

IGFBP2 Is Neither Sufficient nor Necessary for the Physiological Actions of Leptin on Glucose Homeostasis in Male *ob/ob* Mice

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The ability of leptin to improve metabolic abnormalities in models of leptin deficiency, lipodystrophy, and even type 1 diabetes is of significant interest. However, the mechanism by which leptin mediates these effects remains ill-defined. Leptin was recently reported to regulate insulin-like growth factor-binding protein-2 (IGFBP2), and adenoviral overexpression of pharmacological levels of IGFBP2 ameliorates diabetic symptoms in many models of diabetes. We sought to determine the role of physiological levels of IGFBP2 in the glucoregulatory action of leptin. To investigate whether physiological levels of IGFBP2 are sufficient to mimic the action of leptin, we treated male *ob/ob* mice with low-dose IGFBP2 adenovirus (Ad-IGFBP2) or low-dose leptin. Despite similar levels of circulating IGFBP2, leptin but not Ad-IGFBP2 lowered body weight and plasma insulin and improved glucose and insulin tolerance. To elucidate the role of IGFBP2 in normal glucose homeostasis, we knocked down IGFBP2 in male C57BL/6 mice using small interfering RNA to determine whether this would recapitulate any aspect of the *ob/ob* phenotype. Despite successful IGFBP2 knockdown, body weight, blood glucose, and plasma insulin were unchanged. Finally, to determine whether IGFBP2 is required for the glucoregulatory actions of leptin, we prevented leptin-mediated increases in IGFBP2 in male *ob/ob* mice using RNA interference. Even though increases in IGFBP2 were blocked, the ability of leptin to decrease body weight, blood glucose, and plasma insulin levels were unaltered. In conclusion, physiological levels of IGFBP2 are neither sufficient to mimic nor required for the physiological action of leptin. (*Endocrinology* 155: 716–725, 2014)

The adipocyte-derived hormone leptin has well-known effects on body weight regulation and also has profound effects on glucose homeostasis (1). Indeed, leptin-deficient *ob/ob* and leptin receptor-deficient *db/db* mice not only are obese but also exhibit hepatic steatosis, hyperinsulinemia, insulin resistance, glucose intolerance, and often fasting hyperglycemia (2–5). The metabolic manifestation of a loss of leptin action in mice is highly similar to that in type 2 diabetes. Humans that lack leptin action due to leptin deficiency or lipodystrophy also exhibit the preceding metabolic abnormalities (6–8). Leptin

replacement treatment can ameliorate these metabolic aberrations in both mice and humans (3, 6–8). Interestingly, many of the metabolic actions of leptin are not due to its well-established effects on body weight (3, 4, 9). Rather, leptin appears to directly regulate metabolism and glucose homeostasis, although the mechanism by which leptin contributes to glucose regulation has not been fully elucidated.

Recently, there has been substantial interest in insulin-like growth factor-binding protein-2 (IGFBP2) as a mediator of leptin action on metabolism. IGFBPs were first

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Abbreviations: Ad, adenovirus; AUC, area under the curve; CMV, cytomegalovirus; DLin-KC2-DMA, 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; FVII, factor VII; gal, galactosidase; IGFBP2, insulin-like growth factor binding protein-2; PEG-c-DMA, N-[(methoxy polyethylene glycol 2000 carbamyl)-1,2-dimyristyloxylpropyl-3-amine; pfu, plaque-forming units; siRNA, small interfering RNA.

characterized by their ability to bind IGFs. These binding proteins may work in an IGF-dependent manner either by increasing IGF half-life or inhibiting IGF action (10) or in an IGF-independent manner by acting through integrin receptors (11, 12). In *ob/ob* mice, which have abnormally low IGFBP2 levels, leptin treatment was found to restore IGFBP2 expression in the liver, a major site of IGFBP2 expression in the adult mouse (13), as well as circulating IGFBP2 levels (4). Overexpression of IGFBP2 by adenoviral transfer normalized fasting blood glucose and insulin levels, glucose tolerance, hepatic insulin sensitivity, and hepatic steatosis in *ob/ob* mice (4). In addition, overexpression of IGFBP2 in other models of type 1 and 2 diabetes, including diet-induced obese, *A^{Y/a}* (agouti), and streptozotocin-treated mice, corrected their hyperglycemia and glucose intolerance (4). Therefore, induction of IGFBP2 can improve aberrant glucose homeostasis in many models of diabetes in a manner similar to that of leptin, suggesting that IGFBP2 may be a critical mediator of leptin action on glucose metabolism. However, in these overexpression studies, IGFBP2 levels were induced to ≥ 6000 ng/mL, whereas *ob/ob* levels were ~ 35 ng/mL and wild-type levels were ~ 350 ng/mL (4). Thus far, only the glucoregulatory effects supraphysiological levels of IGFBP2 have been reported, and the metabolism-normalizing function of IGFBP2 may not reflect the physiological role of IGFBP2 in response to leptin.

Although pharmacological levels of IGFBP2 evidently have therapeutic value, it is currently unclear what role IGFBP2 plays in maintaining normal metabolic physiology. Characterization of IGFBP2^{-/-} mice revealed only a subtle metabolic phenotype (14–16). IGFBP2^{-/-} mice are neither glucose intolerant nor insulin resistant as measured by glucose tolerance and insulin tolerance tests (14). IGFBP2^{-/-} mice also display no difference in the rate of body weight gain or absolute body weight at 8 weeks of age compared with wild-type littermates (14). However, at 16 weeks of age, male IGFBP2^{-/-} mice were mildly heavier than wild-type mice due to greater lipid mass (14). The lack of a profound phenotype observed in IGFBP2^{-/-} mice may be due to compensation by other IGFBPs, which are up-regulated in IGFBP2^{-/-} mice (14, 15). In contrast, some studies investigating the effect of modest overexpression of IGFBP2 support a protective role of IGFBP2 in glucose metabolism. For example, female transgenic mice overexpressing human IGFBP2 under its native promoter have 2.2-fold higher circulating IGFBP2 and do not have reduced fasting glucose or insulin levels but are protected from age- and diet-induced obesity and insulin resistance (17). Taken together, these studies suggest that lower than normal IGFBP2 levels may be permissive for obesity de-

velopment and that an increase in IGFBP2 levels may prevent obesity, but these effects are modest.

