Leptin Deficiency Causes Insulin Resistance Induced by Uncontrolled Diabetes

Running title: Leptin deficiency and insulin resistance

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**Objective**- Depletion of body fat stores during uncontrolled, insulin-deficient diabetes (uDM) results in markedly reduced plasma leptin levels. This study investigated the role of leptin deficiency in the genesis of severe insulin resistance and related metabolic and neuroendocrine derangements induced by uDM.

**Research design and methods**- Adult male Wistar rats remained non-diabetic or were injected with the β-cell toxin, streptozotocin (STZ) to induce uDM and subsequently underwent subcutaneous implantation of an osmotic minipump containing either vehicle or leptin at a dose (150μg/kg/d) designed to replace leptin at non-diabetic plasma levels. To control for leptin effects on food intake, another group of STZ-injected animals were pair-fed to the intake of those receiving leptin. Food intake, body weight and blood glucose levels were measured daily, with body composition and indirect calorimetry performed on Day 11 and an insulin tolerance test to measure insulin sensitivity performed on Day 16. Plasma hormone and substrate levels, hepatic gluconeogenic gene expression and measures of tissue insulin signal transduction were also measured.

**Results**- Physiological leptin replacement prevented insulin resistance in uDM via a mechanism unrelated to changes of food intake or body weight. This effect was associated with reduced total body fat and hepatic triglyceride content, preservation of lean mass, and improved insulin signal transduction via the insulin receptor substrate-phosphatidylinositol-3-OH kinase (IRS-PI3K) pathway in the liver, but not in skeletal muscle or adipose tissue. Although physiological leptin replacement lowered blood glucose levels only slightly, it fully normalized elevated plasma glucagon and corticosterone levels and reversed the increased hepatic expression of gluconeogenic enzymes characteristic of rats with uDM.

**Conclusions**- We conclude that leptin deficiency plays a key role in the pathogenesis of severe insulin resistance and related endocrine disorders in uDM. Treatment of diabetes in humans may benefit from correction of leptin deficiency as well as insulin deficiency.
Recent evidence implicates leptin not only in the regulation of energy balance but in glucose homeostasis as well. In addition to hyperphagia and obesity, insulin resistance is a prominent feature of animal models characterized by reduced leptin signaling (1), and leptin administration improves insulin sensitivity and glucose metabolism in these models (2,3) independently of its effects on energy homeostasis (4). Investigation into the role of leptin in glucose metabolism has focused largely on genetic models of impaired leptin signaling (e.g., leptin-deficient \( \text{ob/ob} \) mice), while other studies have employed pharmacological doses of leptin (1-6). In this study we investigated the physiological role of leptin in glucose metabolism by determining the contribution made by leptin deficiency to the severe insulin resistance and associated metabolic and endocrine dysfunction characteristic of uncontrolled, insulin-deficient diabetes (uDM).

Severe leptin deficiency is a well-documented consequence of uDM that occurs following destruction of insulin-secreting \( \beta \)-cells (7,8). As insulin is required for the synthesis and storage of triglyceride in adipose tissue, weight gain cannot occur in uDM and the associated loss of body fat is accompanied by markedly reduced plasma leptin levels. This effect, in turn, is implicated in the mechanism whereby uDM increases food intake (9), since exogenous leptin administration at doses that prevent a fall in plasma leptin levels also prevent hyperphagia in uDM (8). Another feature of uDM in humans is progressive, severe insulin resistance (10-12), an effect also observed in streptozotocin (STZ)-induced diabetes in rats (13). Although insulin deficiency clearly underlies hyperglycemia and weight loss in uDM, the contribution of markedly reduced plasma leptin levels to insulin resistance and related metabolic and endocrine derangements in this setting remains to be determined. Since plasma levels of leptin as well as insulin are normalized by insulin treatment of STZ-diabetes, at least some of the beneficial effects that have been ascribed to insulin treatment could result from restoring leptin action to normal (7). Indeed, hyperleptinemia generated either by pharmacological administration of leptin (5) or with adenoviral gene therapy (14) ameliorates hyperglycemia and associated increases of plasma glucagon levels in STZ-induced diabetes, despite persistently low insulin levels. These data raise the possibility that deficient endogenous leptin signaling may underlie at least some manifestations of uDM.

