

# Leptin Therapy Reverses Hyperglycemia in Mice With Streptozotocin-Induced Diabetes, Independent of Hepatic Leptin Signaling

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**OBJECTIVE**—Leptin therapy has been found to reverse hyperglycemia and prevent mortality in several rodent models of type 1 diabetes. Yet the mechanism of leptin-mediated reversal of hyperglycemia has not been fully defined. The liver is a key organ regulating glucose metabolism and is also a target of leptin action. Thus we hypothesized that exogenous leptin administered to mice with streptozotocin (STZ)-induced diabetes reverses hyperglycemia through direct action on hepatocytes.

**RESEARCH DESIGN AND METHODS**—After the induction of diabetes in mice with a high dose of STZ, recombinant mouse leptin was delivered at a supraphysiological dose for 14 days by an osmotic pump implant. We characterized the effect of leptin administration in C57Bl/6J mice with STZ-induced diabetes and then examined whether leptin therapy could reverse STZ-induced hyperglycemia in mice in which hepatic leptin signaling was specifically disrupted.

**RESULTS**—Hyperleptinemia reversed hyperglycemia and hyperketonemia in diabetic C57Bl/6J mice and dramatically improved glucose tolerance. These effects were associated with reduced plasma glucagon and growth hormone levels and dramatically enhanced insulin sensitivity, without changes in glucose uptake by skeletal muscle. Leptin therapy also ameliorated STZ-induced hyperglycemia and hyperketonemia in mice with disrupted hepatic leptin signaling to a similar extent as observed in wild-type littermates with STZ-induced diabetes.

**CONCLUSIONS**—These observations reveal that hyperleptinemia reverses the symptoms of STZ-induced diabetes in mice and that this action does not require direct leptin signaling in the liver. *Diabetes* 60:1414–1423, 2011

Since the discovery of insulin in 1922 (1), insulin therapy has been the predominant treatment for type 1 diabetes. However, the adipocyte-derived hormone leptin has been found to reverse hyperglycemia and prevent mortality when administered to several rodent models of type 1 diabetes (2–7). Leptin has well known glucose-lowering effects in leptin-deficient *ob/ob* mice, and has been established as an important regulator of glucose metabolism. Yet it is surprising that

leptin can restore euglycemia in insulin-deficient rodents, and the mechanism underlying this effect is unclear.

Insulin-deficient diabetes is associated with elevated circulating glucagon levels, which contributes to hyperglycemia (8–11). Interestingly, Yu et al. (2) demonstrated that exogenous leptin can reverse hyperglucagonemia in rats with streptozotocin (STZ)-induced diabetes and in NOD mice; this effect of leptin therapy may contribute to the restoration of euglycemia in these animals. In addition to hyperglucagonemia, insulin resistance is another common characteristic of untreated human and rodent insulin deficiency (12–15). Exogenous leptin can improve insulin sensitivity in rats with STZ-induced diabetes (4,15,16). Therefore the insulin sensitizing effect of leptin may also contribute to lowering blood glucose in type 1 diabetic rodents.

The liver is a key organ that controls glucose flux in response to many metabolic cues and is a major regulator of lipid metabolism and ketone body production. Therefore disturbed hepatic nutrient metabolism is likely a major contributor to hyperglycemia, dyslipidemia, and hyperketonemia in insulin-deficient diabetes. Attenuated insulin action on the liver alone contributes to perturbations in glucose homeostasis (17). Leptin has well-known insulin-sensitizing effects on the liver (18–20), and the long signaling leptin receptor isoform (LepRb) is expressed in the liver and hepatic cell lines (19,21). Intriguingly, we and others have found that direct action of leptin on hepatocytes can modulate hepatic insulin action (18,20,22,23).

We hypothesized that in mice with STZ-induced diabetes, exogenous leptin may act directly on the liver to lower blood glucose and reverse the metabolic consequences of insulin-deficient diabetes. Peripheral leptin therapy in the STZ-diabetic mouse model has not yet been examined; therefore, to test our hypothesis we first characterized the effect of hyperleptinemia on metabolism in mice with STZ-induced diabetes. After this, we examined whether direct leptin action on the liver is required for the therapeutic effect of leptin therapy in insulin-deficient diabetes by administering exogenous leptin to mice with STZ-induced diabetes that have a hepatic-specific disruption of leptin signaling.

## RESEARCH DESIGN AND METHODS

**Animals.** C57Bl/6J male mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice with a tissue-specific disruption of leptin signaling (referred to as *Lepr<sup>flox/flox</sup> Albcre*) and their wild-type littermate controls (*Lepr<sup>flox/flox</sup>*) were generated as previously described (23). Mice were fed a chow diet (5015 Laboratory Diet) and were housed with a 12-h:12-h light-dark cycle with ad libitum access to food and water. All procedures with animals were approved by the University of British Columbia Animal Care Committee and carried out in accordance with the Canadian Council on Animal Care guidelines.

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**STZ administration.** Ten-week-old C57Bl/6J mice were administered 200 mg/kg STZ (Sigma Aldrich) (prepared in acetate buffer, pH 4.5) via intraperitoneal (i.p.) injection 5 days before implantation of mini-osmotic pumps (described below). Nondiabetic control mice were treated with acetate buffer alone. *Lepr<sup>flax/flax</sup>* Albcre and *Lepr<sup>flax/flax</sup>* mice (10–18 weeks old) were given 180 mg/kg i.p. STZ 4 days before pump implantation.

**Leptin therapy via mini-osmotic pumps.** Unless specified otherwise, STZ-leptin mice received 10  $\mu$ g/day mouse recombinant leptin (National Hormone and Peptide Program) prepared in PBS via Alzet 14-day pumps (DURECT) implanted subcutaneously on day 0 (day 0 refers to the day that pumps were first implanted). STZ-saline and nondiabetic controls received pumps loaded with PBS only.

**Plasma analyte measurements.** Body weight, blood glucose, and plasma analytes were measured after a 4-h fast unless specified otherwise. Random-fed samples were collected at 11:00 P.M., 5 h into the dark cycle. Blood glucose was monitored via a One Touch Ultra Glucometer (Life Scan, Burnaby, Canada) from the saphenous vein. Glucagon (Mouse Glucagon RIA; Millipore), free fatty acids (HR Series NEFA HR[2] Kit; Wako Chemical),  $\beta$ -hydroxybutyrate ( $\beta$ -Hydroxybutyrate LiquiColor Test; Stanbio), growth hormone (Rat/Mouse Growth Hormone ELISA; Millipore), and total corticosterone (Corticosterone ELISA; ALPCO) were measured in plasma. Plasma leptin, insulin (23), triglycerides, and cholesterol (24) were measured as previously described. For C57Bl/6J mice, plasma was obtained from blood collected via cardiac puncture on day 14 unless specified otherwise. Plasma from STZ-treated *Lepr<sup>flax/flax</sup>* Albcre and *Lepr<sup>flax/flax</sup>* mice was obtained from saphenous vein blood on day 9, and pre-STZ samples were collected from the same mice on day -6. Insulin tolerance tests and i.p. glucose tolerance tests (IPGTTs) were performed by monitoring blood glucose after an i.p. injection of insulin (0.65 units/kg; Novolin) or dextrose (1.5 g/kg, 30% solution; Fisher Scientific), respectively at time = 0.