In this study, our aim was to further investigate the role of IGFBP2 as a mediator of leptin function on glucose metabolism. We first assessed whether IGFBP2 alone is sufficient to mimic the actions of leptin when expressed at levels comparable to those achieved in response to leptin treatment. We then sought to observe whether IGFBP2 is necessary for normal metabolism via acute small interfering RNA (siRNA) knockdown of IGFBP2 in wild-type mice. Finally, we assessed whether IGFBP2 is required for leptin-mediated normalization of metabolism in *ob/ob* mice. Our results support the notion that physiological levels of IGFBP2 are neither sufficient nor required for the action of leptin on glucose homeostasis.

Materials and Methods

Animals

Male *ob/ob* (stock 000632) mice, wild-type colony controls, and C57BL/6 mice (stock 000664) were obtained from The Jackson Laboratory and acclimatized on arrival for at least 1 week. Mice were housed on a 12:12-hour light-dark cycle with ad libitum access to food (no. 2918; Harlan Laboratories) and water. All experiments were approved by the University of British Columbia Animal Care Committee and performed in accordance with the Canadian Council on Animal Care guidelines.

Overexpression of IGFBP2 by adenoviral transfer

To generate the adenovirus (Ad)-IGFBP2 construct, the mIGFBP2 open-reading frame was removed from pCMV SPORT6 mIGFBP2 (Open Biosystems) with EcoRI and NotI, and subcloned into pShuttle (Clontech) downstream of the cytomegalovirus (CMV) promoter and a heterologous intron (rabbit β globin intron 2). The transgene was then excised with I-CeuI and PI-SceI and ligated into pAdeno-X (Clontech). The adenovirus was generated and propagated by transfecting HEK293 cells with ligated construct, and culture supernatant was sent to ViraQuest Inc for further expansion and purification by a CsCl gradient. The Ad- β -galactosidase (gal) adenovirus expressing lac-Z under the CMV promoter was generated as described previously (18).

To investigate the effect of IGFBP2 expression in vivo, 9-week-old male *ob/ob* mice were injected iv via the tail vein with either 10^8 plaque-forming units (pfu) (low dose) or 10^9 pfu (high dose) of Ad-IGFBP2 or Ad- β -gal.

Administration of leptin by mini-osmotic pump implantation

Nine-week-old male *ob/ob* mice were given a dose of either 0.8 or 5 μ g/d of recombinant mouse leptin (PeproTech) prepared in sterile water and administered via Alzet 1004 mini-osmotic pumps (DURECT Corporation). Water (vehicle)-filled pumps were used as controls. Pumps were incubated at 37°C in sterile saline for 48 hours before subcutaneous implantation.

Preparation of lipid nanoparticle siRNA systems and in vivo delivery

IGFBP2 siRNAs were purchased as Stealth RNAi siRNA (Invitrogen). The sequences are as follows: IGFBP2 siRNA 1, sense 5'-GCCAUCUCUUCUACAACGAGCAGCA-3' and antisense 5'-UGCUGCUCGUUGAGAAGAGAUGGC-3'; IGFBP2 siRNA 2, sense 5'-GCAGUGCAAGAUGUCUCUGAACGGA-3' and antisense 5'-UCCGUUCAGACAUUCGACACUGC-3'; and IGFBP2 siRNA 3, sense 5'-CCCACAGCAGGUUGCAGACAGUGAU-3' and antisense 5'-AUCACUGUCUGCAACCUGCUGUGGG-3'. Factor VII (FVII) siRNA was used as a control and was purchased from Integrated DNA Technologies. The sequence is as follows: FVII siRNA, sense 5'-GGAucAucucAAGucuuAct*T-3' and antisense 5'-GuAAGAcuuGAGAuGaucT*T-3'. Lowercase letters indicate 2'-fluoro modifications, and the asterisks indicate phosphothioate linkages.

The lipids, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol, were obtained from Avanti Polar Lipids. The ionizable lipid 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA) and *N*-[(methoxy polyethylene glycol 2000 carbamyl)-1,2-dimyristyloxylpropyl]-3-amine (PEG-c-DMA) were purchased from AICana Technologies.

Lipid nanoparticle siRNA systems were synthesized by rapid mixing of lipid components with siRNA using a microfluidic micromixer as described previously (19). In brief, a lipid mixture in ethanol composed of DLin-KC2-DMA/DSPC/cholesterol/PEG-c-DMA at a molar ratio of 50:10:39:1 was rapidly combined with 3 volumes of siRNA dissolved in 25 mM acetate (pH 4.0) in a microfluidic micromixer provided by Precision Nanosystems with a combined final flow rate of 4 mL/min. The siRNA to lipid ratio was maintained at 0.06 (wt/wt). Ethanol was removed by 4-hour dialysis of lipid nanoparticle formulations in 50 mM 2-(*N*-morpholino)ethanesulfonic acid-sodium citrate buffer (pH 6.7) followed by overnight dialysis in 1× PBS (pH 7.4). All lipid nanoparticles have mean diameters of ~60 nm in number mode as measured by a Zetasizer Nano ZS. The siRNA concentration was determined by measuring absorbance at 260 nm, and the lipid concentration was measured by using the Cholesterol E assay.

For in vivo delivery, 9-week-old male *ob/ob* mice or 13-week-old male C57BL/6 mice were injected (via the tail vein) with 5 mg/kg lipid nanoparticle encapsulating 1 of 3 IGFBP2 siRNAs or FVII siRNA. Empty particles and PBS were also used as controls.

Plasma analyte measurements

Body weight, blood glucose, plasma insulin, and plasma IGFBP2 were measured after a 4-hour fast. Blood glucose was measured using a OneTouch Ultra Glucometer (LifeScan) via the saphenous vein. Plasma insulin levels were measured using an Ultrasensitive Mouse Insulin ELISA (ALPCO). Plasma IGFBP2 levels were measured using a Mouse/Rat IGFBP-2 ELISA (ALPCO). Plasma leptin levels were measured using a Mouse Leptin ELISA (Crystal Chem).