Based on these considerations, we sought to determine the extent to which deficiency of endogenous leptin contributes to insulin resistance and related endocrine dysfunction in STZ-induced diabetes. To accomplish this goal, we subcutaneously infused either vehicle or leptin at a dose that prevents leptin deficiency in rats with STZ-induced uDM. We found that physiological leptin replacement prevented the development of insulin resistance in uDM via a mechanism independent of its effects on food intake and body weight. Moreover, this leptin effect was associated with normalization of elevated plasma levels of glucagon and corticosterone, with the reversal of increased hepatic expression of the gluconeogenic genes, glucose-6-phosphatase (\( G6Pase \)) and phosphoenolpyruvate kinase (\( \text{Pepck} \)), and with improved insulin signal transduction via the insulin receptor substrate-phosphatidylinositol-3-OH kinase (\( \text{IRS-PI3K} \)) pathway in the liver, but not in skeletal muscle or adipose tissue. By comparison, physiological leptin replacement only modestly reduced hyperglycemia in STZ-induced diabetic rats and did not alter the potent up-regulation of hepatic \( \text{Igfbp2 mRNA} \).
levels previously reported (15). Taken together, these data suggest that reduced leptin levels contribute to the progressive, severe insulin resistance characteristic of uDM via a mechanism that appears to predominantly involve the liver.

RESEARCH DESIGN AND METHODS

Animals. Adult male Wistar rats (Harlan, Indianapolis, IN) were housed in individual cages under specific-pathogen free (SPF) conditions, maintained in a temperature-controlled room with a 12:12h light:dark cycle and provided with ad libitum access to water and standard laboratory chow (PMI Nutrition International Inc) unless otherwise stated. All procedures were performed in accordance with NIH Guidelines for the Care and Use of Animals and were approved by the Animal Care Committee at the University of Washington.

Effect of physiological leptin replacement during STZ-induced leptin deficiency. To induce uDM, rats received two consecutive daily subcutaneous (sc) injections (40mg/kg) of freshly prepared streptozotocin (STZ; Sigma) dissolved in ice-cold sodium citrate (NaCit, pH 4.5). Subsequently, under isoflurane anesthesia, two groups of diabetic animals (n=7-9/group) received a subcutaneously implanted osmotic minipump (Alzet Model 2ML4; DURECT Corporation) containing either vehicle (STZ-veh) or leptin (STZ-lep) at a dose (150 μg/kg/d) calculated to restore plasma leptin levels to non-diabetic control values. Leptin was obtained from Dr. A.F. Parlow (National Hormone & Peptide Program) and diluted in phosphate buffered saline (PBS) (pH 7.9). A control non-diabetic group (n=7-9/group) received sodium citrate, rather than STZ, followed by implantation of a sc osmotic minipump containing vehicle (PBS). Finally, to control for the effect of leptin to reduce food intake in rats with STZ-diabetes (8), an additional group of diabetic animals receiving vehicle were pair-fed (STZ-veh-PF) to match the intake of STZ-lep-treated animals as previously described (6). Food intake, body weight and blood glucose levels were measured daily during the mid-light cycle for a total of 18 days.

The experimental paradigm described above was subsequently repeated in separate groups of animals, with the exception that an indwelling catheter was inserted into the left carotid artery as previously described (16), prior to STZ-administration to permit blood sampling from conscious, unstressed rats for determination of plasma glucagon, catecholamine and corticosterone levels.

Body composition and indirect calorimetry. Determinations of body lean and fat mass were made on a separate group of animals using Quantitative Magnetic Resonance 11 days following STZ-administration (EchoMRI-700TM; Echo Medical Systems, Houston, TX). Locomotor activity was assessed by the infrared beam breaks using an Opto-Varimetrix-3 sensor system (Columbus Instruments, Columbus, OH). Indirect calorimetry was performed on this same group of animals using a computer-controlled calorimetry system (Oxymax; Columbus Instruments, OH) in habituated animals as previously described (17).

Measurement of insulin sensitivity. To determine the effect of leptin replacement on STZ-induced insulin resistance, animals from each group were subjected to an insulin tolerance test (ITT) on Day 16 following STZ injection. Briefly, following a 3-hr fast mid-light cycle, animals received an injection of insulin (2U/kg i.p.; Humulin R; Lilly), and glucose levels were determined on tail-vein blood samples using a hand-held glucometer (Accu-Chek, Roche) at time zero and at 30 min intervals over 120 min.

