described (19, 26). Briefly, at the end of the 2-h incubation, 200 μl medium was transferred to an open 1.5-ml tube, the tube was placed into a capped scintillation vial containing 0.5 ml unlabeled water, and the vial was kept at 50 C for 18 h. During this period, tritiated water in the tube equilibrated with unlabeled water in the vial. To determine the equilibration coefficient, 200 μl medium containing a known quantity of tritiated water (10 μCi) was incubated similarly to other samples. After the 18-h incubation, 10 ml scintillation fluid was added to the vials, and the samples were counted in duplicate in a Beckman scintillation counter. The rate of palmitate oxidation was calculated from the specific activity of the starting fatty acid solution.

ApoB-containing lipoproteins in perfusate (8 ml) or plasma (200 μl; 1:3 diluted with saline) were precipitated by adding 5% 2 g/liter dextran sulfate and 10% 2.25 M magnesium sulfate (27). After gentle mixing, a 10-min incubation at room temperature, and centrifugation at 2000 × *g* for 10 min, the supernatant was removed, and the pellet was dissolved in 200 μl of a high-salt buffer (containing 2 M NaCl, 2 mM EDTA, and

50 mM $NaP_i$), 200 μl *tert*-butanol, and 100 μl of a methanol-Triton X-100 mixture (1:1), each added separately followed by a 30-sec vortex. After centrifugation at 4000 × *g*, TG in the upper phase of the supernatant was analyzed spectrophotometrically as described above. β-Hydroxybutyrate in perfusate samples was measured enzymatically using the β-hydroxybutyrate liquicolor kit (Stanbio, Boerne, TX).

Leptin was measured using a rat-specific RIA kit (Linco Research, Inc., St. Charles, MO). PI3-kinase activity and insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation were assayed as previously described (7). Hypothalamic phospho-signal transducer and activator of transcription 3 (phospho-STAT3) and total STAT3, hepatic phospho-Akt ($Ser^{473}$), Akt, and hepatic phospho-ACC ($Ser^{79}$) were measured using standard immunoblotting techniques [α-phospho-STAT3 catalogue no. 9131, α-STAT3 catalog no. 9132; α-phospho-Akt catalog no. 9271, and α-Akt catalog no. 9272 (Cell Signaling Technology, Beverly, MA); and α-phospho-ACC catalog no. 07-303 (Upstate Biotechnology, Inc., Lake Placid, NY); all used at a 1:1000 dilution]. Immunoblots were quantified by densitometry.

5′-AMP-activated protein kinase activity (AMPK) was measured by quantifying the incorporation of $^{32}P$ into a synthetic substrate peptide AMARAASAAALARRR as previously described (28). Briefly, AMPK was extracted from a liver homogenate using the polyethylene glycol precipitation method (28). The protein concentration was measured colorimetrically. Subsequently, AMPK activity was measured in a 25-μl total volume in the presence of a buffer containing 40 mM HEPES-NaOH (pH 7.0), 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM dithiothreitol, and [$\gamma$-$^{32}P$]ATP/Mg at 30 C for 10 min.

### *Statistical methods*

All results are expressed as the mean ± SE. Statistical significance was determined by unpaired *t* test using the Systat statistical program (Evanston, IL). Statistical significance was assumed at $P < 0.05$.

## Results

### *Acute iv leptin delivery substantially decreases liver TG levels and activates PI3-kinase in lean, but not DIO, rats*

In a previous study (7) we demonstrated that leptin rapidly decreases liver TG levels in a perfused liver model and that these effects are absent in diet-induced obesity. However, it is unknown whether similar effects or defects of leptin action on liver TG levels are present *in vivo*. Furthermore, it is unclear whether these rapid effects on liver TG levels are replicated in other peripheral tissues, such as skeletal muscle. To address these issues, leptin was infused into lean animals (body weight, 286 ± 5 g) at a rate that increased the plasma leptin concentration from 0.4 ± 0.1 to 14.2 ± 1.2 ng/ml (Fig. 1), values within the physiological range of leptin concentrations in rodents. This plasma leptin concentration decreased liver TG levels by 53 ± 3% ($P = 0.001$) compared with vehicle-infused controls (Fig. 2A). Furthermore, leptin increased liver PI3-kinase activity (Fig. 2B) by 341 ± 95% ($P = 0.01$) and IRS-1 tyrosine phosphorylation by 393 ± 136% ($P = 0.03$), consistent with our previous observations in isolated perfused liver (7). Interestingly, leptin did not decrease skeletal muscle TG levels (Fig. 2A). In DIO rats (body weight, 462 ± 10 g), hepatic TG levels were increased by 129 ± 6% compared with standard chow-fed controls [$P < 0.0001$; Fig. 2 (leptin −) *vs.* Fig. 3 (leptin −)], and the fasting plasma leptin concentration was increased by approximately 10-fold compared with lean animals (Fig. 1, 0 min point). However, a leptin infusion that resulted in a plasma concentration of 22.9 ng/ml had no effect on liver TG levels, the activity of liver PI3-kinase, or IRS-1 tyrosine phosphorylation in DIO rats (Fig. 3).