**Glucose uptake measurements.** Five days after pump implantation, in vivo glucose uptake was assessed by intravenous flash tail vein injection of 40  $\mu$ Ci/kg 2-deoxy-D-[<sup>14</sup>C]glucose (PerkinElmer Life and Analytical Sciences, Waltham, MA) with or without 0.3 units/kg insulin (Novolin; prepared in 10 mmol/L sodium citrate, 0.03% BSA) in 4-h fasted mice anesthetized with an injectable cocktail of acepromazine (Atravet), midazolam (Versed), and fentanyl (Hynorm). Mice were killed 20 min postinjection by cervical dislocation, and the soleus muscle was isolated for measurement of deoxy-[<sup>14</sup>C]glucose-6-phosphate accumulation as previously described (25) and normalized to tissue mass.

**RT-PCR analysis of leptin receptor transcripts.** Liver tissue was homogenized in TRIzol (Invitrogen) via tissue homogenizer (Tissue Tearor; Biospec Products). RNA was extracted following manufacturer instructions, and 1  $\mu$ g of RNA was used for cDNA synthesis using an iScript cDNA Synthesis kit (Bio-Rad). Primers used to amplify cDNA are described previously (23).

**Statistical analysis.** All data are represented as means  $\pm$  SEM, and significance was set at  $P \leq 0.05$ . Statistical analyses were done using a Student *t* test unless otherwise stated.

## RESULTS

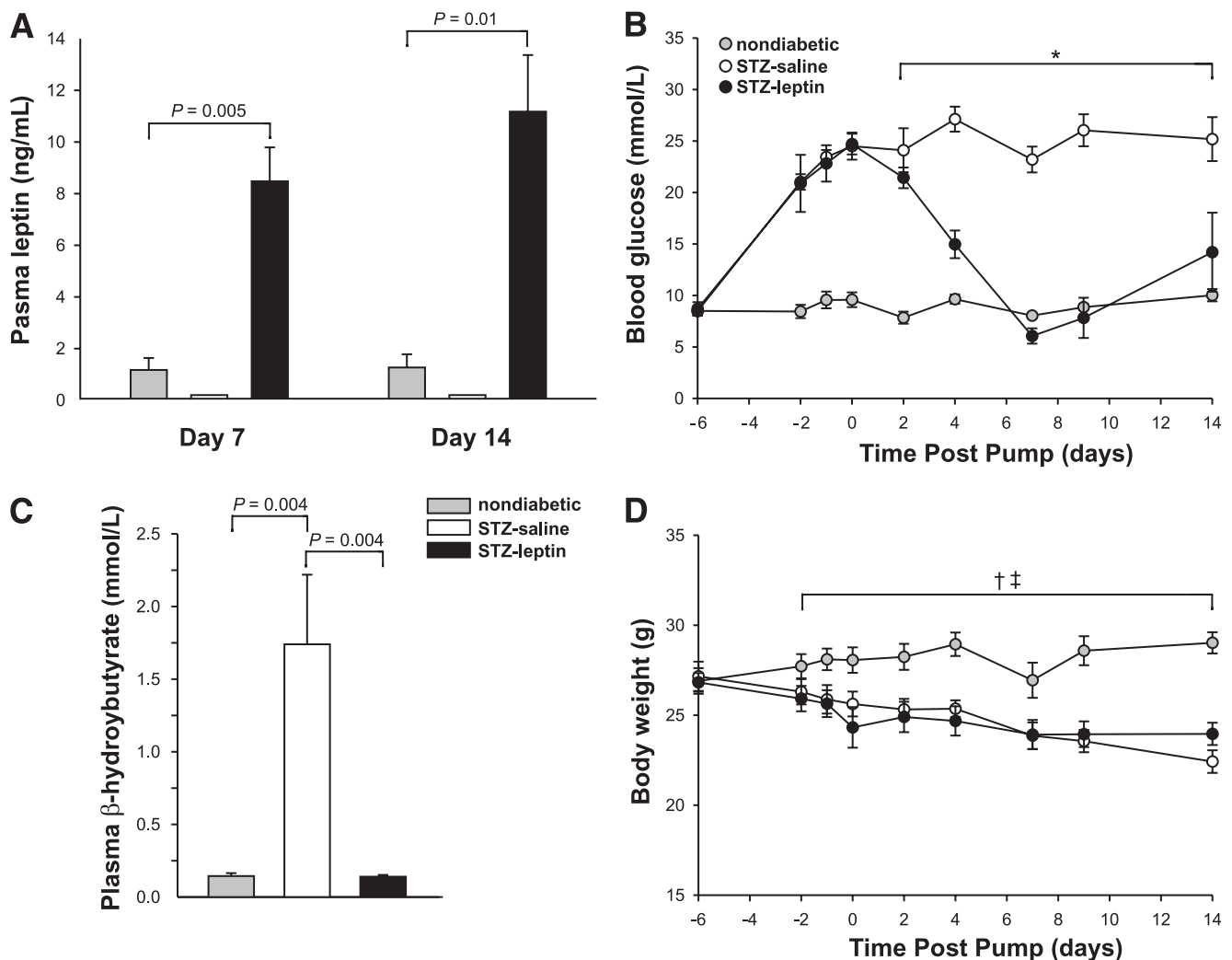
**Leptin therapy ameliorates hyperglycemia in mice with STZ-induced diabetes.** We first characterized the effects of leptin therapy on metabolic parameters in mice with STZ-induced diabetes. C57Bl/6J mice with STZ-induced diabetes were given 10  $\mu$ g/day recombinant mouse leptin (STZ-leptin) or saline (STZ-saline), delivered continuously for 14 days by a subcutaneous osmotic pump, and were also compared with mice that were injected with vehicle only instead of STZ (nondiabetic). STZ administration depleted 4-h fasted plasma leptin levels from  $1.2 \pm 0.5$  ng/mL in nondiabetic controls to below the detection limit ( $<0.2$  ng/mL) in STZ-saline mice (Fig. 1A). STZ-leptin mice were hyperleptinemic, with an approximately sevenfold increase in plasma leptin compared with nondiabetic controls. Leptin levels on day 14 were not significantly different from values obtained on day 7. Seven days after initiation of treatment, 4-h fasted blood glucose concentrations in STZ-leptin mice were reduced to nondiabetic levels ( $6.1 \pm 0.7$  mmol/L STZ-leptin,  $8.0 \pm 0.2$  mmol/L nondiabetic,  $23.2 \pm 1.3$  mmol/L STZ-saline; Fig. 1B) and remained significantly lower than STZ-saline controls for the duration of the study. Interestingly, on day 7 blood glucose levels in STZ-leptin

mice were significantly lower than nondiabetic controls ( $P = 0.018$ ). Although blood glucose levels rose in some individual STZ-leptin mice on day 14, average blood glucose was not significantly different compared with nondiabetic controls on day 14 ( $P = 0.16$ ). We next examined whether leptin therapy ameliorated hyperketonemia.  $\beta$ -Hydroxybutyrate, a predominant ketone body, was drastically elevated in plasma from 4-h fasted STZ-saline mice compared with nondiabetic controls, an effect that was fully reversed by leptin therapy (Fig. 1C). Because high doses of leptin can reduce body weight in some rodent models, we investigated whether leptin treatment exacerbated weight loss in mice with STZ-induced diabetes. STZ administration caused a steady decline in body weight (Fig. 1D); however, STZ-leptin mice maintained similar body weight throughout the study to their STZ-saline controls.

