

¹³C NMR study of the effects of leptin treatment on kinetics of hepatic intermediary metabolism

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ABSTRACT The recent discovery of leptin receptors in peripheral tissue raises questions about which of leptin's biological actions arise from direct effects of the hormone on extraneural tissues and what intracellular mechanisms are responsible for leptin's effects on carbohydrate and lipid metabolism. The present study is focused on the action of leptin on hepatic metabolism. Nondestructive ¹³C NMR methodology was used to follow the kinetics of intermediary metabolism by monitoring flux of ¹³C-labeled substrate through several multistep pathways. In perfused liver from either *ob/ob* or lean mice, we found that acute treatment with leptin *in vitro* modulates pathways controlling carbohydrate flux into ¹³C-labeled glycogen, thereby rapidly enhancing synthesis by an insulin-independent mechanism. Acute treatment of *ob/ob* liver also caused a rapid stimulation of long-chain fatty acid synthesis from ¹³C-labeled acetyl-CoA by the *de novo* synthesis route. Chronic leptin treatment *in vivo* induced homeostatic changes that resulted in a tripling of the rate of glycogen synthesis via the gluconeogenic pathway from [2-¹³C]pyruvate in *ob/ob* mouse liver perfused in the absence of the hormone. Consistent with the ¹³C NMR results, leptin treatment of the *ob/ob* mouse *in vivo* resulted in significantly increased hepatic glycogen synthase activity. Chronic treatment with leptin *in vivo* exerted the opposite effect of acute treatment *in vitro* and markedly decreased hepatic *de novo* synthesis of fatty acids in *ob/ob* mouse liver. In agreement with the ¹³C NMR findings, activities of hepatic acetyl-CoA carboxylase and fatty acid synthase were significantly reduced by chronic treatment of the *ob/ob* mouse with leptin. Our data represent a demonstration of direct effects of leptin in the regulation of metabolism in the intact functioning liver.

Leptin's ability to normalize food intake is mediated largely by receptors in the hypothalamus (1). The *ob/ob* mouse model of obesity and insulin resistance has an intact leptin receptor, but is leptin deficient owing to mutation of the *ob* gene (2). In the *ob/ob* mouse the lipid-depletion effects of chronic leptin treatment exceed that explained by reduced food intake and weight loss alone (3, 4). Discovery of leptin receptors in peripheral tissue (1, 5) raises questions about which of leptin's biological actions arise from direct effects of the hormone on extraneural tissues, and what intracellular mechanisms are responsible for leptin's effects on carbohydrate and lipid metabolism. Using nondestructive ¹³C NMR methodology to follow the kinetics of intermediary metabolism, we define the action of leptin directly on liver. By monitoring flux through several multistep pathways simultaneously in the intact hepatocyte, ¹³C NMR spectroscopy helped identify the metabolic pathways controlled; assays of enzymic activities supported the NMR findings. We found that leptin up-regulates hepatic

glycogen synthesis in *ob/ob* mouse liver with both acute and chronic treatment. Simultaneously, we found that acute treatment of *ob/ob* liver with leptin up-regulated fatty acid synthesis. Chronic treatment of the *ob/ob* mouse *in vivo*, however, produced the opposite effect of a sharp decrease in the hepatic rate of lipogenesis.

MATERIALS AND METHODS

Perfused Mouse Liver for NMR Studies. The isolated perfused liver technique and apparatus designed for liver perfusion in the NMR magnet were as described previously (6, 7). C57BL/6J *ob/ob* or lean C57BL/6J mice, 8 weeks old, of either sex received chow (Purina Diet 5010) and water ad libitum. Perfusion experiments were started during the middle of the dark part of the 12-h/12-h lighting cycle. Four *ob/ob* mouse liver groups were studied: (i) The *ob/ob* mouse group treated chronically with leptin *in vivo* received leptin twice daily, s.c., 0.5 mg/kg body weight, for the 5 days before excision and perfusion of the liver. No leptin was added during perfusion of these livers. (ii) The *ob/ob* mouse control group was sham-treated for 5 days but received no exposure to leptin, either *in vivo* or *in vitro*. (iii) To test the effects of acute leptin treatment *in vitro*, livers from sham-treated *ob/ob* mice received leptin added directly to the perfusion medium every 30 min throughout the perfusion at a level sufficient to match the serum levels measured on day 5 of the leptin *in vivo* protocol. (iv) The effects of inactivated leptin (trypsinization) were studied under the leptin *in vitro* conditions; *ob/ob* mouse livers treated with inactivated leptin behaved the same as the *ob/ob* control group that received no leptin. Two lean mouse perfused liver groups were studied: livers in the lean mouse control group had no exposure to exogenous leptin, and the effects of acute leptin treatment *in vitro* were tested in perfused lean mouse liver by the direct addition of leptin to the perfusion medium as described for the corresponding *ob/ob* group. All livers were perfused in the absence of added insulin. Either 176 min (*ob/ob* liver) or 150 min (lean liver) after initial addition of ¹³C-labeled substrate to the perfused liver, glucagon (0.1 nM) was added to initiate breakdown of glycogen to glucose for quantitation purposes. Several assessments of hepatic viability were made. Only preparations with ATP levels of $\approx 3 \mu\text{mol/g}$ liver wet weight (glww), stable over the first 30 min of ³¹P NMR, were used. After 4–4.5 h of perfusion, ATP levels were either unchanged or decreased by <15%. Lactate dehydrogenase (LDH) release was monitored. Perfusate LDH levels were 0.01 units/ml at $t = 0$ and about 0.10–0.12 units/ml at $t = 4$ h. A rigorous test of liver function is the ability to synthesize glucose at acceptable, linear rates from substrates that enter the pathway at the level of pyruvate. Livers from the *ob/ob* mouse control, leptin *in vitro*, and leptin