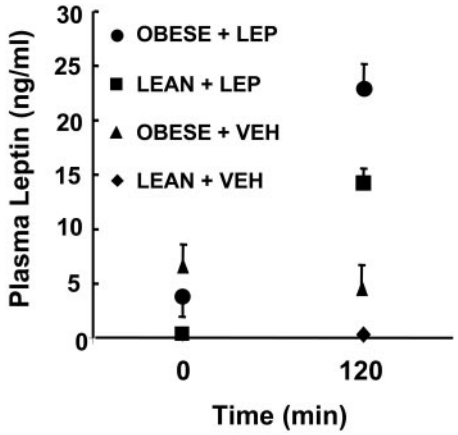


FIG. 1. Plasma leptin concentration before and after vehicle or leptin infusion. Fasted (18 h) standard chow (LEAN) or high-fat-fed (OBESE) rats received a continuous iv infusion of either vehicle (VEH) or leptin (LEP) at a rate of 0.2 μg/kg·min after a priming dose of 2 μg/kg·min in a volume of 5 ml/kg·h for 120 min. Plasma samples were taken before (0 min) and after (120 min) the infusions, and leptin was measured by RIA. Results are presented as the mean ± SE ($n \geq 5$/group).

### *Leptin decreases liver TG secretion*

Whole body TG homeostasis is critically dependent on the secretion of TG as components of the apoB-containing VLDL particle. The demonstration that leptin decreases liver TG levels in lean animals raises the possibility that leptin may alter TG secretion from liver. Thus, we next undertook three experiments to address this hypothesis (Fig. 4). First, the plasma TG concentration was determined subsequent to a 120-min leptin infusion (Fig. 4A). TG concentrations in leptin-infused animals were reduced by 20 ± 7% compared with vehicle-infused animals ($P = 0.017$). To determine whether leptin was decreasing TG appearance or disposal, we next inhibited the clearance of plasma TG using tyloxapol and subsequently measured the appearance of TG in plasma in the absence or presence of a leptin infusion. Leptin infusion in the presence of tyloxapol reduced the rate of VLDL-TG appearance in plasma from 58 ± 3 to 43 ± 4 mg/kg·min ($P = 0.003$, leptin *vs.* vehicle; Fig. 4B). Finally, to rule out changes in the TG content of other apoB-containing lipoproteins and other possible confounding *in vivo* variables, we performed an experiment that assessed the effects of leptin on TG secretion from isolated perfused liver (Fig. 4C). As suggested by our *in vivo* experiment, leptin decreased perfusate VLDL-TG by 51 ± 8% ($P = 0.004$, leptin *vs.* vehicle).

### *Leptin increases lipid oxidative metabolism in liver*

We have demonstrated that leptin decreases total liver TG and TG secretion. However, these observations give no indication of the metabolic fate of the depleted liver TG. It is well established that leptin increases fatty acid oxidation. Thus, one potential mechanism to decrease liver TG would be to increase the rate of fatty acid oxidation subsequent to TG hydrolysis. Therefore, we next examined the effects of leptin on the activity of lipid oxidative pathways in liver. Livers taken from animals infused with leptin for 120 min oxidized exogenous palmitate at a rate 49 ± 7% ($P = 0.001$)