**Leptin therapy acutely restores euglycemia in mice with STZ-induced diabetes.** We next examined whether the glucose-lowering effect of hyperleptinemia in insulin-deficient mice persisted after cessation of leptin therapy. C57Bl/6J mice with STZ-induced diabetes were treated with 5  $\mu$ g/day recombinant leptin via 14-day osmotic pumps (Fig. 2). Blood glucose gradually declined over the treatment period, and hyperglycemia was ameliorated by day 14 of leptin therapy. Hyperglycemia rapidly returned in the STZ-leptin group after pump removal. At this point, the STZ-saline group had to be killed because of deteriorated body condition. When leptin was readministered to STZ-leptin mice at a dose of 24  $\mu$ g/day with a second pump implant, hyperglycemia resolved to a similar extent as observed with the previous dose, but within just 3 days of treatment. These data suggest the maintenance of euglycemia in this model requires continuous leptin administration and that the rate of glucose lowering via leptin administration may be dose dependent.

**Hyperleptinemia improves postprandial glucose metabolism in diabetic mice.** We next investigated whether glucose metabolism was improved in the postprandial state. Random-fed blood glucose levels were  $8.1 \pm 0.3$  mmol/L in nondiabetic mice, whereas the majority of STZ-saline mice had glucose levels above the limit of detection (33.3 mmol/L). Random-fed blood glucose levels in STZ-leptin mice were  $23.9 \pm 3.3$  mmol/L, indicating at least an  $\sim 26\%$  reduction in fed blood glucose compared with STZ-saline mice (Fig. 3A). To examine this more thoroughly we performed an IPGTT (Fig. 3B). STZ-saline controls had poor glucose control after a glucose challenge, and this was markedly improved in STZ-leptin mice. Surprisingly, glucose clearance in STZ-leptin mice was similar to nondiabetic controls, demonstrating that hyperleptinemia dramatically improves glucose metabolism in the absorptive state.

**Leptin therapy reverses STZ-induced dyslipidemia.** We next sought to determine whether hyperleptinemia reversed dyslipidemia in mice with STZ-induced diabetes by measuring 4-h fasted plasma lipids. STZ administration increased triglyceride and cholesterol levels in STZ-saline mice compared with nondiabetic controls, whereas free fatty acids were unaltered by STZ (Fig. 4A–C). Leptin (10  $\mu$ g/day) markedly reduced plasma triglycerides, free fatty acids, and cholesterol in mice with STZ-induced diabetes to levels significantly lower than both STZ-saline and nondiabetic controls. Collectively, these data indicate that leptin can alleviate dyslipidemia in mice with STZ-induced diabetes.

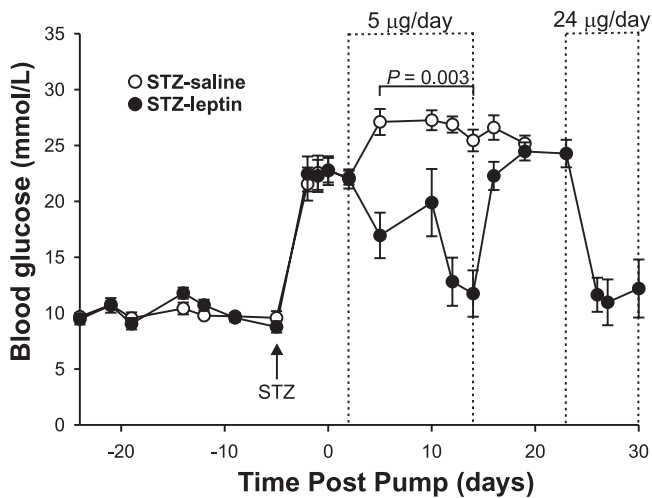


**FIG. 1.** Leptin therapy reverses hyperglycemia and hyperketonemia in diabetic C57Bl/6J mice. Four-hour fasted parameters in STZ-leptin-treated (black), STZ-saline (white), and nondiabetic (gray) mice are shown. Mice received subcutaneous 14-day osmotic pump implants containing a 10  $\mu$ g/day dose of leptin or saline on day 0. **A:** Plasma leptin levels on day 7 and day 14 after pump implantation ( $n = 5$ ). Day 7 samples were collected from the saphenous vein. Leptin was undetectable in plasma samples from STZ-saline mice. **B:** Blood glucose ( $n = 5$ ). **C:**  $\beta$ -Hydroxybutyrate levels on day 14 ( $n \geq 4$ ). **D:** Body weight ( $n = 5$ ). **B** and **D:** Statistical analyses were performed by one-way ANOVA multiple comparison procedure with Holm-Sidak post hoc testing (Sigma Stat, SYSTAT Software, Inc.). \* $P < 0.01$  STZ-leptin vs. STZ-saline mice; † $P < 0.01$  STZ-leptin vs. nondiabetic mice; ‡ $P < 0.01$  STZ-saline vs. nondiabetic mice. Data are expressed as means  $\pm$  SEM.

**Hyperleptinemia suppresses glucagon and growth hormone levels and improves insulin sensitivity in mice with STZ-induced diabetes.** Although it is apparent that leptin mimics the effect of insulin on glucose metabolism in mice with STZ-induced diabetes, the mechanism for this is unclear. As previously reported (2), hyperleptinemia may lower blood glucose in STZ-induced diabetes by alleviating the hyperglucagonemia associated with insulin deficiency. Indeed, STZ-saline mice had an  $\sim 2.5$ -fold increase in 4-h fasted plasma glucagon levels compared with nondiabetic controls, whereas STZ-leptin mice had plasma glucagon levels that were similar to nondiabetic controls (Fig. 5A). Four-hour fasted plasma growth hormone was increased  $\sim 28$ -fold in STZ-saline mice compared with nondiabetic mice and, similar to glucagon, growth hormone levels were markedly decreased in response to leptin therapy, although remaining significantly higher than nondiabetic levels (Fig. 5B). In contrast, plasma corticosterone was increased in mice with STZ-induced diabetes compared with nondiabetic controls but was unaltered by leptin therapy

(Fig. 5C). Therefore, the leptin-mediated suppression of growth hormone and glucagon in mice with STZ-induced diabetes likely contributes to glucose lowering.