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Abbreviations: glww, gram liver wet weight; GS_T, total glycogen synthase; GS_A, *a*-form of glycogen synthase; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; PDH, pyruvate dehydrogenase.

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in vivo groups metabolized [2-¹³C]pyruvate to produce ¹³C-glucose over 3 h at rates of 0.56, 0.75, and 1.18 μmol/glw per min, respectively.

Leptin. Human leptin was expressed in *Escherichia coli*, purified from inclusion bodies, and refolded (8). Larger-scale refolding was achieved by using an Amicon spiral-wound membrane cartridge for buffer exchange. Perfusate leptin levels were determined by RIA. Perfusate leptin levels were sampled every 15 min and were maintained in a range of 4.0–5.0 ng/ml.

NMR Conditions and Analysis. ¹³C and ³¹P NMR spectra of perfused liver at 37°C were measured at 8.46 T as previously described (7). Four ³¹P spectra were accumulated, each 200 scans over 8 min. One natural abundance ¹³C spectrum of 800 scans was accumulated over 11 min. Initial addition of ¹³C-labeled substrate was followed by accumulation of 19–22 ¹³C spectra under isotopic steady-state conditions. All ¹³C spectra were measured under the conditions used for the natural abundance spectrum. At 246–279 min after measurement of the initial ³¹P spectrum, two final ³¹P spectra were measured. Peak areas in ¹³C NMR spectra of perfused liver were determined by fitting spectra to appropriate line shapes with subsequent integration using the CF module of NMR1 software; appropriate guidelines (9) were used for the glycogen resonance. Areas of ¹³C NMR signals of metabolites in each perfused liver were converted from absolute integral units to μmol of ¹³C enrichment at individual carbons by standard approaches (6, 7, 10) using information from three assays of perfusate glucose. Total glucose (¹²C + ¹³C enriched) was measured by the glucose oxidase method in perfusate aliquots sampled first at baseline before substrate addition, then at the time of peak ¹³C-glycogen formation, and again when the ¹³C spectra indicated complete breakdown of glycogen to glucose. Ratios of ¹³C/¹²C at C-1 of glucose produced by gluconeogenesis from labeled substrate and endogenous substrates were obtained by ¹H NMR at 400 MHz for perfusate samples prepared by precipitating proteins with 4% perchloric acid, followed by lyophilization of the supernatant and reconstitution in D₂O. Relative ¹³C enrichments at the individual carbon atoms of glucose produced by gluconeogenesis were measured in perfusate samples by ¹³C NMR at 11.75 T.

Enzyme and mRNA Assays. Livers used for enzyme and mRNA assays were from *ob/ob* mice that were dosed with leptin *in vivo* under the same regimen used for the NMR studies or from control *ob/ob* mice that were sham-treated in the same way. Livers were excised in the middle of the dark part of the 12-h/12-h lighting cycle and immediately were freeze-clamped without perfusion. Standard procedures were used to assay the activities of glycogen synthase (total and active *a*-form, GS_T and GS_a, respectively) (EC 2.4.1.11) (11), glycogen phosphorylase *a*-form (EC 2.4.1.1) (12), fatty acid synthase (FAS) (EC 2.3.1.85) (13), and acetyl-CoA carboxylase (ACC) (EC 6.4.1.2) (14). Total RNA was prepared by using Trizol reagent (GIBCO/BRL). mRNAs were probed with a digoxigenin (DIG)-labeled specific antisense probe (rat ACC gene sequence, nucleotides 994–2420) (15) (GenBank/EMBL accession no. J03808) with bands detected according to the DIG protocol using chemiluminescence (Boehringer Mannheim). Northern blot was reprobed with a DIG-labeled mouse β-actin cDNA probe as control.

Statistical Analysis. ANOVA was performed on each response to assess differences among the groups. Follow-up comparisons were performed where indicated by using least-squares means. *P*-values of 0.05 or lower were deemed significant for all tests and comparisons. All values are given as mean ± SEM.