Oral glucose tolerance and insulin tolerance tests

Mice were fasted for 4 hours and given either an oral gavage of 1.5 g/kg 30% dextrose (Fisher Scientific) or an ip injection of 1 U/kg insulin (Novolin ge Toronto; Novo Nordisk). Blood glucose was sampled from the saphenous vein and measured using a OneTouch Ultra Glucometer (LifeScan). During the Ad-

IGFBP2 study, some glucometer readings for *ob/ob* mice were out of range (>33.3 mM); these readings were assigned a value of 33.3 mM, and statistical analysis was not performed. During the oral glucose tolerance test in the siRNA + leptin study, samples that fell over the limit of detection were diluted with a non-diabetic blood sample of known glucose concentration and re-assayed, and original blood glucose levels were calculated.

RT-quantitative PCR analysis

Liver was collected after a 4-hour fast and stored in RNAlater (QIAGEN) overnight at 4°C. Then the RNAlater was removed and tissues were stored at -80°C. To extract RNA, liver was homogenized using a Tissue Tearor in TRI reagent according to the manufacturer's instructions (Ambion). Next, RNA was DNase treated (New England Biolabs), and cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad). *B2m* was used as a reference gene that was selected from 6 potential reference genes based on stability between experimental groups assessed by geNorm software (20) and PCR efficiency. The SYBR Green method was used with SsoFast EvaGreen Supermix with Low ROX (Bio-Rad), and relative transcript levels were calculated using the Pfaffl method (21). The primer sequences used are as follows: IGFBP1, forward 5'-GATCGCCGACCTCAAGAAATG-3' and reverse 5'-CCTCTAGTCTCCAGAGACCCAG-3'; IGFBP2, forward 5'-ACCCCTTGCCAGCAGGAGT-TGGA-3' and reverse 5'-TCCCTGGATGGGCTTCCCAGGT-3'; IGFBP3, forward 5'-GACAGAATACGGTCCCTGCC-3' and reverse 5'-GGAGCATCTACTGGCTCTGC-3'; IGFBP4, forward 5'-CCACCCCAAACAGTGTACC-3' and reverse 5'-CTCAGACTCCAAGCCAGGTC-3'; IGFBP5, forward 5'-CGACTGTTGTCATTTGCCAGC-3' and reverse 5'-CTTTGTGTTGCTCCATGTTCCG-3'; and IGFBP6, forward 5'-GGGATGCAGACTGGTTGTCG-3' and reverse 5'-CCTCC TTGGGGTTTGCTCTC-3'.

Statistics

Data are presented as means ± SEM and were analyzed using either a one-way or two-way ANOVA with a Bonferroni post hoc test using Prism 6.01 (Graph Pad Software). Significance was set at a value of $P < .05$.

Results

Induction of IGFBP2 to levels similar to those after leptin treatment does not mimic the actions of leptin in *ob/ob* mice

We first sought to overexpress IGFBP2 in *ob/ob* mice without leptin treatment, aiming to achieve plasma IGFBP2 levels similar those induced by low-dose, metabolically significant leptin treatment in these mice. To serve as a reference point, *ob/ob* mice were treated with a low dose (0.8 μg/d) of leptin via a mini-osmotic pump. We and others have shown that because *ob/ob* mice are very sensitive to leptin, they do not require the levels of leptin found in wild-type mice to reduce body weight and plasma insulin levels (3, 4, 9, 22, 23). This dose of leptin increased

plasma leptin levels from undetectable (<0.2 ng/mL) in the vehicle group to 0.8 ± 0.2 ng/mL in the leptin-treated group, which was subphysiological compared with those for wild-type controls, which had levels of 5.7 ± 1.0 ng/mL (Figure 1A). IGFBP2 delivery was achieved by administration of either a low dose (10^8 pfu) or high dose (10^9 pfu) of an adenovirus expressing IGFBP2 (Ad-IGFBP2). A control adenovirus expressing β -galactosidase (Ad- β -gal) was administered to a separate group of *ob/ob* mice. As expected, before leptin treatment, IGFBP2 levels were lower in *ob/ob* mice, averaging ~ 35 ng/mL compared with ~ 325 ng/mL in wild-type mice (Figure 1B). However, plasma IGFBP2 levels in the leptin-treated group were significantly higher than those in the vehicle group from day 5 posttreatment onward ($P < .05$) and by 15 days after leptin treatment had increased to 173 ± 8 ng/mL (Figure 1B). Importantly, the low-dose Ad-IGFBP2 treatment induced plasma IGFBP2 levels at a rate and extent similar to those for leptin treatment (Figure 1B). IGFBP2 levels were significantly higher in the low-dose Ad-IGFBP2 group than in the low-dose Ad- β -gal control group from day 5 onward, and by day 15 posttreatment had increased to 202 ± 60 ng/mL ($P < .05$). In contrast, a high dose of Ad-IGFBP2 increased plasma IGFBP2 levels even further, to $33\,046 \pm 5082$ ng/mL by day 2 ($P < .05$ vs high dose Ad- β -gal) and remained at supraphysiologi-

cal levels throughout the experiment (4080 ± 294 ng/mL on day 15) (Figure 1B). These plasma IGFBP2 levels are similar to those achieved by Hedbacker et al (4), who reported IGFBP2 levels >6000 ng/mL 1 week after administration of IGFBP2 adenovirus. Given the induction of similar IGFBP2 levels in the leptin-treated and the low-dose Ad-IGFBP2 groups, we used these 2 groups of animals to further investigate the physiological contribution of IGFBP2 to the glucoregulatory actions of leptin.

We next sought to determine whether IGFBP2 alone could mimic the body weight-reducing effects of leptin treatment in *ob/ob* mice. Before treatment, obese *ob/ob* mice weighed ~ 44 g, whereas wild-type controls weighed ~ 24 g. As expected, leptin treatment attenuated weight gain, such that body weight was significantly lower than that of vehicle-treated controls by 4% on day 2 and by 13% by day 15 ($P < .05$) (Figure 1C). Although the low Ad-IGFBP2-treated mice had plasma IGFBP2 levels similar to those of leptin-treated mice, their body weights did not differ from those of the low Ad- β -gal group (Figure 1C). High-dose Ad-IGFBP2 treatment did reduce body weight compared with the high-dose Ad- β -gal control (by 6% at day 15; $P < .05$) (Figure 1C), although weight loss occurred more slowly and to a lesser degree than in the leptin-treated group. These data suggest that although high levels of IGFBP2 can induce weight loss in *ob/ob* mice, IGFBP2 at physiological levels does not induce weight loss in *ob/ob* mice.