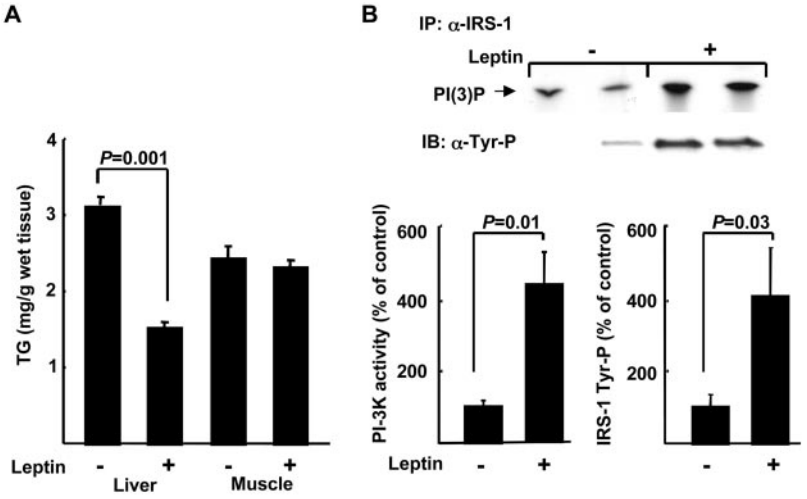


FIG. 2. Effects of iv leptin infusion on liver and skeletal muscle TG levels and liver PI3-kinase activity and IRS-1 tyrosine phosphorylation in lean rats. Lean rats were infused with vehicle (Leptin −) or leptin (Leptin +) as described in Fig. 1. Subsequently, liver and skeletal muscle (soleus) were isolated, and TG levels were assessed (A). For the measurement of liver PI3-kinase activity and IRS-1 tyrosine phosphorylation (B), animals were infused with vehicle (Leptin −) or leptin (Leptin +) for 15 min, then the liver was isolated. PI3-kinase activity and tyrosine phosphorylation of IRS-1 were measured as described in *Materials and Methods*. B (*Upper*), Representative autoradiograph of PI3-kinase activity and tyrosine phosphorylation of IRS-1. B (*Lower left*), Quantification of PI3-kinase activity measured as phosphatidylinositol 3-phosphate [PI(3)P] production and derived from scintillation counting of $^{32}$P-containing PI(3)P on TLC plates. B (*Lower right*), Quantification of tyrosine phosphorylation of IRS-1 measured by densitometry. Significant differences between groups are indicated. Results are presented as the mean ± SE (n = a minimum of 6 livers or muscles/group for TG and a minimum of 5 livers/group for PI3-kinase and IRS-1 tyrosine phosphorylation). IP, Immunoprecipitated; IB, immunoblotted.

greater than the rate of fatty acid oxidation in livers taken from vehicle-infused animals (Fig. 5A). Furthermore, leptin increased ketogenesis, another important oxidative pathway in liver, in perfused liver by 69 ± 15% ($P = 0.004$) compared with vehicle-perfused control livers (Fig. 5B). In the same experiment we evaluated palmitate incorporation into lipids and found no differences between leptin- and vehicle-treated livers, suggesting that esterification of fatty acids into the TG pool was not altered by leptin (data not shown). Increased rates of oxidation induced by leptin were matched by decreases in the activity (as measured by phosphorylation) of ACC, demonstrating a biochemical mechanism for leptin action on fatty acid oxidation (Fig. 6C). We also examined potential biochemical mechanisms of leptin action by assessing the responses of Akt and AMPK to a leptin stimulus. Leptin increased phosphorylation of the PI3-kinase downstream target Akt by 73 ± 15% (Fig. 6A; $P = 0.01$). However, similar to our previous observations in isolated liver (7), leptin had no effect on AMPK activity after a 120-min leptin stimulus (Fig. 6B) or a 15-min leptin infusion (data not shown).