RESULTS AND DISCUSSION

In this study, rates of gluconeogenesis, glycogen synthesis, and synthesis of long-chain fatty acids via the *de novo* synthesis

pathway are measured in liver from *ob/ob* mice treated chronically with leptin *in vivo*, but perfused without addition of leptin, and are compared with the rates measured in liver from untreated control *ob/ob* mice, or to rates observed for *ob/ob* liver treated with leptin *in vitro* during the perfusion period. In this way, a direct comparison was made of homeostatic changes, which were induced in liver by chronic treatment of the animal with leptin, with changes in metabolism that occurred in liver from untreated animals in response to direct addition of the hormone to the perfusion medium during the NMR study. The spectra shown in Fig. 1 are of a liver perfused under approximately metabolic and isotopic steady-state conditions with the gluconeogenic substrate [2-¹³C]pyruvate (6.7 mM) + NH₄Cl (2.7 mM), which is interconverted to [2-¹³C]alanine in the liver. The natural abundance of ¹³C is only 1%, permitting our use of specifically labeled pyruvate that was highly (≥99%) enriched with ¹³C at carbon 2. In the Krebs cycle, the ¹³C-label is randomized; the ¹³C-distribution at specific carbons in metabolites synthesized from this substrate gives information on fluxes through metabolic pathways (6, 10, 16, 17). By accumulating a spectrum of the ¹³C natural abundance of each liver before addition of ¹³C-labeled substrate and then subtracting this spectrum from each spectrum accumulated after the addition of ¹³C-labeled substrate, we selectively quantitated only those metabolites that could be traced back directly to [2-¹³C]pyruvate, such as glutamate, glutamine, glucose, and glycogen (Figs. 1 and 2).

Effects of Leptin *in Vivo* and *in Vitro* on Glycogen Synthesis. Our ¹³C spectra follow the gluconeogenic pathway from [2-¹³C]pyruvate to the branch point at glucose-6-P, and thereby either directly to ¹³C-labeled glucose via glucose-6-phosphatase activity, or to ¹³C-labeled glycogen by the glyco-

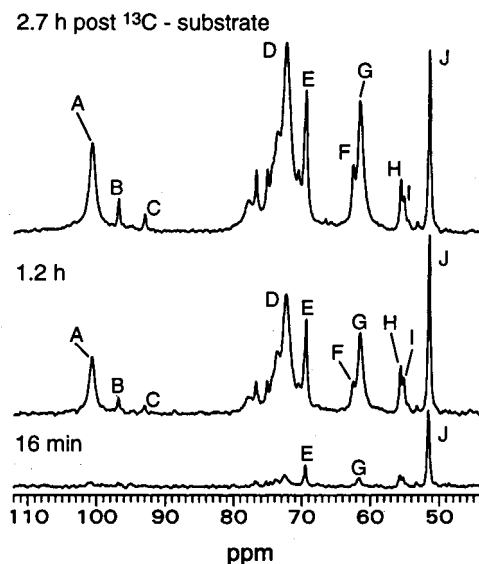


FIG. 1. ¹³C NMR spectra of a representative perfused liver from an *ob/ob* mouse treated chronically with leptin *in vivo*. No leptin was added during perfusion of the liver. Three spectra measured at the indicated times postsubstrate are shown. The 105–60 ppm region contains signals of newly synthesized ¹³C-labeled glucose and glycogen, which increased monotonically as the level of substrate, seen as [2-¹³C]alanine, was held approximately constant. The ¹³C natural abundance background spectrum of the liver, measured under identical conditions before addition of substrate, was subtracted from each of the spectra shown. (A) C-1 of glucosyl units in glycogen. (B and C) C-1 of β and α anomers of glucose, respectively. (D) C-2 to C-5 of glucosyl units in glycogen. (E) Lactate C-2 and C-2 of the glycerol backbone of triacylglycerols (TG). (F) C-1 + C-3 of TG glycerol. (G) C-6 of glucose and of glucosyl units of glycogen. (H) Glutamate C-2. (I) Glutamine C-2. (J) Alanine C-2. Label in TG glycerol arises from both exchange into endogenous pools and synthesis.

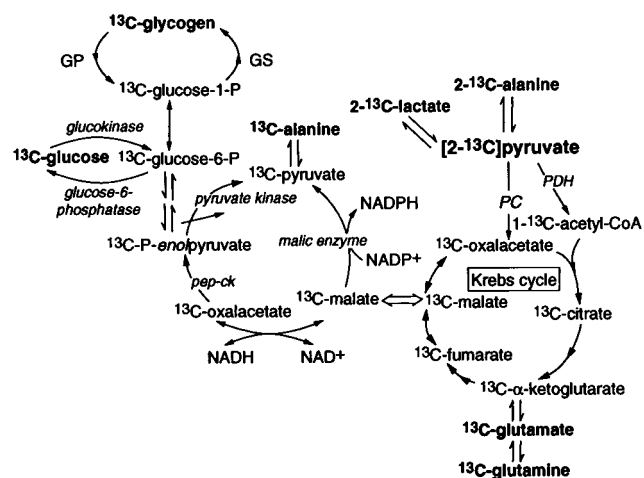


FIG. 2. Simplified gluconeogenic pathway from [2-¹³C]pyruvate. GS, glycogen synthase; GP, glycogen phosphorylase. ¹³C-metabolite denotes the presence of more than one isotopomer owing to randomization of the original ¹³C label in the Krebs cycle. Randomized ¹³C-pyruvate can reenter the Krebs cycle (not shown).