To ascertain the effect of IGFBP2 induction on glucose homeostasis, we measured 4-hour fasted blood glucose and plasma insulin. At baseline, *ob/ob* mice were not overtly hyperglycemic, and blood glucose levels did not change in response to leptin therapy or treatment with Ad-IGFBP2 or control adenovirus (Figure 1D). However, despite fasting euglycemia, *ob/ob* mice were hyperinsulinemic compared with wild-type controls (~ 10 ng/mL vs ~ 1 ng/mL insulin before treatment) (Figure 1E). As expected, by day 2 leptin-treated mice had lower insulin levels than vehicle-treated controls (3.2 ± 0.4 ng/mL vs 11.9 ± 1.8 ng/mL, $P < .05$), which was maintained until day 12 (3.6 ± 0.6 ng/mL vs 11.3 ± 2.2 ng/mL, $P < .05$) (Figure 1E). No difference in insulin levels was observed between the low Ad-

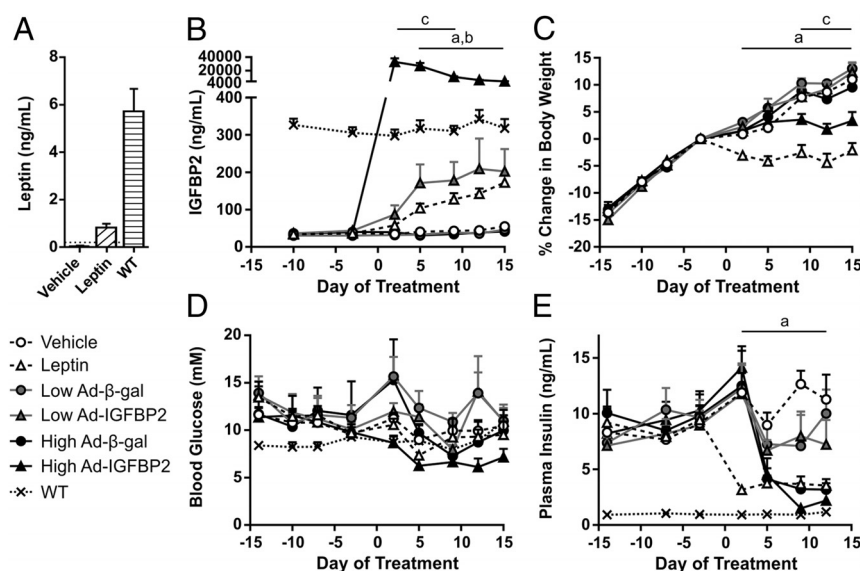


Figure 1. Low-dose Ad-IGFBP2 treatment does not mimic the body weight and plasma insulin-reducing effects of leptin. Nine-week-old male *ob/ob* mice were treated (day 0) with either 0.8 μ g/d leptin or vehicle as a control via mini-osmotic pumps or either low- or high-dose Ad-IGFBP2 or Ad- β -gal as a control. Wild-type (WT) mice not receiving any treatment served as a reference point. Plasma leptin levels were measured on day 12 after a 4-hour fast (A). Plasma IGFBP2 (B), body weight (C) (expressed as percent change over baseline), blood glucose (D), and plasma insulin (E) were measured after a 4-hour fast. Statistical analyses were performed using two-way ANOVA multiple comparisons with Bonferroni post hoc testing. The following groups were compared: a, $P < .05$ leptin ($n = 6$) vs vehicle ($n = 5$); b, $P < .05$ low Ad-IGFBP2 ($n = 4$) vs low Ad- β -gal ($n = 6$); and c, $P < .05$ high Ad-IGFBP2 ($n = 6$) vs high Ad- β -gal ($n = 5$). Data are means \pm SEM; $n = 6$ for wild-type controls.

IGFBP2 and low Ad- β -gal groups or between the high Ad-IGFBP2 and high Ad- β -gal groups (Figure 1E). We did observe a decrease in insulin levels in the high Ad-IGFBP2 group between day 2 and day 5 (from 14.2 ± 1.9 to 4.5 ± 1.5 ng/mL), but we attribute this to nonspecific virus effects because the high Ad- β -gal control group displayed the same decrease (from 12.5 ± 3.2 ng/mL at day 2 to 4.1 ± 1.0 ng/mL at day 5) (Figure 1E). Therefore, although exogenous leptin treatment and Ad-IGFBP2 treatment induced similar plasma IGFBP2 levels, treatment with leptin but not Ad-IGFBP2 reduced plasma insulin. These results indicate that IGFBP2 alone is not sufficient to mimic the insulin-lowering effects of leptin in *ob/ob* mice.

We next assessed the effect of IGFBP2 expression on glucose tolerance (Figure 2, A and B). Mice treated with low-dose leptin had lower and earlier peak blood glucose levels than vehicle-treated mice, all of which had glucose levels exceeding the maximum detection limit (33.3 mM) of the glucometer (26.5 mM at 10 minutes for leptin-treated group vs 33.3 mM at 20 minutes for vehicle-treated animals) (Figure 2A). The areas under the curve (AUCs) were similar between the groups (Figure 2B), although this may in part be due to limitations in our ability to accurately quantify high glucose levels with the glucometer. Mice treated with low-dose Ad-IGFBP2 or Ad- β -gal had glucose levels and glucose excursion comparable to those for the vehicle-treated group, whereas mice treated with high dose Ad-IGFBP2 had markedly improved glucose tolerance compared with that in control virus-treated mice (Figure 2B).

In addition to impaired glucose tolerance, all *ob/ob* groups had notably impaired insulin tolerance compared with wild-type controls (Figure 2C). In response to insulin treatment, blood glucose levels in wild-type mice dropped to 26% of baseline by 30 minutes after injection, whereas none of the groups of *ob/ob* mice exhibited this response. Leptin treatment did not restore normal insulin sensitivity in *ob/ob* mice, although leptin-treated mice did display a trend toward having lower blood glucose levels at 20 and 30 minutes after injection compared with all other *ob/ob* groups, this trend did not reach significance. Insulin tolerance profiles were superimposable between the low and high Ad-IGFBP2 groups compared with their respective controls. Therefore, induction of low levels of IGFBP2 did not improve glucose or insulin tolerance in a manner similar to that for low-dose leptin treatment.