### *Acute ICV leptin delivery does not replicate the effects of iv leptin delivery on liver TG levels*

A number of studies have demonstrated the effects of ICV delivery of leptin on carbohydrate (29) and lipid (24, 30) metabolism. To determine the role of central mechanisms in mediating the acute effects of leptin on liver TG levels *in vivo*, we delivered leptin by the ICV route for 120 min. ICV leptin

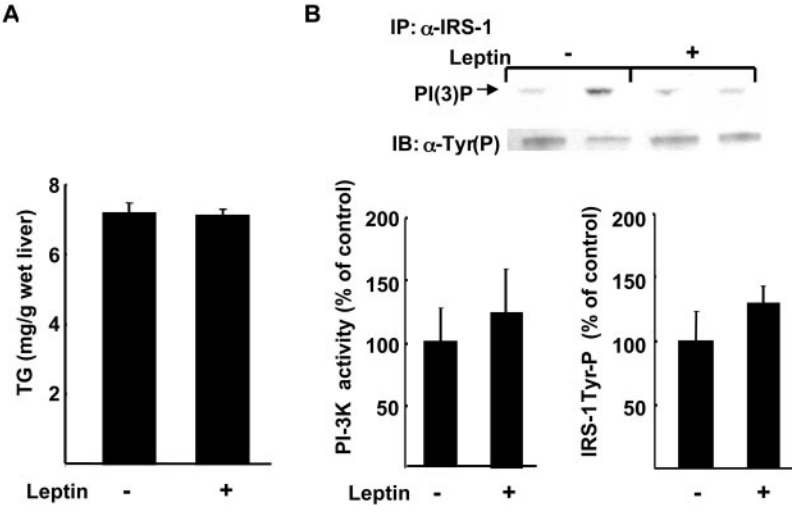


FIG. 3. Effects of iv leptin infusion on liver TG levels, liver PI3-kinase activity, and IRS-1 tyrosine phosphorylation in obese rats. Obese rats were infused with vehicle (Leptin −) or leptin (Leptin +) as described in Fig. 1. Subsequently, liver was isolated, and TG levels were assessed (A). For the measurement of liver PI3-kinase activity and IRS-1 tyrosine phosphorylation (B), animals were infused with vehicle (Leptin −) or leptin (Leptin +) for 15 min, then the liver was isolated. PI3-kinase activity and tyrosine phosphorylation of IRS-1 were measured as described in *Materials and Methods*. B (*Upper*), Representative autoradiograph of PI3-kinase activity and tyrosine phosphorylation of IRS-1. B (*Lower left*), Quantification of PI3-kinase activity measured as described in Fig. 2. B (*Lower right*), Quantification of tyrosine phosphorylation of IRS-1measured by densitometry. Results are presented as the mean ± SE (n = a minimum of 5/group for all measurements). IP, Immunoprecipitated; IB, immunoblotted.

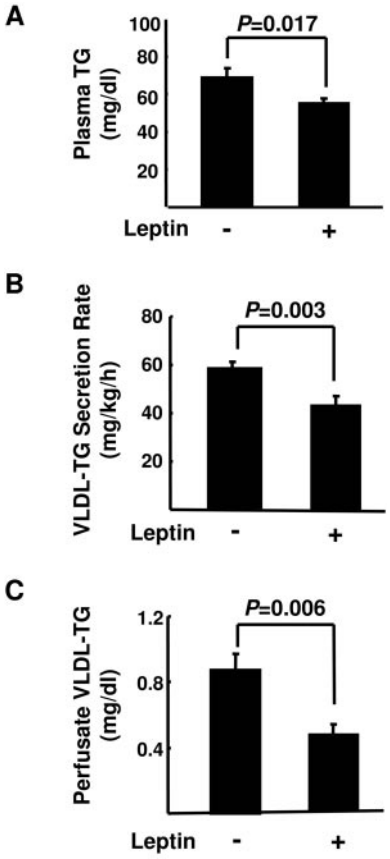


FIG. 4. Effects of leptin on plasma TG and liver TG secretion. A, Plasma samples were taken from lean rats after vehicle (Leptin −) or leptin (Leptin +) infusion as described in Fig. 1. The TG concentration was measured as described in *Materials and Methods*. B, Lean rats were infused with vehicle (Leptin −) or leptin (Leptin +) for 120 min as described in Fig. 1. Subsequently, a bolus injection of tyloxapol (Triton WR1339; 300 mg/kg) was given iv, and vehicle or leptin infusions were continued for an additional 90 min. VLDL-TG was measured, and secretion rates were calculated as described in *Materials and Methods*. Preliminary experiments determined that the rate of TG secretion was linear over the 90-min experiment (data not shown). C, Livers from fed lean rats were isolated and perfused with either vehicle (Leptin −) or leptin (Leptin +) for 90 min. Subsequently, perfusate VLDL-TG were measured as described in *Materials and Methods*. Significant differences between groups are indicated. Results are expressed as the mean ± SE [n = 12 for plasma TG (A), and a minimum of 6 livers/group for tyloxapol (B) and perfused liver (C) experiments].