Blood and urine collection and assay. Urinary glucose was measured using a GM9D glucose direct analyzer (Analox Instruments, United Kingdom) on urine samples obtained from animals 14 days following STZ-
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adminstration. Tail vein blood samples were collected in chilled EDTA-treated tubes for measurement of plasma insulin and leptin on Days 0, 1, 2, 3, 4, 7 and 16, and non-esterified free fatty acids (NEFA) on Day 16. On Day 16, arterial blood for catecholamine, corticosterone and glucagon assays was collected from conscious, freely moving animals and placed into tubes containing EGTA/glutathione, EDTA and benzamidine (10μl of 1M)/heparin (1U), respectively. Whole blood was centrifuged at 1500 rpm for 20 min, plasma removed, aliquoted and stored at -20°C for subsequent analysis. Plasma immunoreactive insulin and leptin levels were determined by ELISA (Crystal Chem). FFAs were measured using a colorimetric assay kit that relies on fatty acid as substrate for enzymatic acylation of CoA (WAKO Chemicals). Catecholamine levels were measured in duplicate using a sensitive and specific radioenzymatic assay (18). Glucagon was assayed with a glucagon RIA Kit (Linco Research), and plasma corticosterone levels were measured using EIA (Diagnostics Systems Laboratories).

Tissue processing and biochemical analysis. To measure tissue insulin sensitivity, 18 days following STZ injection, animals were fasted 4-h mid-light cycle, injected with either vehicle or insulin (2U/kg i.p.) and sacrificed 15 min later. Liver, skeletal muscle (tibialis anterior), and white adipose tissue (WAT; epididymal depot) were excised and snap-frozen for subsequent analysis.

Tissues were homogenized in T-Per lysis buffer (10μl/mg tissue) (Pierce) supplemented with protease and phosphatase inhibitor cocktails (Roche). Homogenates were centrifuged, pellets discarded and supernatants retained for determination of protein content using a Micro BCA protein assay kit (Pierce) and equal amounts of protein were used for each condition in each assay. Insulin-induced activation of PI3K was assessed in tissues by measuring serine phosphorylation of Akt (residue 473) using an ELISA assay (Invitrogen). Liver tissue triglyceride content was determined using quantitative magnetic resonance with the Echo 3-in-1 MRI machine (Echo Medical Systems).

RT-PCR. Total RNA was extracted from liver and pancreas using TRIzol B (MRC), quantified by spectrophotometry (Nanodrop 1000, Thermo Scientific, IL) and reverse-transcribed (1μg) with AMV reverse transcriptase (Promega). Real-time PCR was then performed on an ABI Prism 7900 HT (Applied Biosystems) using SYBR Green master mix (Applied Biosystems). PCR data were analyzed using the Sequence Detection System software (SDS Version 2.2; Applied Biosystems). Expression levels of each gene were normalized to a house-keeping gene (18S RNA) and expressed as % of veh-veh controls. Non-template controls were incorporated into each PCR run.

Statistical analysis. All results are expressed as mean ± SEM. Statistical analyses were performed using Statistica (Version 7.1; StatSoft, Inc). A one-way analysis of variance with a LSD post-hoc test was used to compare mean values between multiple groups and a two-sample unpaired student’s t-test was used for two-group comparisons. In all instances, probability values of <0.05 were considered significant.

RESULTS

Effect of physiological leptin replacement on glucose and energy homeostasis during STZ-induced diabetes. As expected (8), plasma insulin levels were dramatically reduced by Day 2 in all animals that received STZ relative to non-diabetic controls, and remained very low throughout the duration of the study (Fig. 1A). The effect of STZ on plasma leptin levels closely paralleled its effect on plasma insulin levels, with marked reductions observed by Day 2 in diabetic animals receiving sc vehicle (Fig. 1B). By
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As expected, the reduction of plasma insulin levels in animals made diabetic by STZ caused marked hyperglycemia (Fig. 1C). Although marked hyperglycemia was also observed in STZ-treated animals that received sc leptin, the magnitude of this effect was modestly reduced compared to diabetic animals receiving vehicle (p<0.05), indicating that leptin deficiency exacerbates (but is not a major cause of) hyperglycemia in rats with uDM. This modest glucose-lowering effect of leptin replacement, however, cannot be explained by reduced food intake, since blood glucose was not reduced in STZ-diabetic animals that were pair-fed to the intake of STZ-diabetic rats receiving sc leptin. By comparison, blood glucose levels remained within the normal range in non-diabetic controls.