As expected, the majority of 4-h fasted STZ-saline and STZ-leptin mice had plasma insulin levels that were below detection limits ( $<0.025$  ng/mL) even using an ultrasensitive mouse insulin ELISA (Fig. 5D). However, using this ultrasensitive assay, insulin levels were detectable in most random-fed STZ-saline and STZ-leptin mice, albeit at  $\sim 9$  and  $\sim 7\%$  that of random-fed nondiabetic controls, respectively. Although these data confirm that hyperleptinemia does not reverse diabetes by increasing insulin concentrations, it is important to note that insulin is not completely eliminated by STZ administration. In light of this, we investigated whether augmented insulin sensitivity may also contribute to the glucose-lowering effect of leptin in mice with STZ-induced diabetes. Both the nondiabetic and STZ-leptin groups exhibited a potent response to exogenous insulin, such that 10 min after insulin injection all but one mouse in both groups had blood glucose levels



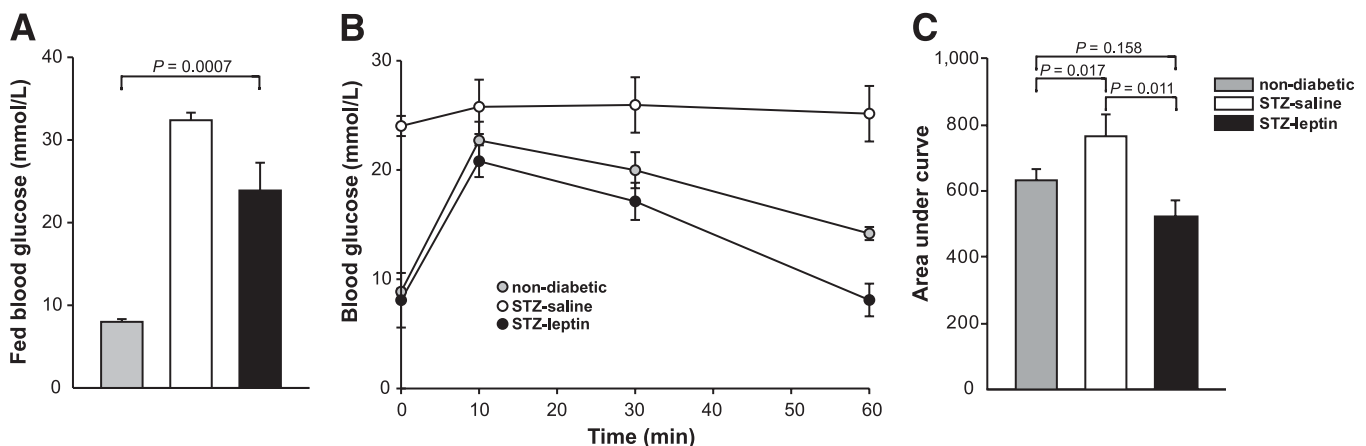
**FIG. 2.** The effects of leptin on hyperglycemia in mice with STZ-induced diabetes are acute. Four-hour fasted blood glucose in STZ-leptin (black) and STZ-saline (white) mice ( $n = 7$ ) is shown. C57BL/6J mice given STZ on day  $-5$  were subsequently treated with  $5 \mu\text{g/day}$  leptin or saline via subcutaneous 14-day osmotic pumps implanted on day 0. Pumps were removed on day 14 after blood glucose measurement. STZ-saline mice were killed on day 19 because of deteriorating body condition. The STZ-leptin group received a 7-day osmotic pump implant delivering  $24 \mu\text{g/day}$  leptin on day 24. Statistical analyses were performed with one-way ANOVA multiple comparison procedure with Holm-Sidak post hoc testing. Data are expressed as means  $\pm$  SEM.

below  $4 \text{ mmol/L}$  and had to be rescued by exogenous glucose administration; thus only one time point for these groups was collected (Fig. 5E). STZ-saline controls were significantly insulin resistant compared with nondiabetic mice, since insulin administration resulted in only a  $\sim 19\%$  drop compared with an  $\sim 56\%$  drop in blood glucose, respectively, 10 min post-insulin injection ( $P = 0.00023$ ). Remarkably, leptin-treated mice displayed an  $\sim 76\%$  drop in blood glucose 10 min after insulin injection and were significantly more insulin sensitive than both STZ-saline mice ( $P = 0.00016$ ) and nondiabetic controls ( $P = 0.019$ ). These data indicate that not only does  $10 \mu\text{g/day}$  leptin reverse the insulin resistance observed in mice with STZ-induced

diabetes but it also augments insulin sensitivity beyond that of nondiabetic mice. Therefore, although insulin levels are not altered, enhanced insulin action in response to hyperleptinemia may contribute to glucose lowering in rodents with STZ-induced diabetes.

To determine whether the improved insulin sensitivity observed in STZ-leptin mice was a result of increased glucose uptake, we evaluated tissue-specific glucose uptake in vivo by intravenous flash injection of 2-deoxy-D- $^{14}\text{C}$ glucose as described previously (25,26). Glucose uptake in the soleus muscle was comparable in STZ-saline and STZ-leptin mice (Fig. 5F). An intravenous insulin bolus increased glucose uptake in the soleus relative to baseline; however, insulin-stimulated glucose uptake was not significantly increased in STZ-leptin mice compared with STZ-saline controls. Because of the extreme depletion of adipose tissue in both STZ-saline and STZ-leptin mice, glucose uptake in adipose could not be determined. These observations suggest that glucose uptake in skeletal muscle and adipose is not sufficient to account for the enhanced insulin sensitivity and restored euglycemia in STZ-leptin mice.

**Generation of mice with a liver-specific attenuation of leptin signaling.** Because leptin signaling can have a direct insulin-like and insulin-sensitizing effect on the liver (18,20,22), we determined whether hepatic leptin signaling was involved in mediating the therapeutic effects of hyperleptinemia in rodents with STZ-induced diabetes. To accomplish this, we used the *Cre-lox* approach to determine whether hyperleptinemia can reverse STZ-induced diabetes in mice with disrupted hepatic leptin signaling (*Lepr<sup>fllox/fllox</sup> Albcre* mice). *Lepr<sup>fllox/fllox</sup> Albcre* mice are homozygous for a floxed leptin receptor allele (*Lepr<sup>fllox</sup>*) and express the *Cre* transgene under control of the albumin promoter, which confers hepatocyte-specific recombination of *Lepr<sup>fllox</sup>* (23,27). Recombination produces a mutated allele (*Lepr<sup>Δ17</sup>*) with a truncated intracellular signaling domain that can no longer mediate leptin-induced JAK-STAT signaling (28) (Fig. 6A). RT-PCR analysis for LepRb mRNA from liver cDNA generated an amplicon similar in size to the 343 base pair product expected from wild-type LepRb transcript in *Lepr<sup>fllox/fllox</sup>* controls; liver cDNA from *Lepr<sup>fllox/fllox</sup> Albcre* mice produced an amplicon consistent



**FIG. 3.** Leptin improves postprandial glucose metabolism in mice with STZ-induced diabetes. **A** and **B**: Random-fed blood glucose on day 13 and i.p. glucose tolerance on day 9 in STZ-leptin (black), STZ-saline (white), and nondiabetic (gray) mice are shown. **A**: All but one STZ-saline mouse had a blood glucose concentration above the limit of detection and was assigned a value of  $33.3 \text{ mmol/L}$  ( $n = 5$ ). **B**: Mice received an i.p. injection of  $1.5 \text{ g/kg}$  glucose at time 0 ( $n \geq 4$ ). **C**: Area under the curve values for the IPGTT. Data are expressed as means  $\pm$  SEM.