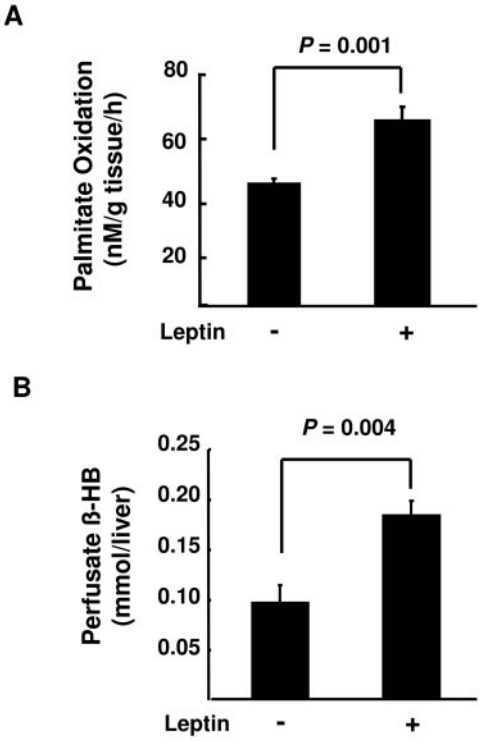


FIG. 5. Effects of leptin on liver fatty acid oxidation and ketogenesis. A, Lean rats were infused with vehicle (Leptin −) or leptin (Leptin +) as described in Fig. 1. Livers were then promptly isolated, and the rate of [$^3$H]palmitate oxidation measured as described in *Materials and Methods*. B, Livers from fed lean rats were isolated and perfused with either vehicle (Leptin −) or leptin (Leptin +) for 90 min as described in *Materials and Methods*. Ketogenesis was measured as accumulated $\beta$-hydroxybutyrate in the perfusate samples taken at the end of the perfusion period. Significant differences between groups are indicated. Results are expressed as the mean ± SE [n = a minimum of 6 livers/group, except for fatty acid oxidation where n = 4 (16 liver sections)/group].

delivery at a rate of 1 $\mu$g/kg·h increased hypothalamic STAT3 phosphorylation by 162 ± 40%; $P = 0.005$) (Fig. 7A), demonstrating activation of central pathways of leptin action, but did not decrease liver TG levels (Fig. 7B). Similar to lean rats, ICV infusion of leptin in obese rats did not alter liver TG levels (data not shown).

## Discussion

The primary goal of the current study was to establish *in vivo* the role of leptin in the acute regulation of liver TG metabolism. A number of novel observations arise from these studies. We demonstrate, first, that iv delivery of leptin for 120 min substantially decreases liver TG levels and activates PI3-kinase in lean, but not DIO, rats; second, that leptin decreases TG secretion from liver, implicating a role for leptin in the regulation of VLDL metabolism; third, that leptin elevates hepatic fatty acid oxidation and ketogenesis, most likely by inhibition of ACC activity; and finally, that ICV leptin administration does not replicate the iv effects of leptin on liver TG levels.

Liver TG metabolism *in vivo* is altered by hormonal, neural, and nutrient signals that regulate the metabolic pathways involved in the synthesis, degradation, and/or secretion of TG. In a previous study (7), we demonstrated that leptin rapidly decreases liver TG levels in the isolated perfused liver. The current study addressed whether there were similar effects of leptin *in vivo* when a physiological hormonal and metabolic milieu was present. We demonstrate that the extent of leptin effects on TG levels is the same *in vivo* and in the perfused liver (~50% depletion in both models). Furthermore, skeletal muscle TG were not reduced by leptin, suggesting that the effects of leptin were specific to liver. A note of caution should be attached to this conclusion, because acute stimulation of TG hydrolysis by leptin in isolated skeletal muscle has been reported (11, 13). However, we would point out that these studies were performed in an *in vitro* system (isolated muscle), and the dose of leptin used was