In diabetic animals receiving sc vehicle, food intake was increased by Day 6 after STZ administration and remained elevated compared to non-diabetic controls (Fig. 1D). In comparison, consistent with previous reports (8), diabetic hyperphagia was prevented by maintaining plasma leptin levels in the physiological range, such that food intake was comparable to that of non-diabetic controls (8) (Fig. 1D).

While body weight gradually increased in non-diabetic rats during the study, it decreased immediately following diabetes onset in STZ-treated rats receiving vehicle, and remained significantly below baseline values despite their pronounced hyperphagia (Fig 2A). STZ-diabetic animals that were pair-fed to the intake of leptin-treated diabetic rats exhibited even greater weight loss than diabetic rats fed ad libitum, owing to their reduced food intake. Interestingly, STZ-induced diabetic rats that received sc leptin lost significantly less weight than STZ-veh-PF animals, maintaining weight comparable to diabetic rats fed ad libitum, despite consuming less food.

To explore the basis for these body weight changes, we subjected a separate cohort of animals to the same protocol, and measured body composition analysis and indirect calorimetry 11 days following STZ-administration. We found that leptin treatment caused a greater loss of body fat mass in STZ-diabetic animals than was observed in those receiving vehicle (Fig 2B), but also spared lean body mass relative to STZ-veh-PF animals (Fig 2C). Moreover, using indirect calorimetry, we found that the rate of O₂ consumption (VO₂) was markedly increased in vehicle-treated STZ-diabetic animals relative to non-diabetic controls, regardless of whether they were fed ad libitum or PF, and that this hypermetabolic effect was attenuated by physiological leptin replacement (Fig 2D). However, the increased metabolic rate in STZ-diabetic animals could not be attributed to changes in ambulatory activity (# beam breaks:2953±243 for veh-veh vs. 1562±87 for STZ-veh vs. 2215±163 for STZ-lep vs. 2927±207 for STZ-veh-PF). Respiratory quotient (RQ) and metabolic heat production are not reported because the assumptions involved in using RQ to estimate heat production are not met in abnormal metabolic states including those characterized by a shift to ketone utilization as a metabolic substrate or during rapid depletion of protein and fat stores (19,20). Nonetheless, these data collectively suggest that increased energy expenditure in STZ-veh-PF relative to STZ-lep-treated animals likely contributes to their excessive weight loss and depletion of lean mass, despite consuming equal amounts of food.

Effect of physiological leptin replacement on insulin sensitivity in rats with STZ-DM.
Consistent with a previous report that insulin resistance is progressive over time in rats with
STZ-induced diabetes (13), we confirmed that the glucose-lowering effect of insulin was markedly diminished on Day 16 compared to Day 7 following STZ-administration (p<0.05) (Fig 3A). To determine whether reduced plasma leptin levels contribute to this progressive insulin resistance, animals that received either vehicle or a physiological replacement dose of leptin were subjected to an ITT 16 days following induction of uDM with STZ. Relative to STZ-treated animals receiving vehicle, the ability of insulin to reduce blood glucose was dramatically enhanced in those that received leptin (p<0.05) (Fig. 3B). To account for differences in the level of hyperglycemia at the onset of the ITT, insulin-induced changes of blood glucose were also analyzed as a percent of basal values (Fig. 3C). Regardless of whether blood glucose levels were analyzed as absolute values or as percent basal, physiological leptin replacement in STZ-induced diabetes markedly increased insulin sensitivity via a mechanism that could not be attributed to changes in food intake, since it was not observed in STZ-veh-PF animals (Fig. 3B-D).

**Effects of STZ-induced diabetes with or without physiological leptin replacement on insulin signal transduction.** To further characterize the impact of physiological leptin replacement on insulin action in uDM, we examined insulin-induced activation of the IRS-PI3K-Akt signal transduction pathway in liver, muscle and WAT. After i.p. saline, there were no differences in levels of pS473-Akt in any tissue between treatment groups, but as expected, systemic insulin injection significantly increased levels of pS473-Akt in liver, muscle and WAT in all groups compared with saline (p<0.05) (Fig. 3). In liver tissue, the ability of insulin to increase levels of pS473-Akt was reduced in STZ-treated animals receiving vehicle compared to non-diabetic controls, and this effect was prevented by physiological leptin replacement (Fig. 4A). Furthermore, since insulin-stimulated production of pS473-Akt was also reduced in STZ-veh-PF livers, the effect of leptin to normalize this response cannot be explained by reduced food intake. In contrast, insulin-induced activation of pS473-Akt in skeletal muscle was not affected by either STZ-induced diabetes or by leptin administration (Fig. 4B). Finally, although insulin significantly increased pS473-Akt in WAT of animals in each group relative to saline-injected controls, the response to insulin was significantly attenuated in all STZ-induced diabetic animals relative to non-diabetic controls (Fig. 4C).