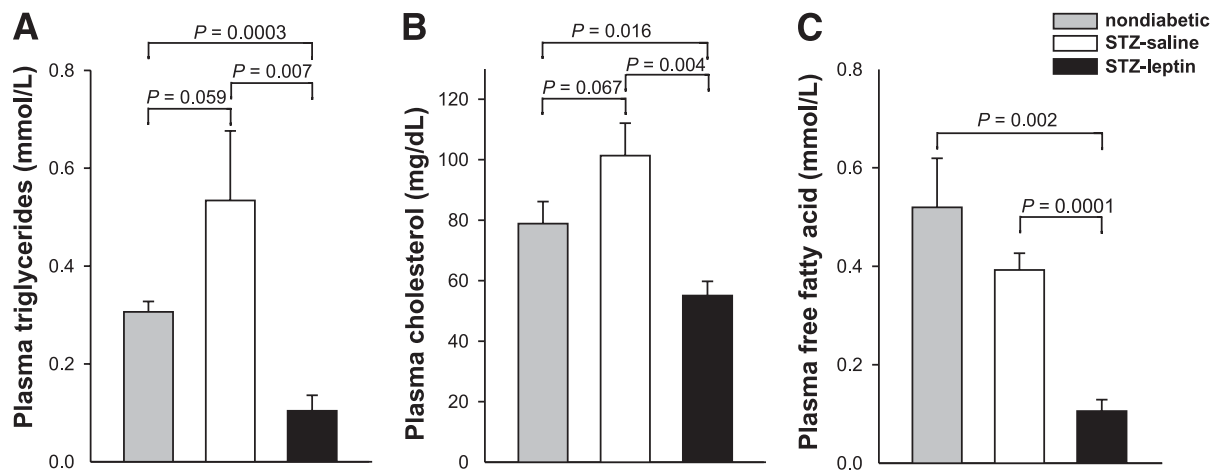


FIG. 4. Leptin reduces plasma lipid levels in mice with STZ-induced diabetes. Four-hour fasted plasma triglycerides (A), cholesterol (B), and free fatty acids (C) in STZ-leptin (black), STZ-saline (white), and nondiabetic (gray) mice on day 14 ( $n \geq 4$ ) are shown. Data are expressed as means  $\pm$  SEM.

with the expected 267 base pair size of the *Lepr*<sup>Δ17</sup> transcript (Fig. 6B). Thus, in the livers of *Lepr*<sup>flox/flox</sup> *Albcre* mice, the majority of leptin receptors no longer contain the JAK-STAT signaling domain.

#### Hepatic leptin signaling is not required for the therapeutic effect of leptin in STZ-induced diabetes.

To determine whether leptin therapy reverses STZ-induced diabetes through direct action on the liver, age-matched male *Lepr*<sup>flox/flox</sup> *Albcre* mice and *Lepr*<sup>flox/flox</sup> littermate controls were first administered STZ to induce a state of insulin deficiency. Following development of hyperglycemia, both *Lepr*<sup>flox/flox</sup> controls and *Lepr*<sup>flox/flox</sup> *Albcre* mice were divided into two groups, one group treated with 10  $\mu$ g/day leptin and the other with saline, delivered via 14 day subcutaneous osmotic pump implants (Fig. 7). Nondiabetic *Lepr*<sup>flox/flox</sup> *Albcre* and *Lepr*<sup>flox/flox</sup> mice had similar body weight, blood glucose, and plasma leptin and insulin levels, as previously described (23). STZ administration produced an  $\sim$ 10-fold reduction in mean plasma leptin in both *Lepr*<sup>flox/flox</sup> *Albcre* and *Lepr*<sup>flox/flox</sup> mice, respectively (Fig. 7A), and treatment with 10  $\mu$ g/day leptin increased plasma leptin levels by  $>19$ -fold in both types of mice.

We then examined whether hyperleptinemia in *Lepr*<sup>flox/flox</sup> *Albcre* mice could lower blood glucose to the same extent as in *Lepr*<sup>flox/flox</sup> controls. After STZ administration, all groups displayed a similar extent of hyperglycemia (Fig. 7B). STZ-saline-treated *Lepr*<sup>flox/flox</sup> *Albcre* mice and STZ-saline-treated littermate controls were severely hyperglycemic for the duration of the study, whereas *Lepr*<sup>flox/flox</sup> *Albcre* and *Lepr*<sup>flox/flox</sup> mice treated with leptin showed a marked reduction in fasting blood glucose. Interestingly, leptin treatment was equally effective at alleviating hyperglycemia in mice with disrupted hepatic leptin signaling as in their wild-type littermates (Fig. 7B). However, unlike our studies in C57Bl/6J mice, a 10  $\mu$ g/day dose of leptin was not able to fully restore fasting blood glucose levels to pre-STZ levels in *Lepr*<sup>flox/flox</sup> mice; this may be explained by differences in genetic background, which can alter leptin-related phenotypes (29,30). Nevertheless, comparing *Lepr*<sup>flox/flox</sup> *Albcre* mice with littermates of identical genetic background reveals that liver leptin signaling is not required for the glucose-lowering action of leptin in STZ-induced diabetes.

In addition to assessing the impact of attenuated hepatic leptin signaling on the glucose-lowering action of leptin, we also examined the impact on the ketone lowering action of leptin. Pre-STZ plasma  $\beta$ -hydroxybutyrate levels were significantly elevated by  $\sim$ 10- and  $\sim$ 5-fold after STZ administration in *Lepr*<sup>flox/flox</sup> *Albcre* and *Lepr*<sup>flox/flox</sup> mice, respectively (Fig. 7C). Similar to C57Bl/6J mice, leptin therapy reduced plasma  $\beta$ -hydroxybutyrate levels by  $\sim$ 85% in *Lepr*<sup>flox/flox</sup> controls to pre-STZ values. This effect was even more pronounced in *Lepr*<sup>flox/flox</sup> *Albcre* mice, which, despite inactivated hepatic leptin signaling, exhibited a  $\sim$ 96% reduction in plasma  $\beta$ -hydroxybutyrate levels in response to leptin therapy. In fact, leptin therapy reduced plasma  $\beta$ -hydroxybutyrate to levels that were significantly lower than pre-STZ values in the *Lepr*<sup>flox/flox</sup> *Albcre* mice ( $P = 0.022$ ). Thus, leptin action on hyperketonemia does not depend on hepatic leptin signaling.