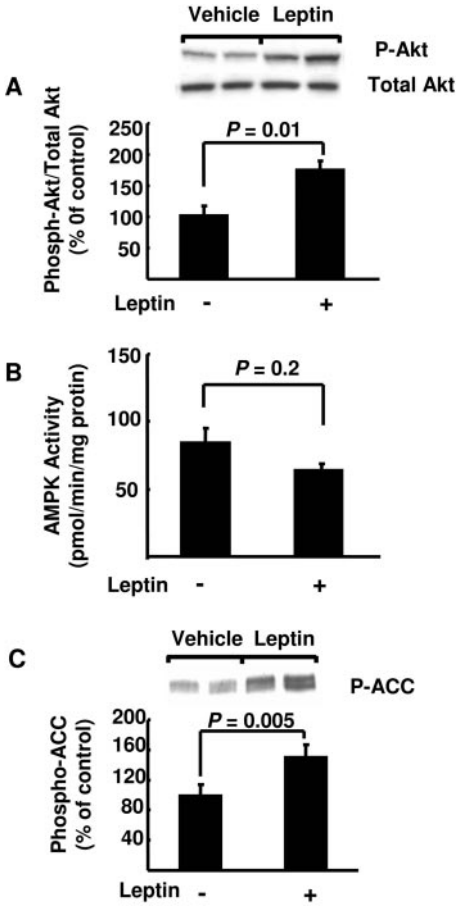


FIG. 6. Effects of leptin on Akt and ACC phosphorylation and AMPK activity. A, Lean animals were infused iv with vehicle (Leptin −) or leptin (Leptin +) for 15 min, then the livers were isolated. Akt phosphorylation ($Ser^{473}$) and total Akt were measured as described in *Materials and Methods*. The *upper panel* shows a representative autoradiograph of phospho-Akt and total Akt. The *lower panel* is a quantification of phospho-Akt normalized to total Akt levels as measured by densitometry (n = 5 animals/group). B, AMPK activity was measured in livers from rats infused with vehicle (Leptin −) or leptin (Leptin +) as described in Fig. 1 and *Materials and Methods*; n = 4 animals/group). C, ACC phosphorylation ($Ser^{79}$) was assessed in livers from rats infused with vehicle (Leptin −) or leptin (Leptin +) as described in Fig. 1 and *Materials and Methods*. The *upper panel* shows a representative autoradiograph of phospho-ACC. The *lower panel* is a quantification of phospho-ACC as measured by densitometry. Significant differences are indicated. Results are presented as the mean ± SE (n = 6 animals/group).

1000 times higher then that used in our study (~10 μg/ml *vs.* ~10 ng/ml). Either of these issues may explain the lack of an effect observed in the current study on muscle TG. Finally, mechanisms similar to those responsible for the actions of leptin in the isolated liver are implicated in the action of leptin *in vivo*. Thus, we previously demonstrated that the effects of leptin on liver TG levels are dependent on the activation of PI3-kinase. Furthermore, Muoio *et al.* (14) reported that the PI3-kinase inhibitor, wortmannin, attenuates the effects of leptin on skeletal muscle lipid metabolism. In the present study leptin substantially increased hepatic PI3-kinase activity and IRS-1 tyrosine phosphorylation, and also increased the serine phosphorylation of Akt, a major metabolic target of PI3-kinase activity. Indeed, the activation of PI3-kinase occurs at a relatively early time point (15 min) in response to a leptin stimulus and is present at even earlier times (5 and 10 min; data not shown). These data confirm previous reports of leptin activation of PI3-kinase in liver (7, 31) and in primary hepatocytes (32). However, although this combination of observations is suggestive, a definitive demonstration that leptin acts through PI3-kinase to regulate liver TG metabolism *in vivo* is still required.

Despite substantial progress, the effects of leptin resistance on the metabolic actions of leptin *in vivo* remain ill defined. A previous study (33) demonstrated decreased leptin regulation of hepatic glucose metabolism in rats on a high-caloric diet. The current study expands these observations to demonstrate that the acute effect of leptin on liver TG levels is absent in DIO rats. These observations are in agreement with our previous study of isolated livers from DIO rats and rats fed the high-caloric diet described above. The mechanism of hepatic leptin resistance in DIO is most likely post receptor, because hepatic leptin receptor expression is not decreased in DIO rats (7). Furthermore, in both isolated liver (7) and *in vivo* (current study), leptin does not activate PI3-kinase in DIO rats. In the broader context of leptin resistance, our observations are consistent with the loss of the effects of leptin on fatty acid oxidation in skeletal muscle isolated from obese humans and DIO rats (11, 34). Taken together, these studies indicate that there is a substantial loss of leptin's ability to regulate lipid and carbohydrate metabolism in obesity. Furthermore, the data support the hypothesis that leptin resistance may contribute to the atherogenic plasma lipid profile (large VLDL and small, dense LDL) and the inappropriate accumulation of lipid in peripheral tissues commonly found in obesity.