Acute knockdown of IGFBP2 in C57BL/6 mice does not alter glucose homeostasis

We next investigated whether acute knockdown of IGFBP2 in wild-type mice would recapitulate any aspects of the *ob/ob* phenotype. To achieve knockdown of plasma

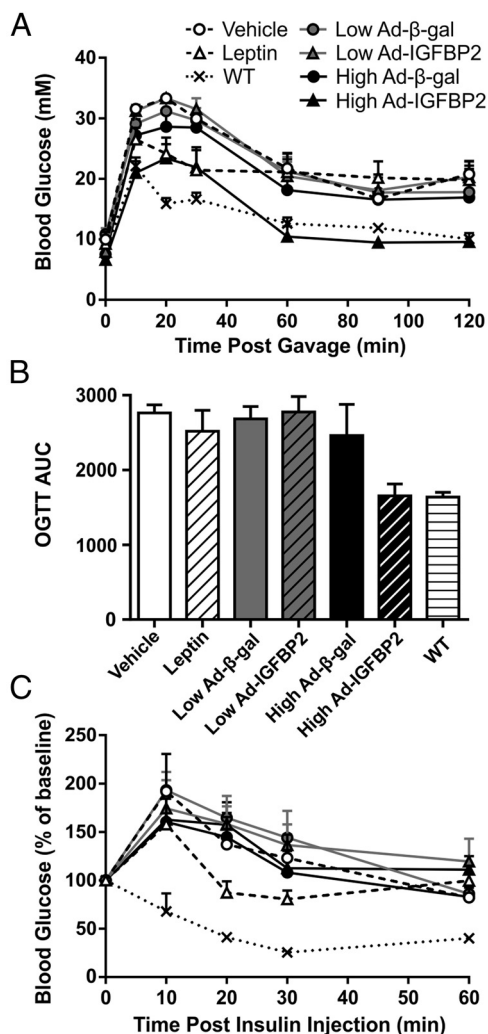


Figure 2. Oral glucose tolerance and insulin tolerance in *ob/ob* mice receiving leptin or Ad-IGFBP2 treatment. Oral glucose tolerance tests (OGTT) and insulin tolerance tests were performed on male *ob/ob* mice receiving either 0.8 μ g/d leptin or vehicle or low-dose (10^8 pfu) or high-dose (10^9 pfu) Ad-IGFBP2 or Ad- β -gal. On day 9 after a 4-hour fast, mice were gavaged with 1.5 g/kg glucose, blood glucose responses were measured (A), and AUC was calculated (B). On day 15 after a 4-hour fast, mice were administered 1 U/kg insulin by ip injection, and blood glucose was measured (C). During the glucose tolerance test, at least 1 sample in every *ob/ob* group fell over the limit of detection and was assigned a value of 33.3 mM; therefore, statistical analysis was not performed in A or B. For the insulin tolerance test, statistical analyses were performed using two-way ANOVA multiple comparisons with Bonferroni post hoc testing. The following groups were compared: leptin ($n = 6$) vs vehicle ($n = 5$); low Ad-IGFBP2 ($n = 4$) vs low Ad- β -gal ($n = 6$); and high Ad-IGFBP2 ($n = 6$) vs high Ad- β -gal ($n = 4$). However, no statistical differences were detected. Data represent means \pm SEM; $n = 6$ for wild-type (WT) controls.

IGFBP2 levels, we assessed the knockdown efficiency of 3 different IGFBP2 siRNAs relative to that of a FVII siRNA control. We selected lipid nanoparticles as a vehicle for IGFBP2 siRNA delivery, because lipid nanoparticles efficiently target siRNA to the liver in vivo (24, 25), and the liver is suspected to be a major source of circulating

IGFBP2 (4, 13). siRNAs were introduced into wild-type mice via tail vein injection at a dose of 5 mg/kg. IGFBP2 siRNA caused a rapid and dramatic reduction in circulating IGFBP2 levels (Figure 3A). By day 1 postinjection IGFBP2 levels were decreased by at least 92% from pretreatment levels (~ 350 ng/mL) in all IGFBP2 siRNA groups. We also observed an acute decrease in IGFBP2 levels in the FVII control group on day 1, although IGFBP2 returned to pretreatment levels by day 10. We attribute this observation to the high dosage of lipid nanoparticles because empty particles produced the same effect (Supplemental Figure 1 published on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>). Significant knockdown of IGFBP2 levels was thus achieved from day 3 to days 20, 24, or 29 in mice treated with IGFBP2 siRNA 1, 2, or 3, respectively, compared with that in the FVII control group ($P < .05$) (Figure 3A). Thus, we concluded that the IGFBP2 siRNA approach was successful in reducing plasma IGFBP2.

Our untreated *ob/ob* mice had a range of plasma IGFBP2 levels from 19 to 76 ng/mL, compared with ~ 350 ng/mL in wild-type mice. Using the *ob/ob* mouse as a model, we defined an IGFBP2 level of < 75 ng/mL as a state of insufficiency. Therefore, IGFBP2 siRNA 1, 2, and 3 produced IGFBP2 insufficiency for 3, 6, and 13 days, respectively. Despite substantially reducing plasma IGFBP2 levels over this time, body weight and glucose homeostasis were not altered by IGFBP2 knockdown in any of the 9

mice treated with IGFBP2 siRNA. The weight of mice treated with IGFBP2 siRNA was indistinguishable from that of control mice over the course of the experiment (Figure 3B). Likewise, 4-hour fasted blood glucose values were unchanged between the groups regardless of plasma IGFBP2 levels (Figure 3C). Maintenance of glucose homeostasis was not a result of elevated plasma insulin levels, because no statistically significant differences in 4-hour fasted plasma insulin levels were observed among any of the IGFBP2 knockdown mice compared with those in controls (Figure 3D). These results suggest that physiological levels of plasma IGFBP2 are not essential for maintenance of normal fasted body weight, blood glucose, or insulin levels.