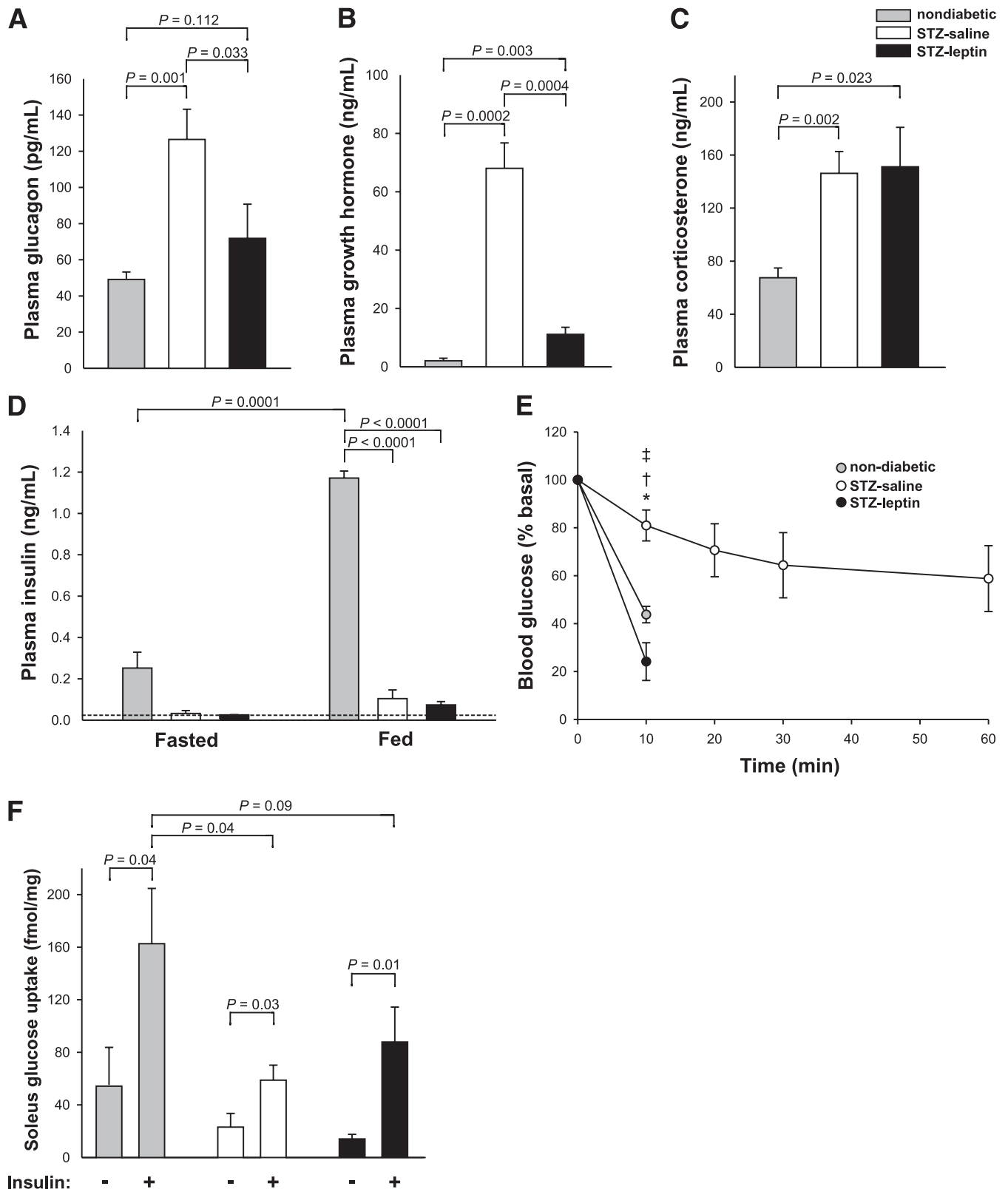
*Lepr*<sup>flox/flox</sup> *Albcre* mice and *Lepr*<sup>flox/flox</sup> controls administered STZ displayed a gradual decrease in body weight, and all groups had similar body weights for the duration of the study (Fig. 7D). STZ administration decreased 4-h fasted insulin levels to  $\sim$ 13 and  $\sim$ 10% of pre-STZ levels in *Lepr*<sup>flox/flox</sup> controls and *Lepr*<sup>flox/flox</sup> *Albcre* mice, respectively, and insulin levels were not restored by leptin therapy (Fig. 7E). Thus, similar to C57Bl/6J mice, the effects of leptin on glucose and ketone levels are not attributable to changes in insulin levels or body weight.

Because hyperleptinemia could alleviate the symptoms of insulin-deficient diabetes in mice with disrupted hepatic leptin signaling, we predicted that enhanced insulin sensitivity would still be observed in these mice in response to leptin therapy. To test this we performed insulin tolerance tests. Both *Lepr*<sup>flox/flox</sup> *Albcre* and *Lepr*<sup>flox/flox</sup> mice with STZ-induced diabetes displayed significantly enhanced insulin sensitivity in response to leptin treatment compared with their saline-treated counterparts ( $P = 0.00081$  and  $P = 0.033$ , respectively; Fig. 7F). Therefore, hepatic leptin signaling is not required for the augmentation of insulin action by leptin therapy.

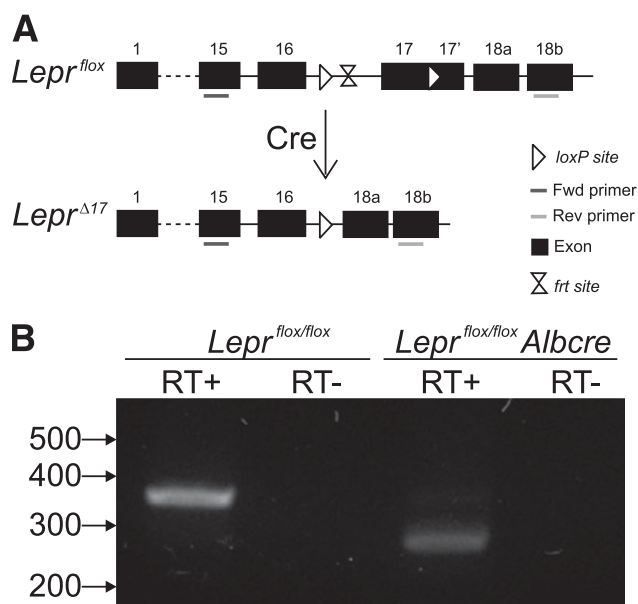
#### DISCUSSION

To investigate the mechanism of leptin action on glucose homeostasis in type 1 diabetes, we examined the effect of hyperleptinemia in mice with STZ-induced diabetes.





**FIG. 5.** Leptin reduces plasma glucagon and growth hormone levels and enhances insulin action in mice with STZ-induced diabetes. Four-hour fasted plasma glucagon (A), growth hormone (B), and corticosterone (C) on day 14 ( $n \geq 4$ ) are shown. D: Four-hour fasted plasma insulin (day 7) and random-fed plasma insulin (day 13) were measured in samples collected from the saphenous vein. Fasted insulin was undetectable in all but one mouse in both the STZ-leptin and STZ-saline groups ( $n \geq 4$ ), whereas random-fed insulin was detectable in most STZ-induced mice tested ( $n \geq 3$ ). The limit of detection (0.025 ng/mL) is shown as a broken line. E: Insulin tolerance test (day 4;  $n = 5$ ). All but one mouse in both the STZ-leptin and nondiabetic groups had to be rescued with exogenous glucose at 10 min after insulin injection. Statistical analyses were performed using Student *t* test on 10-min values. F: Glucose uptake measured in soleus muscle collected 20 min after intravenous injection of 2-deoxy-D- $^{14}$ C]glucose with and without insulin ( $n = 3-5$ ). Glucose uptake was normalized to tissue weight. STZ-leptin (black), STZ-saline (white), and nondiabetic (gray) are shown. \* $P < 0.05$  STZ-leptin vs. STZ-saline mice; † $P < 0.05$  STZ-leptin vs. nondiabetic mice; ‡ $P < 0.05$  STZ-saline vs. nondiabetic mice. Data are expressed as means  $\pm$  SEM.