**Potential mechanisms whereby leptin deficiency causes insulin resistance in STZ-induced diabetes.** Uncontrolled diabetes is associated with increased circulating levels of both glucagon and corticosterone, and these responses are implicated in diabetes manifestations including insulin resistance, hyperglycemia and hyperphagia (21-23). Based on recent evidence that adenovirally-induced hyperleptinemia ameliorates hyperglycemia via normalization of elevated plasma glucagon levels (14), we determined whether maintaining physiological leptin levels also is sufficient to prevent diabetes-induced increases of plasma glucagon, and broadened the hypothesis to include other counter-regulatory hormones. Consistent with the aforementioned findings (14), plasma glucagon levels were elevated in STZ-treated animals receiving vehicle relative to non-diabetic controls, and this elevation was prevented by physiological leptin replacement (Fig. 5A). Thus, leptin deficiency appears to be required for the effect of uDM to raise glucagon levels. Similarly, plasma corticosterone levels were increased in STZ-treated rats, and this effect was also attenuated by leptin replacement (Fig. 5B). In contrast, plasma norepinephrine (NE) and epinephrine (EPI) levels were similar across treatment groups (Fig. 5C,D). Normalization
of both plasma glucagon and corticosterone levels may therefore contribute to improved hepatic insulin sensitivity and modest reduction of plasma glucose induced by leptin replacement, but catecholamines are unlikely to be involved.

To investigate whether changes of plasma or tissue lipid accumulation might contribute to the effect of leptin on insulin sensitivity, we measured both plasma NEFA levels and triglyceride content in liver. We found no differences in either plasma NEFA levels (Day 16) or liver triglyceride content in STZ-veh treated animals compared to non-diabetic controls, regardless of whether they were fed ad libitum or pair-fed, but these levels were both reduced in diabetic animals that received leptin (Fig. 6A,B).

To investigate the role of leptin deficiency in the effect of uDM to increase hepatic glucose production and hepatic insulin resistance (24), we used real-time PCR to measure hepatic expression of mRNA encoding G6Pase and Pepck in the absence of insulin injection. As expected, levels of both mRNA species were elevated in diabetic animals that received vehicle relative to non-diabetic controls (Fig. 6C,D). In contrast, hepatic expression of both G6Pase and Pepck genes were comparable between diabetic animals receiving leptin and non-diabetic controls, and were significantly below vehicle-treated diabetic animals. This effect is not attributable to reduced food intake, since pair-feeding was without effect (Fig. 6C,D). A similar pattern of hepatic expression was seen for mRNA encoding peroxisome proliferator-activated receptor-γ-coactivator-1α (PGC-1α), a transcriptional co-regulator implicated in the activation of both G6Pase and Pepck. Thus, leptin deficiency is required for increased hepatic expression of gluconeogenic genes in uDM. By comparison, Igfbp2, a recently reported hepatic gene implicated in the action of leptin to suppress hepatic glucose production (15), was increased >20-fold in STZ-diabetic rats relative to non-diabetic controls, and this diabetes-induced up-regulation was not effected by leptin treatment (Fig. 6F).

DISCUSSION
Growing evidence suggests that either impaired or deficient leptin signaling results in the development of insulin resistance and impaired glucose metabolism (1,4-6,25). Here, we report that leptin deficiency also contributes to the development of progressive insulin resistance and associated neuroendocrine derangements in uDM. We found that systemic administration of exogenous leptin at a dose that maintains normal physiological plasma leptin levels prevented the development of severe, progressive insulin resistance in rats with uDM, and that this effect could not be explained by leptin-induced changes of food intake or body weight. Moreover, the mechanism underlying this effect appears to preferentially involve the liver, as physiological leptin replacement in uDM reduced hepatic triglyceride content and gluconeogenic gene expression, and also restored insulin signal transduction to normal in liver, but not in skeletal muscle or WAT. In addition, physiological leptin replacement in uDM reduced body fat while sparing lean mass, attenuated the increased energy expenditure that accompanies uDM (26), and normalized elevated levels of plasma glucagon and corticosterone. Taken together, these findings implicate leptin deficiency as a cause of wide-ranging metabolic and neuroendocrine derangements associated with uDM.