IGFBP2 does not contribute to leptin-mediated improvements in body weight, blood glucose, and plasma insulin of *ob/ob* mice

We next investigated whether plasma IGFBP2 is necessary for the metabolic actions of leptin by treating *ob/ob* mice with leptin while knocking down IGFBP2. Mice were injected with IGFBP2 siRNA 3 or FVII siRNA 1 day before implantation of mini-osmotic pumps delivering either 5 μ g/d leptin or vehicle. Both leptin-treated groups had elevations in plasma leptin levels from undetectable amounts to levels similar to those of wild-type mice on day 11 (Figure 4A). Upon FVII siRNA injection and leptin treatment, plasma IGFBP2 levels increased linearly and eventually surpassed wild-type levels, reaching 691 ± 62 ng/mL by day 11 (Figure 4C). In contrast, *ob/ob* mice treated with IGFBP2 siRNA were unable to mount this response to leptin treatment and maintained lower IGFBP2 levels compared with those of controls throughout the study ($P < .05$ vs FVII siRNA from day 5 onward) (Figure 4C). On day 11, IGFBP2 levels in leptin-treated, IGFBP2 knockdown mice were only slightly elevated compared with those of vehicle-treated controls (142 ± 23 ng/mL compared with 60 ± 3 ng/mL; $P < .05$) (Figure 4C). This result was consistent with hepatic transcript levels as the FVII siRNA group displayed a 17 ± 3 -fold increase in IGFBP2 mRNA levels over vehicle, and this increase was blunted in the IGFBP2 siRNA group, which experienced only a 3 ± 1 -fold increase over that with vehicle ($P < 0.05$) (Figure 4B). Therefore, we successfully reduced the leptin-mediated increase in IGFBP2.

Despite blocking an increase in plasma IGFBP2, leptin was able to reduce body weight and improve glucose metabolism. By day 11 of leptin treatment, body weight had decreased by $\sim 17\%$ in both the IGFBP2 and FVII siRNA groups (Figure 4D). Upon leptin treatment, both IGFBP2 and control knockdown groups experienced a similarly modest decrease in fasting blood glucose, which were sta-

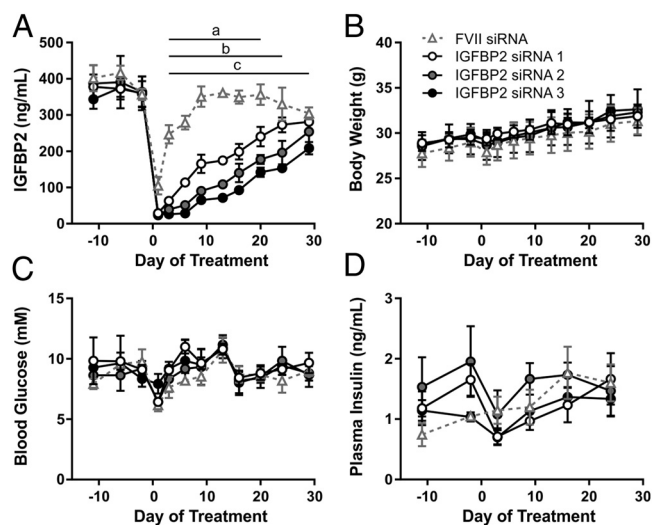


Figure 3. Acute knockdown of IGFBP2 does not affect body weight, blood glucose, or plasma insulin. Thirteen-week-old male C57BL/6 mice were treated with 5 mg/kg lipid nanoparticles encapsulating 1 of 3 IGFBP2 siRNAs or FVII siRNA by tail-vein injection on day 0. Plasma IGFBP2 (A), body weight (B), blood glucose (C), and plasma insulin (D) were measured after a 4-hour fast. Statistical analyses were performed using two-way ANOVA multiple comparisons with Bonferroni post hoc testing. The following groups were compared: a, $P < .05$ FVII vs IGFBP2 siRNA 1; b, $P < .05$ FVII vs IGFBP2 siRNA 2; and c, $P < .05$ FVII vs IGFBP2 siRNA 3. Data are means \pm SEM; n = 3 per group.

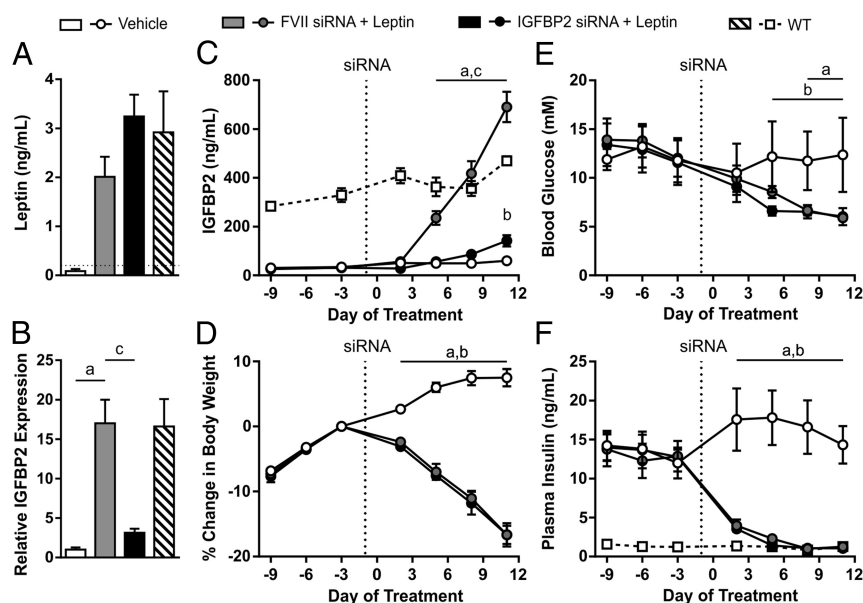


Figure 4. Prevention of the IGFBP2 response to leptin does not block leptin-mediated effects on body weight, blood glucose, or plasma insulin. On day -1 , 9-week-old male *ob/ob* mice were treated with 5 mg/kg lipid nanoparticles encapsulating either FVII siRNA or IGFBP2 siRNA 3 by tail-vein injection (the vehicle group did not receive siRNA). On day 0, mice were implanted with mini-osmotic pumps delivering 5 μ g/d leptin or vehicle. Wild-type (WT) mice did not receive either treatment. Plasma leptin levels (A) and IGFBP2 transcript levels (B) were measured on day 11 after a 4-hour fast. Plasma IGFBP2 (C), body weight (D) (expressed as percent change over baseline), blood glucose (E), and plasma insulin (F) were measured after a 4-hour fast. Statistical analyses were performed using one-way (A and B) or two-way (C–F) ANOVA multiple comparisons with Bonferroni post hoc testing. The following groups were compared: a, $P < .05$ vehicle vs FVII siRNA; b, $P < .05$ vehicle vs IGFBP2 siRNA; and c, $P < .05$ FVII siRNA vs IGFBP2 siRNA. Data are means \pm SEM; $n = 6$ per *ob/ob* group, and 4 for wild-type controls.