**FIG. 6.** The long form of the leptin receptor is truncated in livers of *Lepr<sup>lox/lox</sup> Albcre* mice. **A:** Schematic representation of *Lepr<sup>lox</sup>* and *Lepr<sup>Δ17</sup>* alleles. **B:** RNA was extracted from livers of *Lepr<sup>lox/lox</sup> Albcre* mice and *Lepr<sup>lox/lox</sup>* controls for use as a template for generating cDNA (RT+). Reactions lacking reverse transcriptase were set up as negative controls (RT-). The cDNA generated was PCR amplified using primers indicated in A. These primers generate a 343 base pair amplicon for the *Lepr<sup>lox</sup>* transcript and a 267 base pair amplicon for the *Lepr<sup>Δ17</sup>* transcript. Arrows on the left denote the migration of molecular weight markers.

Hyperleptinemia normalized fasting blood glucose and ketone levels, reduced plasma lipids, and normalized glucose tolerance in response to an IPGTT. These findings support previous observations in insulin-deficient rodents (2–5).

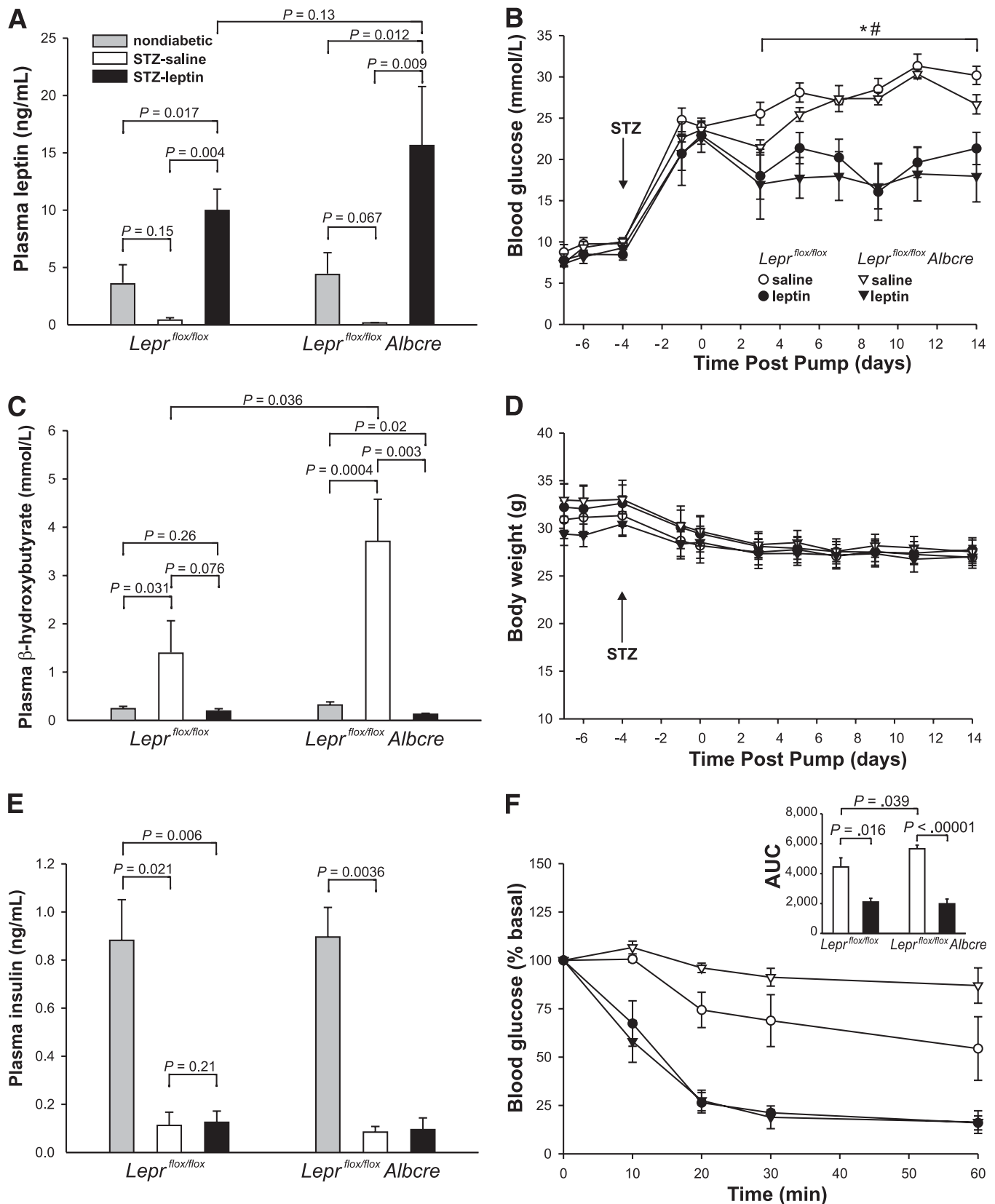
The mechanism responsible for the effects of leptin in an insulin-deficient state is unclear. Insulin deficiency causes marked hyperphagia (31), and leptin replacement normalizes food intake in rats with STZ-induced diabetes (15,32). Therefore, suppression of hyperphagia could lower blood glucose by reducing nutrient intake. However, pair-feeding studies indicate that decreased food intake cannot account for the restoration of euglycemia in leptin-treated insulin-deficient rodents (2–5). Despite the anorexogenic actions of leptin, mice with STZ-induced diabetes treated with leptin did not display reduced body weight. This has been reported previously in insulin-deficient rodents treated with physiological and supraphysiological doses of leptin (2,4,5,15); in fact, others have reported that over prolonged treatment periods, weight loss was actually attenuated in hyperleptinemic rodents with type 1 diabetes compared with diabetic controls (2). Yet similar doses of leptin have been found to reduce body weight in both nondiabetic C57Bl/6J mice and *ob/ob* mice (33), indicating that the weight-reducing effect of leptin may be context dependent. Diabetic animals pair-fed to leptin-treated animals display pronounced weight loss compared with both leptin-treated and untreated diabetic animals fed ad libitum (4,5,15). Attenuated weight loss in leptin-treated diabetic animals may be because of the improved health of the animals, which may counter the anorexogenic action of leptin. Supporting this phenomenon, German et al. (15) recently found that leptin replacement actually inhibits an STZ-induced increase in energy expenditure. These studies suggest that in the context of insulin-deficient

diabetes, hyperleptinemia has an anabolic effect that opposes the anorexogenic action of leptin.

In addition to confirming that hyperleptinemia lowers plasma glucagon levels in STZ-induced diabetes (2), the current study reveals that leptin therapy alleviates the increase in plasma growth hormone associated with uncontrolled diabetes. Chronically elevated growth hormone levels can decrease insulin sensitivity in rodents and humans (34,35). Therefore, leptin-mediated suppression of growth hormone may contribute to the improvement in whole body insulin sensitivity observed in STZ-leptin mice. We postulate that reduced glucagon and growth hormone levels along with enhanced insulin action may mediate the restoration of euglycemia in leptin-treated animals with STZ-induced diabetes. German et al. (15) recently demonstrated that in rats with STZ-induced diabetes, a physiological dose of leptin normalizes plasma glucagon and reverses insulin resistance, yet does not restore euglycemia. Our observations do not contradict these findings; here, mice with STZ-induced diabetes treated with a supra-physiological dose of leptin were more insulin sensitive than nondiabetic controls, whereas German et al. (15) found that rats with STZ-induced diabetes treated with a physiological dose of leptin had similar insulin sensitivity to nondiabetic controls. This suggests that to restore euglycemia in insulin-deficient diabetes, the dose of leptin must be high enough to reach a threshold of insulin sensitivity that can compensate for dramatically lowered insulin levels.