Although insulin resistance is a well-documented complication of uDM (10-12,27), few studies have sought to identify the underlying mechanism. Some investigators have postulated a role for hyperglycemia, based on a model in which “glucose toxicity” impairs insulin signal transduction in
peripheral tissues (28,29). Consistent with this, insulin resistance in T1D individuals is attenuated when glycemic control is improved (30,31) whereas uncontrolled hyperglycemia induces insulin resistance in these individuals (32,33). Our finding that physiological leptin replacement prevented uDM-induced insulin resistance with only very modest effects to reduce diabetic hyperglycemia, however, suggests that in this study, hyperglycemia per se is unlikely to play a major role. Although reduced food intake might also be expected to contribute to leptin’s insulin-sensitizing effects, this possibility is inconsistent with our finding that diabetic animals pair-fed to the intake of those receiving leptin failed to exhibit an improvement of insulin sensitivity.

In light of the potent insulin-sensitizing effect of leptin replacement in rats with uDM, it is reasonable to ask why the effect on hyperglycemia was so modest. Presumably, this reflects the severe insulin deficiency characteristic of this model, such that improved insulin sensitivity has little impact on ambient glucose levels in the absence of insulin therapy. This observation also highlights an important distinction between the effect of physiological leptin replacement we observed in the current work, and recent work examining the effect of pharmacological hyperleptinemia in uDM (5,14). In the latter, induction of very high circulating leptin levels in rodents with uDM resulted in a full normalization of hyperglycemia, whereas this clearly was not the case in our study. Thus, normalization of hyperglycemia in uDM cannot be achieved simply by physiological replacement of endogenous leptin but apparently requires pharmacological levels of leptin.

Complementing our finding that physiological leptin replacement enhances insulin-mediated glucose lowering in STZ-induced diabetic animals, we found that this intervention also restored insulin signaling in liver, but not in skeletal muscle or adipose tissue, as measured by insulin-induction of pAkt. The mechanisms underlying this improvement of hepatic insulin signaling remain uncertain, but could involve reductions of body fat mass and/or liver triglyceride content. Alternatively, we recently reported that leptin action in the hypothalamic arcuate nucleus improves hepatic insulin action via a mechanism involving the hepatic vagus nerve (6). Thus, additional studies are warranted to determine whether this autonomic mechanism might also contribute to the improved insulin sensitivity following physiological leptin replacement in uDM.

uDM is associated with increased plasma glucagon levels and elevated hepatic expression of the gluconeogenic genes, G6Pase and Pepck and these responses are implicated in the pathogenesis of insulin resistance (34,35) and diabetic hyperglycemia (21,22). Our finding that physiological leptin replacement in uDM dramatically improved insulin sensitivity and normalized elevated levels of both plasma glucagon and hepatic expression of these gluconeogenic genes is consistent with this hypothesis. A recent study reported that hyp erleptinemia induced by an adenoviral gene therapy approach ameliorated hyperglycemia in STZ-induced diabetes, and this was hypothesized to occur via a suppression of hyperglucagonemia and consequent reduction of hepatic gluconeogenic gene expression (14). Previous studies, however, have not established the extent to which hyperglycemia in uDM might be driven by increases of glucagon and associated changes of glucose production. In this context, we note that although plasma glucagon levels and hepatic expression of G6Pase and Pepck were normalized by physiological leptin replacement in our study, hyperglycemia was only slightly improved compared to diabetic animals that received vehicle. These data suggest that normalization of plasma
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glucagon levels plays only a minor role in leptin’s anti-diabetic effects, although it remains possible that hyperglucagonemia contributes to progressive insulin resistance in uDM. Further, our findings identify for the first time a role for leptin deficiency in the effect of uDM to raise circulating glucagon levels.