tistically lower than that for vehicle-treated controls on day 11 (12 ± 3.8 , 6.0 ± 0.9 , and 5.9 ± 0.3 mM for vehicle, IGFBP2 siRNA, and FVII siRNA respectively; $P < .05$) (Figure 4E). In a similar manner, leptin treatment normalized fasting plasma insulin levels in *ob/ob* mice irrespective of whether IGFBP2 was knocked down. Within 2 days of leptin administration, plasma insulin levels in both IGFBP2 and FVII siRNA groups were significantly lower than those of the vehicle-treated *ob/ob* mice and had reached near-wild-type levels ($P < .05$) (Figure 4F), well before plasma IGFBP2 levels had been restored to wild-type levels (Figure 4C). Therefore, leptin treatment of *ob/ob* mice decreased body weight, fasting blood glucose and fasting plasma insulin to the same extent irrespective of whether IGFBP2 levels had been knocked down.

Finally, we assessed glucose tolerance in *ob/ob* mice in response to leptin treatment with or without IGFBP2 knockdown (Figure 5). As expected, leptin treatment improved glucose tolerance. Whereas vehicle-treated *ob/ob* mice had impaired glucose tolerance compared with that of wild-type mice, leptin-treated FVII knockdown *ob/ob* mice showed a glucose excursion that was more similar to that of wild-type controls than vehicle-treated *ob/ob* mice, with significantly lower glucose levels than those for ve-

hicle from 60 minutes onward (Figure 5A), although the trend toward a lower integrated AUC did not reach statistical significance ($P = .11$ for FVII siRNA vs vehicle) (Figure 5B). Similarly, the mice given leptin in conjunction with IGFBP2 siRNA had a trend toward improved glucose tolerance over that for the vehicle-treated *ob/ob* mice, although neither individual glucose measurements nor integrated AUC reached statistical significance (Figure 5).

Mice with a lifelong ablation of IGFBP2 display 2- to 4-fold increases in hepatic IGFBP-1, -3, -4, -5, and -6 mRNA levels, and it has been suggested that the lack of metabolic phenotypes in these mice may be due to this compensation by other IGFBPs (14). Therefore, we measured other IGFBP transcript levels in the liver of our acute IGFBP2 knockdown model (Figure 6). We did not find any statistical differences between the *ob/ob* groups, indicating that up-regulation of other IGFBPs does not occur in this model (Figure 6).

Discussion

The ability of leptin to improve various metabolic abnormalities in models of leptin deficiency (3, 7), lipodystrophy (8), and type 1 diabetes (26, 27) has generated interest in understanding the mechanism of these effects. Hedbacker et al (4) uncovered IGFBP2 as a leptin-regulated gene and determined that vast overexpression of IGFBP2 alone using adenoviral transfer can induce effects similar to those of leptin. However, the supraphysiological plasma levels of IGFBP2 induced in these studies may not reflect physiological actions of IGFBP2, and the reported effects could have been a result of pharmacological doses. Therefore, we sought to delineate the physiological role of IGFBP2 in glucose metabolism using 3 complementary approaches. First, we assessed whether physiological levels of IGFBP2 were sufficient to recapitulate the glucose-normalizing effects of leptin in *ob/ob* mice. Using an adenoviral vector, we induced IGFBP2 expression in *ob/ob* mice to obtain plasma levels of IGFBP2 similar to those induced by leptin treatment. Second, we assessed whether IGFBP2 is essential for glucose homeostasis in wild-type mice by siRNA-

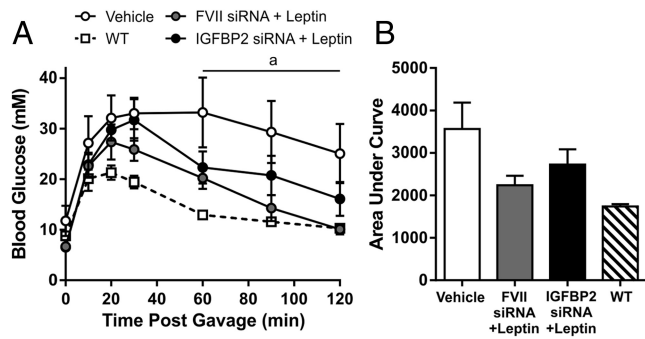


Figure 5. Oral glucose tolerance in *ob/ob* mice receiving leptin and either IGFBP2 or FVII siRNA. Nine-week-old male *ob/ob* mice received 5 mg/kg lipid nanoparticles encapsulating containing either FVII or IGFBP2 siRNA on day -1 followed by 5 μ g/d leptin (or vehicle as a control in mice that did not receive siRNA) on day 0. On day 8 posttreatment, mice were gavaged with 1.5 g/kg glucose after a 4-hour fast, blood glucose levels were monitored (A) and AUC was calculated (B). For mice that had blood glucose values greater than the limit of detection of 33.3 mM, a sample of blood was mixed with an equal volume sample of nondiabetic blood and measured again to assign a new calculated value. Statistical analyses were performed using two-way (A) or one-way ANOVA (B) multiple comparisons with Bonferroni post hoc testing. For A: a, $P < .05$ vehicle vs FVII siRNA; no significant differences were observed between vehicle vs IGFBP2 siRNA nor FVII siRNA vs IGFBP2 siRNA. No statistical differences were observed in B. Data represent means \pm SEM; $n = 6$ per *ob/ob* group and 4 for wild-type (WT) controls.

mediated IGFBP2 knockdown. Finally, we investigated whether IGFBP2 is required for the therapeutic effects of leptin treatment in *ob/ob* mice by blocking leptin-mediated increases in IGFBP2 using siRNA. Collectively, these studies reveal that physiological levels of IGFBP2 are neither necessary nor sufficient for normal glucose homeostasis in the fasting state or for the therapeutic actions of leptin replacement therapy.