gen synthase route (Fig. 2). The kinetics of net glycogen synthesis (as a gluconeogenic process) can be monitored by measurement of ¹³C enrichment at C-1 of the glucosyl units in glycogen by ¹³C NMR. As seen in Fig. 1, glycogen C-1 gives rise to ¹³C NMR signals free of overlapping peaks. The rate of glycogen synthesis (Fig. 3A) by *ob/ob* mouse liver was 3-fold greater after chronic administration of leptin *in vivo* as compared with the *ob/ob* control liver rate ($P < 0.001$) and 1.9-fold greater as compared with the rate in *ob/ob* liver treated with

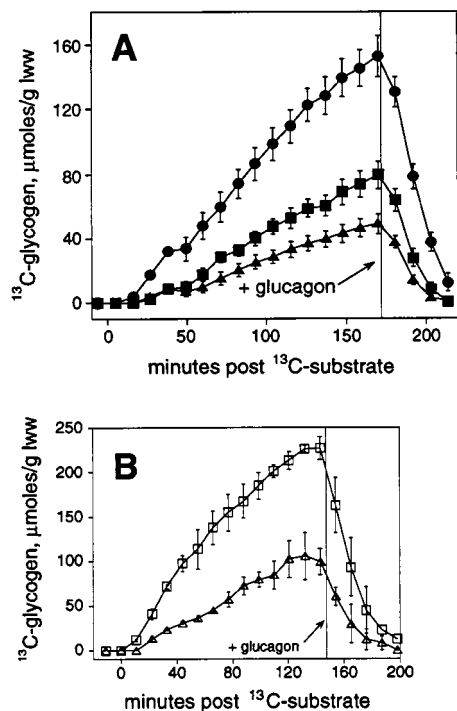


FIG. 3. Quantitation of the effects of leptin treatment on kinetics of glycogen synthesis in *ob/ob* mouse liver (A) and lean mouse liver (B). ¹³C enrichments in the glucosyl units ($\mu\text{mol/g lww}$) are shown over time after initial addition of [2-¹³C]pyruvate and NH₄Cl. For *ob/ob* mouse liver (A), the effects of leptin treatment *in vivo* (●) ($n = 7$) or *in vitro* (■) ($n = 5$) are compared with control (▲) ($n = 9$). For lean mouse liver (B), the effect of leptin treatment *in vitro* (□) is compared with control (△), $n = 2-3$ each. Where not visible, error bar is smaller than symbol.

leptin *in vitro* ($P < 0.01$) (Table 1). When leptin was administered directly to *ob/ob* perfused liver *in vitro*, glycogen synthesis (Fig. 3A) was 1.6-fold greater compared with untreated control *ob/ob* liver ($P < 0.05$) (Table 1). Leptin treatment *in vitro* also markedly enhanced glycogen synthesis in liver from normal lean mice (Fig. 3B). This enhancement is reflected in a 2-fold increase in the rate of glycogen synthesis with direct administration of leptin to perfused lean mouse liver (Table 1). Our NMR analysis methodology, which is based on ¹³C NMR of perfused liver and both ¹³C (Table 2) and ¹H NMR of perfusate samples, allowed quantitation of the effects of leptin treatment on contributions of both endogenous and ¹³C-labeled carbon sources to the total glucose output (Table 1). There was no significant difference in the contribution of endogenous substrates to total hepatic glucose output among the three *ob/ob* treatment groups. However, gluconeogenesis from the ¹³C-labeled substrate was increased significantly in livers from *ob/ob* mice chronically treated with leptin *in vivo* as compared with either *ob/ob* control livers or to *ob/ob* livers treated with leptin *in vitro* (Table 1). These findings are consistent with increased flux through phosphoenolpyruvate carboxykinase (PEPCK) in *ob/ob* liver after leptin treatment *in vivo*. PEPCK is rate limiting for gluconeogenesis and is unusual in that regulation of its activity is exerted only at the level of gene expression.

Our results establish that leptin up-regulates hepatic glycogen synthesis by two mechanisms. The larger increase in glycogen synthesis measured in livers from *ob/ob* mice chronically treated with leptin suggests expression level changes affecting regulation of the activities of one or more of the pathway enzymes beyond the glucose-6-phosphate branch-point because these changes persisted throughout several hours of subsequent perfusion postexcision without addition of either leptin or insulin to the perfusion medium. The finding of enhanced hepatic glycogen synthesis after short-term leptin treatment *in vitro* is intriguing as this rapid response is consistent with leptin modulating signal transduction pathways controlling hepatic glycogen synthesis in an insulin-independent manner. As shown in Table 3, leptin treatment of the *ob/ob* mouse *in vivo* resulted in significantly increased hepatic glycogen synthase activity as well as in significant activation of the enzyme as indicated by an increase in the ratio of GS_a to GS_r. Thus, leptin treatment *in vivo* results in long-term remodeling of hepatic glycogen synthase activity as well as activation of the enzyme (Table 3). These findings are consistent with our ¹³C NMR results that demonstrated increased glycogen synthesis with leptin treatment of *ob/ob* mouse liver *in vitro* and an even larger increase in glycogen synthesis in liver from *ob/ob* mice treated chronically with leptin. Hepatic glycogen phosphorylase activity was unchanged by leptin treatment *in vivo* (Table 3).