Despite enhanced whole body insulin sensitivity, glucose uptake in skeletal muscle was not altered by leptin therapy, indicating that leptin-induced changes in insulin sensitivity are likely mediated through other target tissues. This supports previous data that under basal conditions, the rate of glucose disappearance in hyperleptinemic rats with STZ-induced diabetes is not increased compared with diabetic controls (4). Several lines of evidence point to the liver as a primary tissue mediating the antidiabetic effects of leptin; leptin-treated rats with STZ-induced diabetes exhibit reduced glucose appearance rates and decreased expression/activation of hepatic gluconeogenic factors (2,4,5). Although leptin can modulate liver glucose metabolism through direct hepatic leptin signaling (18,36), the current study reveals that this pathway is not required for leptin therapy to ameliorate STZ-induced hyperglycemia or hyperketonemia, or to enhance insulin sensitivity. Although it is possible that some hepatocytes retain expression of functional leptin receptors that compensate for attenuated hepatic leptin signaling, RT-PCR analysis indicated that wild-type LepRb expression is efficiently knocked out in the liver of *Lepr<sup>lox/lox</sup> Albcre* mice. Furthermore, *Lepr<sup>lox/lox</sup> Albcre* mice trended toward a more potent response to hyperleptinemia in regard to plasma  $\beta$ -hydroxybutyrate and blood glucose levels than wild-type littermates, as opposed to an attenuated response.

Although direct action on the liver is not required for the glucose-lowering action of leptin therapy, leptin-induced effects on liver metabolism through the brain may be important. Reconstitution of leptin receptors in the hypothalamus of leptin receptor-deficient rats enhances the inhibitory action of insulin on hepatic glucose production (30). Furthermore, central delivery of leptin was found by Hidaka et al. (5) to reverse hyperglycemia in rats with STZ-induced diabetes, a finding that has now been confirmed by others (6,7,37); interestingly, central leptin therapy can inhibit hepatic glucose production in rats with STZ-induced diabetes (37). The effects of leptin on circulating factors



**FIG. 7.** Leptin relieves the symptoms of STZ-induced diabetes in *Lepr*<sup>flox/flox</sup> Albcre mice. Four-hour fasted parameters in *Lepr*<sup>flox/flox</sup> Albcre and *Lepr*<sup>flox/flox</sup> mice that were STZ administered on day -4, and received subcutaneous 14-day osmotic pumps delivering 10 μg/day leptin or saline on day 0, are shown. **A:** Plasma leptin ( $n \geq 4$ ). **B:** Blood glucose ( $n \geq 4$ ). **C:** Plasma β-hydroxybutyrate ( $n \geq 4$ ). **D:** Body weight ( $n \geq 4$ ). **E:** Plasma insulin ( $n \geq 4$ ). Only two STZ-leptin-treated *Lepr*<sup>flox/flox</sup> Albcre mice had detectable levels of insulin. **F:** Insulin tolerance test on day 11 ( $n \geq 3$ ). Area under the curve values are shown in the inset. **A, C, and E:** Pre-STZ (gray), STZ-saline (white), and STZ-leptin (black). **B, D, and F:** *Lepr*<sup>flox/flox</sup> Albcre mice given STZ-leptin (▼) or STZ-saline (▽). *Lepr*<sup>flox/flox</sup> controls given STZ-leptin (●) or STZ-saline (○) are shown. **B and D:** Statistical analyses were performed using one-way ANOVA multiple comparison procedure with Holm-Sidak post hoc testing on values. \* $P < 0.0001$  *Lepr*<sup>flox/flox</sup> Albcre STZ-leptin vs. STZ-saline mice; # $P < 0.0001$  *Lepr*<sup>flox/flox</sup> STZ-leptin vs. STZ-saline mice. Data are expressed as means ± SEM.



that influence hepatic insulin sensitivity may also contribute to the therapeutic action of leptin. By decreasing free fatty acid levels, possibly through increased lipid oxidation in adipose tissue (38), hyperleptinemia may enhance hepatic insulin sensitivity (39). Furthermore, the suppression of glucagon and growth hormone likely reduces hepatic glucose output in STZ-induced diabetes. Therefore, direct and/or indirect leptin action on other tissues including adipocytes and pancreatic  $\alpha$ -cells may also be important for the therapeutic action of leptin (40–42). Although the  $\beta$ -cell is a key target of leptin (24), it is unlikely to mediate the therapeutic action of leptin after STZ-mediated  $\beta$ -cell depletion. The inhibitory effect of leptin on insulin secretion (24,43) would not benefit insulin-deficient rodents, and hyperleptinemia does not appreciably alter insulin levels or  $\beta$ -cell mass in rodents with type 1 diabetes (2). Elucidating the direct target tissues, and the intracellular pathways required for the glucose-lowering effects of leptin in type 1 diabetes, will provide important insight into both physiological and therapeutic mechanisms of leptin action.

An important consideration for the use of leptin as a therapeutic for type 1 diabetes will be the immune modulating actions of leptin. Treatment of young, prediabetic NOD mice with leptin accelerates the onset of insulinitis and diabetes (44). Moreover, insulinitis and T-effector cell activation is suppressed in NOD mice with a naturally occurring loss-of-function mutation in the leptin receptor (45,46). In contrast, hyperleptinemia has been found to prevent the onset of virally induced autoimmune diabetes in the BBDR rat (47). Therefore, the potential proautoimmune effects of leptin therapy in patients with type 1 diabetes must be thoroughly investigated.

Based on observations from the current study and by others, leptin may prove to be a promising therapy for type 1 diabetes. Interestingly, some of the metabolic disturbances associated with insulin deficiency may be caused by the hypoleptinemia in uncontrolled insulin-deficient diabetes (32,48). In rats with STZ-induced diabetes, leptin replacement reverses insulin resistance, hyperglucagonemia, and hyperphagia (15,32). Thus, leptin replacement may prove a rational therapy for type 1 diabetes. Furthermore, leptin therapy may have some advantages over insulin therapy. We observed that in *Lep<sup>r</sup><sup>fllox/fllox</sup>* mice, although hyperglycemia was only partially rescued with leptin therapy, hyperketonemia was completely reversed. The disappearance of ketone bodies in response to leptin may be due to leptin action on systemic free fatty acids or changes in hepatic metabolic pathways. This is provocative since resolution of diabetic ketoacidosis with continuous insulin administration in human patients is reported to occur after hyperglycemia is reversed (49,50). A potential pitfall of leptin administration in conjunction with insulin is increased risk of hypoglycemia. In our studies, a single dose of insulin that modestly lowered blood glucose in untreated diabetic animals caused rapid and severe hypoglycemia in leptin-treated animals. Thus, although leptin in combination with insulin may dramatically improve treatment for patients with type 1 diabetes, further studies must more rigorously assess the increased risk of hypoglycemia.

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