Studies in normal animals (8, 9), leptin-deficient humans (1, 2), and transgenic mice (10) demonstrated substantial leptin-induced decreases in plasma TG. Increased uptake of TG into peripheral tissues or decreased synthesis/export of TG from liver are two possible mechanisms that could result in a decrease in the circulating concentration. The current study addressed the acute effects of leptin on the export of TG from liver, based on the hypothesis that a decrease in total liver TG levels may also decrease hepatic TG secretion. We offer three independent lines of evidence to support a role for leptin in the regulation of whole body TG and, by extension, VLDL metabolism. We demonstrate, first, that plasma TG are decreased by approximately 20% in response to a 120-min leptin infusion; second, that the accumulation of VLDL-TG after tyloxapol administration is reduced by approximately 26%, suggesting that it is liver export, rather than peripheral uptake, of TG that is altered by leptin; and third that leptin decreases TG secretion from the perfused liver by approximately 50%. We also addressed the effects of leptin on plasma apoB levels and associated cholesterol, and found no differences between leptin- and vehicle-infused animals (Huang, W., N. Dedousis, and R. O'Doherty, unpublished observations). However, these measurements included cholesterol and apoB associated with LDL; for that reason, these data are not reported in the current study. Additional studies are planned that will address the acute effects of leptin on intrahepatic VLDL metabolism.

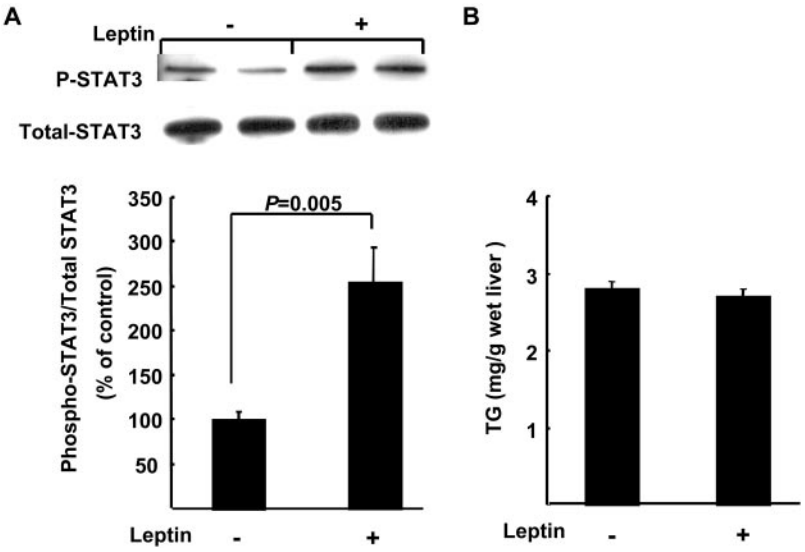


FIG. 7. Effects of ICV leptin infusion on liver TG levels. Lean rats with indwelling third cerebral ventricle cannulas received an artificial cerebrospinal fluid (Leptin −) or leptin (Leptin +) infusion for 120 min as described in *Materials and Methods*. Subsequently, the hypothalamus and liver were isolated and flash-frozen. Hypothalamus STAT3 phosphorylation and total STAT3 protein and liver TG were assessed as described in *Materials and Methods*. A (*Upper panels*), Representative autoradiographs of phospho-STAT3 and total STAT3. The *lower panel* is a quantification of phospho-STAT3 normalized to total STAT3 as measured by densitometry. B, TG content in the livers taken from the same animals. Significant differences are indicated. Results are presented as the mean ± SE (n = 6 animals/group).