Effects of Leptin *In Vivo* and *In Vitro* on Long-Chain Fatty Acid Synthesis. Our ¹³C spectra also followed the kinetics of long-chain fatty acid synthesis (Fig. 4A). In perfused liver, the substrate [2-¹³C]pyruvate is converted to [1-¹³C]acetyl-CoA, which in the *de novo* synthesis pathway labels alternate carbons in fatty acids (Fig. 4B). With the exception of the repeating methylene groups, the labeled carbons gave rise to signals distinguishable on the basis of chemical shift from signals of the adjacent fatty acyl carbons that should not show ¹³C enrichment under these conditions (10). Thus, measurement of ¹³C enrichment at several specific sites in fatty acyl chains in time-resolved spectra of liver perfused under these steady-state conditions offers a way to follow the effects of leptin treatment on the kinetics of fatty acid synthesis (Fig. 5A-C). Hepatic fatty acid synthesis, which is best measured at the repeating methylene carbons (Fig. 5A), was decreased 2.6-fold in perfused liver from *ob/ob* mice treated with leptin *in vivo* as compared with the *ob/ob* control group ($P < 0.05$). Leptin was not added during perfusion of livers from mice treated with

Table 1. Effect of leptin treatment on carbon source contribution to total hepatic glucose output in *ob/ob* mouse liver and rate of glycogen synthesis in *ob/ob* and lean mouse liver

Group	From endogenous sources	From ¹³ C-pyruvate $\mu\text{mol glucose/glww}$	Total glucose output	Net rate of glycogen synthesis* $\mu\text{mol per glww/min}$
<i>ob/ob</i> control (7)	151 \pm 12	101 \pm 8	252 \pm 11	0.36 \pm 0.04
<i>ob/ob</i> leptin <i>in vitro</i> (5)	134 \pm 7	136 \pm 14	271 \pm 10	0.57 \pm 0.06 [†]
<i>ob/ob</i> leptin <i>in vivo</i> (7)	115 \pm 16	216 \pm 20 ^{‡§}	331 \pm 28 [¶]	1.07 \pm 0.10 ^{‡§}
Lean control (3)				0.84 \pm 0.17
Lean leptin <i>in vitro</i> (2)				1.71 \pm 0.03

Leptin *in vivo* group received leptin, s.c., 0.5 mg/kg body weight, twice a day for 5 days before liver excision, with no leptin added during perfusion. Livers from the the leptin *in vitro* group were from untreated mice and received leptin added to the perfusate at a level that matched day 5 serum levels of the leptin *in vivo* protocol. Control livers received no leptin treatment. Number of experiments is in parentheses.

*Rate of incorporation of ¹³C-labeled glucosyl units/glww per min.

[†]*P* < 0.05 versus control group.

[‡]*P* < 0.001 versus control group.

[§]*P* < 0.01 versus leptin *in vitro* group.

[¶]*P* < 0.01 versus control group.

leptin *in vivo*. When leptin was administered directly *in vitro* to *ob/ob* liver from untreated mice, stimulation of fatty acid synthesis rather than reduction was observed (Fig. 5A). Fatty acid synthesis was 1.6-fold greater with short-term leptin treatment of the perfused *ob/ob* mouse liver compared with untreated control *ob/ob* liver (*P* < 0.05) and 4-fold greater than fatty acid synthesis in liver from *ob/ob* mice treated with leptin *in vivo* (*P* < 0.005). From the data shown in Fig. 5A, the rates of long-chain fatty acid synthesis from [1-¹³C]acetyl-CoA were 0.24 \pm 0.06, 1.01 \pm 0.12, and 0.64 \pm 0.16 $\mu\text{mol/glww}$ per min for the leptin *in vivo*, leptin *in vitro*, and control *ob/ob* liver groups, respectively. Fatty acid synthesis in lean mouse liver was barely measurable.

Our finding of enhanced hepatic long-chain fatty acid synthesis in *ob/ob* mouse liver after short-term leptin treatment *in vitro* is consistent with leptin modulating signaling pathways involved in stimulation of fatty acid synthesis from acetyl-CoA by ACC, the key regulatory enzyme of *de novo* synthesis of fatty acids (18), possibly by activation of phosphatidylinositol 3-kinase (19). The decrease in lipogenesis induced by chronic treatment of *ob/ob* mice with leptin is consistent with decreases in the total activities of lipogenic enzymes (18). We tested this last hypothesis by measuring the effect of leptin treatment *in vivo* on the activities of FAS and ACC and on the activation state of ACC. As shown in Table 3, hepatic FAS and ACC activities both were significantly reduced by leptin treatment of

the *ob/ob* mouse *in vivo*. However, the activation state of ACC as estimated by assaying the ratio of activity at 0 and 4 mM citrate (14, 20) was unchanged by chronic leptin treatment. Within the limits of detection there was no significant difference in mRNA levels for hepatic ACC in control and chronically treated *ob/ob* mice (data not shown), implying posttranslational regulation of this enzyme by chronic treatment. Our findings on the effects of leptin treatment *in vivo* on hepatic FAS and ACC activities support the ¹³C NMR results showing decreased *de novo* synthesis of fatty acids in perfused liver from *ob/ob* mice treated chronically with leptin.