Hyperglucagonemia and the energy cost associated with increased gluconeogenesis are implicated in the increased metabolic rate in poorly controlled T1D patients, an effect that is reversed by insulin treatment (26). Such a mechanism may also explain the marked increase of energy expenditure in STZ-diabetic rats relative to non-diabetic controls, as this cannot be explained by changes in activity. Changes of food intake are also unlikely to explain this metabolic response to uDM, since metabolic rate was increased similarly in STZ-veh and STZ-veh-PF groups. Although leptin has previously been demonstrated to increase energy expenditure, at least in part via sympathetic activation of brown adipose tissue (36), we found that the elevated metabolic rate induced by uDM was attenuated by leptin replacement. Although the mechanism underlying this leptin effect awaits further study, suppression of elevated rates of gluconeogenesis and perhaps other futile cycles seems likely. To our knowledge, this is the first demonstration of an effect of leptin to lower metabolic rate and reinforces the concept that the effect of leptin on metabolic regulation is highly context-dependent. These data also help to explain why leptin-treated diabetic animals lose weight in similar amounts to those receiving vehicle, despite consuming much less food. Indeed, matching the intake of STZ-veh animals by pair-feeding to that of leptin-replaced rats produced excessive weight loss. These findings collectively suggest that leptin deficiency is a major determinant of hypermetabolism induced by uDM.

uDM is also characterized by increased glucocorticoid secretion and activation of the HPA axis (37,38). Since glucocorticoids both inhibit peripheral glucose uptake in muscle and adipose tissue and induce hepatic expression of G6Pase and Pepck (39), HPA activation in uDM likely contributes to insulin resistance in this setting. Our finding that leptin replacement normalized elevated plasma corticosterone levels in STZ-induced diabetic rats provides a plausible mechanism to explain the observed improvement of insulin sensitivity, but again suggests that increased circulating glucocorticoid levels are not a dominant cause of hyperglycemia in uDM. Also of interest here is the implication that leptin deficiency causes HPA activation in uDM, as has been suggested in other conditions (40). In contrast, we observed no effect of leptin replacement on plasma catecholamines.

Recently, it was reported that systemic administration of leptin upregulates hepatic expression of Igfbp2, and adenovirally-induced overexpression of IGFBP2 normalizes blood glucose levels in insulin-resistant and uDM mice (15). Our finding that hepatic expression of Igfbp2 was increased ≥20-fold in leptin-deficient STZ-diabetic animals relative to non-diabetic controls, however, suggests that increased hepatic expression of this gene is unlikely to exert salutary effects in the setting of uDM, an impression confirmed by the absence of any effect of leptin replacement on Igfbp2 mRNA levels. Thus, hepatic Igfbp2 is unlikely to explain the effect of physiological leptin replacement to improve insulin sensitivity in uDM.

Several lines of evidence suggest that insulin resistance occurs in patients with T1D (10-12,27) and is an independent risk factor for micro- and macro-vascular complications in these patients (41,42). However, therapeutic approaches currently available for treating insulin resistance in T1D are quite
limited. Although intensive insulin therapy in individuals with T1D improves insulin sensitivity and glycemic control, the Diabetes Control and Complication Trial (DCCT) reported that while intensive insulin therapy preserves pancreatic β-cell function and improves retinopathy and neuropathy, these benefits are offset by an increased frequency of severe hypoglycemia and weight gain (43-45). In contrast, treating T1D subjects with lifestyle modifications, such as diet (46) and exercise (47) or pharmaceutical approaches using thiazolidinediones (TZD) (48) or metformin (49), yield improvements in insulin sensitivity in T1D subjects, but not glycemic control. Our current data raise the possibility that a combination therapy approach using both insulin and leptin treatment in T1D patients may reduce the amount of insulin required to achieve good glycemic control and also limit weight gain (50).

In summary, we report that STZ-induced diabetes causes a rapid and severe reduction of plasma leptin levels that precedes the development of severe insulin resistance. The latter effect was prevented by restoration of plasma leptin to normal physiological levels which also reduced hepatic triglyceride content and restored normal insulin signal transduction in the liver. Moreover, physiological replacement of plasma leptin levels suppressed the characteristic increase of plasma glucagon and corticosterone levels and elevated hepatic expression of the gluconeogenic genes Pepck and G6Pase in uDM, but only modestly reduced the extent of hyperglycemia. These findings also implicate leptin deficiency as a cause of hyperglucagonemia and suggest that while normalization of elevated plasma glucagon levels may contribute to the anti-diabetic effects of pharmacological leptin therapy, other factors must also be involved. We conclude that leptin deficiency plays a major role in the progressive, severe insulin resistance characteristic of uDM. This novel finding also raises the therapeutic possibility that supplementing insulin treatment with leptin may be a useful adjunct in the management of T1D.