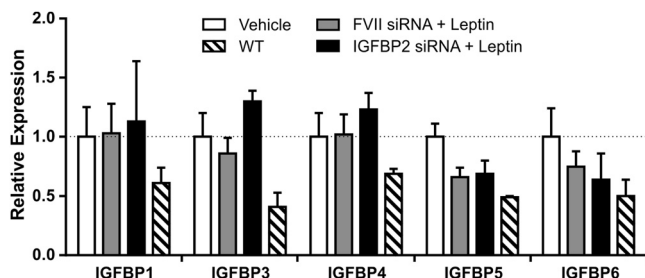


Figure 6. Hepatic IGFBP-1, -3, -4, -5, and -6 mRNA levels do not change in *ob/ob* mice receiving leptin and either IGFBP2 or FVII siRNA. Nine-week-old male *ob/ob* mice received 5 mg/kg lipid nanoparticles encapsulating either FVII or IGFBP2 siRNA on day -1 followed by 5 μ g/d leptin (or vehicle as a control in mice that did not receive siRNA) on day 0. Liver samples were collected on day 11 and IGFBP-1, -3, -4, -5, and -6 mRNA levels were measured. Statistical analyses were performed using one-way ANOVA multiple comparisons with Bonferroni post hoc testing. The groups compared were vehicle vs FVII siRNA, vehicle vs IGFBP2 siRNA, and FVII siRNA vs IGFBP2 siRNA; however, no statistical differences were detected. Data represent means \pm SEM; $n = 6$ per *ob/ob* group and 4 for wild-type (WT) controls.

In contrast to physiological levels of IGFBP2, treatment with supraphysiological levels of IGFBP2 affects some aspects of glucose metabolism. The results from our high-dose Ad-IGFBP2 study are largely in agreement with those of Hedbacker et al (4): in our study high-dose Ad-IGFBP2 treatment induced a reduction in body weight compared to controls by day 9 of overexpression, similar to Hedbacker et al (4), who found that significant weight loss was not achieved until 1 week after IGFBP2 overexpression. It is noteworthy that weight loss was delayed and diminished in magnitude in Ad-IGFBP2-treated mice compared with leptin-treated mice, which had lost significant weight by day 2. This suggests that the mechanism of weight loss induced by leptin and IGFBP2 in *ob/ob* mice may be at least partially distinct. The high-dose Ad-IGFBP2 treatment of *ob/ob* mice also decreased fasted plasma insulin levels; however, an identical decrease was observed in the high-dose Ad- β -gal control group, suggesting a nonspecific adenovirus effect on glucose homeostasis as has been reported previously (28) or related to protein overexpression in the liver. Hedbacker et al (4) did not report virus-induced hypoinsulinemia; this difference compared with the current results may be related to our use of a control virus using a construct expressing lac-Z under the CMV promoter instead of an empty virus containing only the CMV promoter. Interestingly the high Ad-IGFBP2 treatment improved glucose tolerance compared with that for control animals, similar to the Hedbacker et al (4) report, although no differences in whole-body insulin tolerance were observed. This finding suggests that in the fed state pharmacological levels of IGFBP2 may modulate glucose handling and is consistent with a previous report demonstrating that transgenic mice expressing human IGFBP2 at levels which increased total circulating IGFBP2 levels by 2.2-fold, were protected from age- and diet-induced obesity and glucose intolerance (17). Therefore, our data add additional support to the notion that pharmacological doses of IGFBP2 may influence glucose metabolism.

The phenotype we observed in mice with siRNA-mediated knockdown of IGFBP2 is consistent with that observed after chronic ablation of IGFBP2 in wild-type mice. Male IGFBP2^{-/-} mice are neither glucose intolerant nor insulin resistant, although they display a mild increase in body fat by 16 weeks of age (14). This lack of a substantial metabolic phenotype may be due to compensation through up-regulation of other IGFBPs (14, 15). Therefore, we aimed to acutely knock down IGFBP2 in wild-type mice to minimize the opportunity for compensation due to lifelong gene ablation. We observed that body weight, fasting blood glucose, and fasting plasma insulin were unchanged after rapid and efficient knockdown of plasma IGFBP2. We anticipated that if IGFBP2 were vital

for physiological leptin action in wild-type mice, we would have observed changes in metabolic control with acute knockdown of IGFBP2. Indeed, acute inhibition of leptin action in wild-type mice via polyethylene glycol (PEG)-ylated mouse leptin antagonist increased body weight (29), plasma insulin, and glucose-stimulated insulin secretion and impaired whole-body insulin sensitivity (30) only after a few days of treatment. Therefore, our study suggests that IGFBP2 is not required for maintenance of glucose homeostasis. In addition to investigating the requirement of IGFBP2 in normal mice, we found that IGFBP2 is not necessary for normalization of metabolism by leptin therapy in *ob/ob* mice. Therefore, we conclude that IGFBP2 is not required either for proper metabolic control or for the gluoregulatory action of leptin.

Our studies and other reports do not support a substantial role for IGFBP2 in maintaining glucose homeostasis, and the function of IGFBP2 remains largely unclear. *IGFBP2*^{-/-} mice do not have a major metabolic phenotype, although they have a ~25% decrease in spleen weight and a ~15% increase in liver weight (16), as well as alterations in bone turnover and architecture (14). Moreover, transgenic mice overexpressing mouse IGFBP2 under the CMV promoter, driving a 3-fold increase in circulating IGFBP2 levels, had only a mild reduction in postnatal body weight gain that was attributed to changes in carcass and organ weights rather than fat mass reductions (31). Among the most compelling studies connecting IGFBP2 to glucose regulation is a report that mice expressing human IGFBP2 under its native promoter are protected from age- and diet-related increases in adiposity and glucose intolerance (17). It has been speculated that these effects of IGFBP2 might arise from its inhibition of IGF-1, which affects organ growth and adipocyte differentiation. Therefore, these studies illustrate that many other perturbations are observed when manipulating IGFBP2 levels that may be entirely independent of leptin action. Although leptin clearly regulates IGFBP2 levels, IGFBP2 does not appear to be a critical mediator of leptin action on glucose and body weight regulation. Nevertheless, IGFBP2 may be physiologically important for axes of leptin action beyond glucose regulation, a possibility that will require further investigation.

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