Glutamate Labeling and Ketone Body Levels. Evidence consistent with decreased generation of NADPH by malic enzyme, an important lipogenic enzyme, after leptin treatment of the *ob/ob* mouse *in vivo* was provided by methodology based on distribution of ¹³C label in key metabolites in liver (data not shown). However, flux through malic enzyme is largely driven by the rate of utilization of NADPH (18). Decreased demand for NADPH reducing equivalents is consistent with the decreased activity of ACC with chronic leptin treatment given in Table 3. Leptin has been shown to lower triacylglycerol content in rat pancreatic islets by increasing oxidation of fatty acids (21). We first tested this hypothesis in liver by examining the ¹³C enrichments in hepatic glutamate. Pyruvate can enter the Krebs cycle (Fig. 2) by two routes: by activity of pyruvate

Table 2. Isotopic distribution in carbons of glucose produced from [2-¹³C]pyruvate substrate by perfused liver from *ob/ob* and lean mice

Relative ¹³ C enrichment of glucose	<i>ob/ob</i> liver (20)*	Lean liver (5) [†]
C-1	73.6 \pm 1.4	78.5 \pm 0.6
C-2	89.7 \pm 1.5	96.0 \pm 0.6
C-3	34.6 \pm 1.3	48.0 \pm 3.0
C-4	38.4 \pm 1.3	51.0 \pm 3.0
C-5	100.0	100.0
C-6	79.0 \pm 1.0	81.0 \pm 3.0
C-123/C-456	0.91 \pm 0.01	0.96 \pm 0.02

Although distributions for the individual livers were used in calculations, mean values are given to indicate the liver-to-liver variation in this distribution.

*Mean values for 20 *ob/ob* livers of which eight were untreated controls, five were treated with leptin *in vitro* only, and seven were perfused in absence of hormone but were from mice treated with leptin *in vivo*. Leptin treatment as given in Table 1.

[†]Mean values for two untreated lean mouse livers and three lean livers treated with leptin *in vitro*.

Table 3. Effects of leptin treatment *in vivo* on the activities of glycogen synthase, glycogen phosphorylase, and ACC in *ob/ob* mouse liver

Enzyme	Control, units/glww	Leptin, units/glww	<i>P</i>
GS _a	0.19 \pm 0.05 (7)	0.58 \pm 0.11 (8)	<i>P</i> = 0.01
GS _r	0.62 \pm 0.10 (7)	1.15 \pm 0.18 (8)	<i>P</i> < 0.05
GS _a /GS _r	0.30 \pm 0.05 (7)	0.50 \pm 0.05 (8)	<i>P</i> < 0.05
GPa*	12.5 \pm 2.0 (7)	11.3 \pm 1.5 (8)	NS
ACC(-cit) [†]	0.37 \pm 0.02 (8)	0.27 \pm 0.04 (8)	<i>P</i> < 0.05
ACC(+cit) [†]	0.72 \pm 0.03 (8)	0.51 \pm 0.05 (8)	<i>P</i> < 0.005
ACC(-/+) [†]	0.52 \pm 0.02 (8)	0.53 \pm 0.02 (8)	NS
FAS [‡]	0.52 \pm 0.04 (10)	0.29 \pm 0.03 (10)	<i>P</i> < 0.001

Leptin treatment as given in Table 1. Units/glww is defined as the conversion of 1 μmol of substrate per min at 30°C per glww. Number of experiments is in parentheses. GPa, the *a*-form of glycogen phosphorylase. NS, not significant.

*For comparison, GPa for lean mouse liver (*N* = 4) was 7.2 \pm 4.2 units/glww.

[†]ACC activity was measured in the absence (-cit) or presence (+cit) of 4 mM citrate. ACC(-/+) is the ratio of activity at 0 and 4 mM citrate.

[‡]For FAS activity is defined as the synthesis of 1 nmol of palmitic acid per min at 30°C per mg of liver protein.