The current study clearly demonstrates that leptin increases the activity of oxidative pathways in the liver. Thus, exogenous fatty acid oxidation by the liver was increased after a 2-h leptin infusion *in vivo*, and leptin increased ketogenesis in the isolated, perfused liver. These data raise a number of discussion points. First, our data are in good agreement with previous observations (7) demonstrating increased oxygen consumption in leptin-perfused livers and increases in fatty acid oxidation in skeletal muscle and cardiac muscle in response to leptin (11–17). Second, one plausible explanation for the metabolic fate of the reduced liver TG level is the partitioning of fatty acids derived from TG into oxidative pathways. This hypothesis is supported by previous studies in skeletal muscle that demonstrate leptin-induced increases in TG hydrolysis and partitioning of fatty acids toward oxidation (12). Also, in the current study the incorporation of palmitate into the total lipid pool, a large portion of which is fatty acid esterification into TG, was unaltered by leptin in the perfused liver (data not shown), suggesting that leptin stimulates the hydrolysis of TG in liver. Third, despite the observation that leptin increased ACC phosphorylation, AMPK activity was unaltered. The current data are in agreement with our previous observations in perfused livers (7). In skeletal muscle there appears to be a clear relationship between leptin-induced increases in AMPK activity and fatty acid oxidation (16). However, this is not the case in cardiac muscle (17). Furthermore, leptin increased Akt activity, as measured by elevated phosphorylation, an event that may be expected to decrease AMPK activity, as previously reported in mouse heart (35). Thus, the role of AMPK in mediating the effects of leptin on fatty acid oxidation in the liver remains unclear. Finally, the combined observations of leptin-induced decreases in liver TG levels and secretion, and increased oxidative metabolism suggest that TG partitioning to oxidative pathways contributes to the effects of leptin on TG homeostasis. This may have implications for a role for leptin in the regulation of the plasma lipoprotein profile and, perhaps more generally, the capacity of increased intrahepatic fatty acid oxidation to improve VLDL profiles in hyperlipidemic states. In support of a role for leptin in the regulation of lipoprotein metabolism are the reports of acute and chronic effects of leptin on liver cholesterol (24, 36), bile acid (37), and high-density lipoprotein catabolism (38).

The role of the hypothalamus in mediating leptin action is well established. The most relevant observations in the context of the current study are the demonstrations that acute (~5 h) ICV delivery of leptin elevates hepatic gluconeogenesis and decreases glycogenolysis (29), that a single low dose of leptin delivered ICV decreases the activity of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase and cholesterol 7$\alpha$-hydroxylase, enzymes that play important roles in the regulation of hepatic cholesterol metabolism (24), and that a 2-wk ICV delivery of leptin substantially reduces hepatic steatosis in a mouse model of lipodystropy (30). Based on these observations, it was somewhat surprising that ICV delivery of leptin in the current study did not decrease liver TG levels. Hypothalamic activation by leptin was apparent, because leptin increased STAT3 phosphorylation. The lack of an effect of leptin could not be explained by the time of infusion, because this was the same for both ICV and iv infusions (120-min), or by the leptin dose, because the ICV infusion rate directly into the third ventricle (1 $\mu$g/kg·h) most likely resulted in a higher concentration of leptin in the hypothalamus than that obtained with an iv infusion (plasma leptin, ~14 ng/ml), although direct measurements of leptin concentration in the hypothalamus were not possible. It should also be noted that the results obtained in the current study are consistent with those obtained in isolated perfused liver (7) and with the study by VanPatten *et al.* (24) that demonstrated unaltered liver TG levels in rats 12 h after receiving an ICV leptin stimulus. Finally, our data are in accord with a number of studies demonstrating metabolic effects of leptin in isolated peripheral tissues and *in vivo*, including observations that the expression of wild-type leptin receptors in Zucker diabetic fatty rat liver decreases liver TG levels (39), and that administration of leptin to gold thioglucose obese mice that lack hypothalamic leptin function decreases hepatic lipogenesis (40).

In conclusion, the current study clearly establishes a role

for leptin in the acute regulation of liver TG levels *in vivo*. Leptin-induced decreases in TG secretion from the liver also suggests a role for leptin in determining the TG content of VLDL, a critical determinant of the plasma lipoprotein profile. The mechanisms responsible for decreased intrahepatic TG stores and TG secretion probably involve nutrient partitioning away from storage, because leptin increases the activity of fat oxidation pathways in liver.

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