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**FIGURE LEGENDS**

**Figure 1.** Physiological leptin replacement attenuates diabetic hyperglycemia and diabetic hyperphagia in STZ-treated rats.
A) Plasma insulin, B) plasma leptin, C) blood glucose, and D) mean daily food intake in STZ-induced diabetic animals receiving either vehicle and fed ad libitum (open squares) or pair-fed (closed diamonds), a physiological replacement dose of leptin (open diamond) or non-diabetic controls (closed triangles). * p<0.05 vs. STZ-lep; # p<0.05 vs. STZ-veh-PF.
Figure 2. Physiological leptin replacement reduces fat mass and attenuates increased energy expenditure in STZ-treated rats.
A) Body weight change, B) percent body fat mass, C) lean body mass and D) VO₂ measured using quantitative magnetic resonance and indirect calorimetry, respectively, in STZ-induced diabetic animals receiving either vehicle and fed ad libitum (open squares) or pair-fed (closed diamonds), a physiological replacement dose of leptin (open diamond) or non-diabetic controls (closed triangles). * p<0.05 vs. veh-veh; # p<0.05 vs. STZ-lep.

Figure 3. Physiological leptin replacement improves insulin sensitivity in STZ-treated rats.
A) Blood glucose levels in STZ-induced diabetic animals on Day 7 (closed triangles) and 16 (open squares) following STZ-injection during an insulin tolerance test (2U/kg). *p<0.05 vs. Day 16. B) Blood glucose levels, C) % basal blood glucose levels and D) the inverse integrated area under the % basal glucose curve in STZ-induced diabetic animals receiving either vehicle and fed ad libitum (open squares) or pair-fed (closed diamonds), a physiological replacement dose of leptin (open diamond) or non-diabetic controls (closed triangles) during an insulin tolerance test (2U/kg) * p<0.05 vs. STZ-lep; # p<0.05 vs. STZ-veh-PF.

Figure 4. Physiological leptin replacement increases hepatic insulin signal transduction.
Effect of intraperitoneal (2U/kg) insulin (closed bars)-induced activation of serine phosphorylation of Akt compared to vehicle (open bars) in A) liver, B) muscle (tibialis anterior) and C) white adipose tissue (epididymal fat) in STZ-induced diabetic animals receiving either vehicle and fed ad libitum (STZ-veh) or pair-fed (STZ-veh-PF), a physiological replacement dose of leptin (STZ-lep) or non-diabetic controls (veh-veh). * p<0.05 vs. veh-veh-veh; # p<0.05 vs. veh-veh-ins.

Figure 5. Physiological leptin replacement reduces hyperglucagonemia and hypercorticosteronemia.
Arterial plasma A) glucagon, B) corticosterone, C) norepinephrine (NE) and D) epinephrine (EPI) levels in non-diabetic controls (open bar) or in STZ-induced diabetic animals receiving either vehicle (solid bar) or a physiological replacement dose of leptin (striped bar). * p<0.05 vs. veh-veh; # p<0.05 vs. STZ-lep.

Figure 6. Physiological leptin replacement reduces plasma and hepatic lipid content and gluconeogenic gene expression.
A) Plasma non-esterified fatty acids (NEFA) obtained from tail-vein samples, hepatic B) triglyceride content and expression of C) G6Pase, D) Pepck, E) Pgc-1α and F) Igfbp2 using real-time PCR in non-diabetic controls (open bar) or in STZ-induced diabetic animals receiving either vehicle and fed ad libitum (solid bar) or pair-fed (hatched bar) or a physiological replacement dose of leptin (striped bar). * p<0.05 vs. veh-veh; # p<0.05 vs. STZ-lep.
Figure 1

A

B

C

D

Figure 2

A

B

C

D

Leptin deficiency and insulin resistance
Figure 4

A

Liver [pS473]Akt / protein

veh veh STZ veh STZ lep STZ veh-PF

*# *

B

WAT [pS473]Akt / protein

veh veh STZ veh STZ lep STZ veh-PF

*# **

C

Muscle [pS473]Akt / protein

veh veh STZ veh STZ lep STZ veh-PF

*
Figure 5

A

B

C

D

Leptin deficiency and insulin resistance