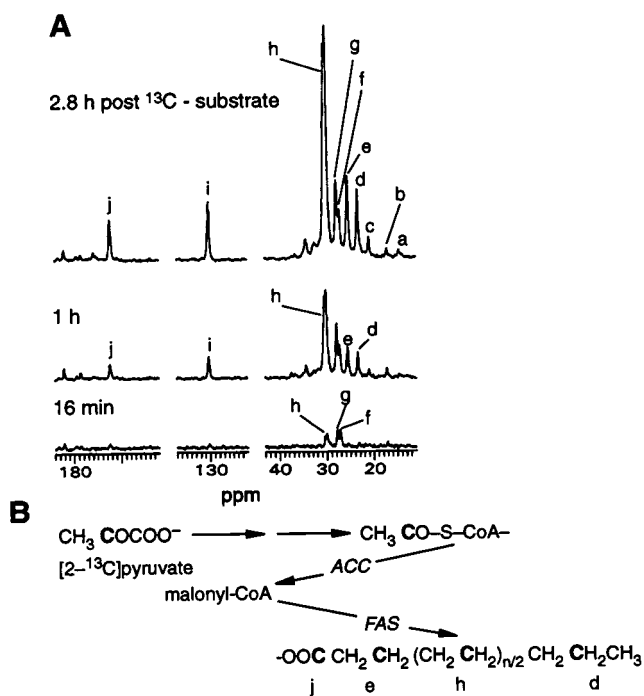


FIG. 4. The kinetics of *de novo* synthesis of long-chain fatty acid from $[1-^{13}\text{C}]$ acetyl-CoA in *ob/ob* mouse liver. ^{13}C NMR spectra (A) of a representative *ob/ob* mouse liver exposed to leptin *in vitro*. The regions containing fatty acyl carbon signals are shown for spectra measured at the indicated times after initial addition of $[2-^{13}\text{C}]$ pyruvate and NH_4Cl substrate. Only signals of metabolites that can be traced back to $[2-^{13}\text{C}]$ pyruvate are seen. NMR conditions are as given for Fig. 1. (a) Fatty acyl terminal methyl carbon. (b) Alanine C-3. (c) Lactate C-3. Fatty acyl carbons include: (d) $\text{CH}_3^{13}\text{CH}_2\text{CH}_2^-$; (e) $^{-13}\text{CH}_2\text{CH}_2\text{CO}^-$; (h), the repeating methylene carbons; (i) $^{-13}\text{CHCH}_2\text{CH}_2^-$; and (j) $^{-13}\text{CH}_2\text{CH}_2^{13}\text{CO}^-$. (f) Glutamine C-3. (g) Glutamate C-3. Randomization of ^{13}C label is seen in slight labeling of a. (B) Simplified *de novo* synthesis pathway showing incorporation of original ^{13}C label of pyruvate into specific sites in fatty acids. C represents ^{13}C .

carboxylase (PC) or via flux through pyruvate dehydrogenase (PDH). The metabolic changes that occur during β -oxidation of fatty acids are known to inactivate PDH (22). The relative proportion of $[2-^{13}\text{C}]$ pyruvate entering the Krebs cycle via PDH is reflected in the ^{13}C enrichment at glutamate C-5, whereas flux through PC is reflected in labeling at glutamate C-2 and C-3 (10). The ratio of ^{13}C -labeling at glutamate C-5 to that at (C-2+C-3) was 1.15 ± 0.12 in *ob/ob* control liver and 0.94 ± 0.15 in *ob/ob* liver treated with leptin *in vitro*, indicating about equal flux through PC and PDH in both groups. In liver from *ob/ob* mice treated with leptin *in vivo*, the corresponding ratio was 1.64 ± 0.09 , which is significantly greater than the ratio in either of the other two *ob/ob* groups ($P < 0.05$); the glutamate ratio measured after chronic leptin treatment indicates 60% greater flux through PDH relative to PC posttreatment. Thus, glutamate labeling suggests that leptin treatment *in vivo* did not inactivate PDH as would be anticipated if hepatic fatty acid oxidation had increased. We then tested the hypothesis by measuring perfusate concentrations of an important product of β -oxidation, β -hydroxybutyrate (β -HB), that might accumulate if leptin increased fatty acid oxidation. However, $[3-^{13}\text{C}]\beta$ -HB was not different in *ob/ob* mouse liver control, leptin *in vitro*, and leptin *in vivo* groups, with perfusate levels of 0.41 ± 0.06 , 0.36 ± 0.15 , and 0.41 ± 0.09 mM, respectively. Acetoacetate (AA) also was not different in the three groups: $[3-^{13}\text{C}]\text{AA}$ accumulated to 0.12 ± 0.04 , 0.14 ± 0.02 , and 0.09 ± 0.04 mM in perfusate of the *ob/ob* control, leptin *in vitro*, and leptin *in vivo* groups, respectively. The ratios

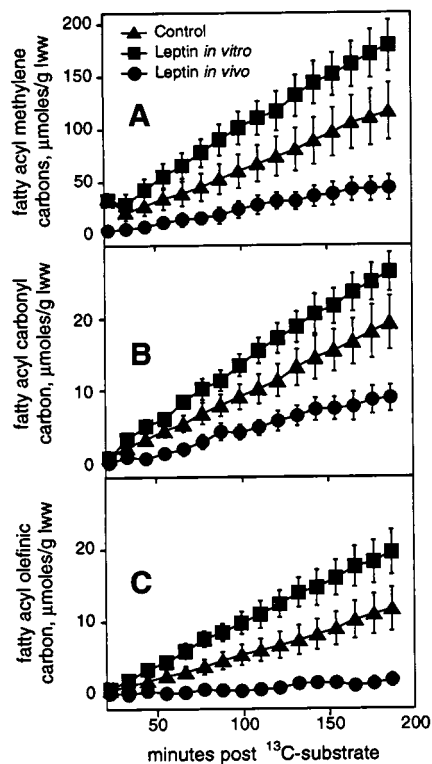


FIG. 5. Quantitation of effect of leptin treatment *in vivo* (●) ($n = 7$) or *in vitro* (■) ($n = 5$), compared with control (▲) ($n = 7$), on kinetics of ^{13}C enrichment at specific carbons of fatty acid synthesized via the *de novo* route in *ob/ob* mouse liver. Leptin was not added during perfusion of livers from mice treated with leptin *in vivo*. ^{13}C enrichments ($\mu\text{mol/g lww}$) are shown over time after initial addition of $[2-^{13}\text{C}]$ pyruvate for the repeating methylene carbons (A), for the fatty acyl carbonyl carbon (B), and for the monounsaturated olefinic carbon (C). Where not visible, error bar is smaller than symbol.

of perfusate AA to β -HB, an indicator of the mitochondrial-free NAD/NADH ratio, were also about the same, 0.29 ± 0.10 , 0.36 ± 0.11 , and 0.25 ± 0.10 , respectively, in the same *ob/ob* mouse liver treatment groups.

Effects on Fatty Acid Chain Length and Unsaturation. Fatty acid synthesis measured at the other carbons labeled in the *de novo* synthesis pathway [i.e., the carbonyl carbon (Fig. 5B) and the chemically unique methylene carbons, d and e in Fig. 4A and B] was decreased 2.2- to 3-fold in liver from *ob/ob* mice treated with leptin *in vivo* as compared with either the *ob/ob* controls ($P < 0.05$) or the *ob/ob* leptin *in vitro* group ($P < 0.001$). Quantitation of the kinetics of ^{13}C enrichment at fatty acyl methylene carbons d and e (data not shown) closely followed the enrichment pattern shown for the carbonyl carbon (Fig. 5B). With our ^{13}C method, contributions of simple chain elongation are distinguishable from *de novo* synthesis as ^{13}C labeling of the carbonyl carbon occurs by both routes, whereas ^{13}C labeling of carbon d in the terminal acetyl unit occurs only by the *de novo* synthesis pathway (10). By this method, the average chain lengths of fatty acids synthesized by *ob/ob* mouse livers in the control, leptin *in vitro*, and leptin *in vivo* groups were 18.8 ± 0.1 , 18.8 ± 0.1 , and 18.0 ± 0.4 , respectively, indicating elongation of the initial pathway product, palmitate. Decreases in ^{13}C enrichment at the monounsaturated olefinic carbon (Fig. 5C) in the *ob/ob* leptin *in vivo* group were even more pronounced, being 5.6-fold lower than in the *ob/ob* control group ($P < 0.01$) and 10-fold lower compared with *ob/ob* mouse livers treated directly with leptin ($P < 0.001$). Comparison of ^{13}C enrichment at the monounsaturated olefinic carbon (i) with the ^{13}C enrichment at the methylene carbon (d) in the terminal acetyl unit indicated a

sharp decrease in $\Delta 9$ desaturase activity in *ob/ob* mouse liver after treatment with leptin *in vivo*. Whereas 66% of fatty acids synthesized by control *ob/ob* mouse livers and 75% of fatty acids synthesized by *ob/ob* livers exposed to leptin *in vitro* were monounsaturated, only 24% of fatty acids synthesized by livers from mice chronically treated with leptin were monounsaturated. Hormonal regulation of $\Delta 9$ desaturase activity is incompletely defined, although insulin appears to be an inducer of the enzyme *in vivo*. Consequently, these data on the control of $\Delta 9$ desaturase by leptin treatment *in vivo* focus attention on understanding regulation of this enzyme. Our results on chain length and percent monounsaturations are consistent with biosynthesis of oleic, stearic, 9-eicosenoic, and arachidic acids, in addition to palmitic acid, all of which are products of the major pathways of fatty acid biosynthesis (23).

Taken together, our ^{13}C NMR data demonstrate two direct effects of leptin on hepatic metabolism: up-regulation of glycogen synthesis in liver from *ob/ob* and lean mice and up-regulation of long-chain fatty acid synthesis by the *de novo* synthesis pathway in *ob/ob* liver. Up-regulation of fatty acid synthesis was demonstrated only for acute treatment. Livers from *ob/ob* mice chronically treated with leptin exhibited down-regulation of fatty acid synthesis. Chronic administration of leptin to *ob/ob* mice induced homeostatic changes that persisted throughout several hours of subsequent liver perfusion postexcision without addition of leptin and resulted in greater enhancement of hepatic glycogen synthesis than was observed with acute treatment. Consistent with our ^{13}C NMR results, leptin treatment of the *ob/ob* mouse *in vivo* resulted in significantly increased hepatic glycogen synthase activity as well as activation of the enzyme. Analysis of ^{13}C and ^1H NMR results for *ob/ob* mouse liver showed a significant increase in flux of ^{13}C -labeled metabolites through the gluconeogenic pathway after chronic, but not acute, administration of leptin. Chronic treatment of the *ob/ob* mouse with leptin *in vivo* markedly decreased hepatic *de novo* synthesis of long-chain fatty acids during perfusion without addition of leptin. In agreement with our ^{13}C NMR results, the activities of hepatic ACC and FAS were significantly reduced by chronic treatment of the *ob/ob* mouse with leptin. Liver's pivotal position in lipid metabolism suggests that our finding that leptin treatment *in vivo* blocks hepatic *de novo* synthesis of long-chain fatty acids from acetyl-CoA, without increasing β -oxidation of fatty acids, is an additional mechanism through which leptin might reduce adiposity in the *ob/ob* mouse. Our data represent a demonstration of direct effects of leptin in the regulation of metabolism in the intact functioning liver and show the ^{13}C NMR method to be useful in investigations of leptin's role as a regulator of metabolism.

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