Brief communication

MOLECULAR METABOLISM

The role of autonomic efferents and uncoupling protein 1 in the glucose-lowering effect of leptin therapy

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ABSTRACT

Objective: Leptin reverses hyperglycemia in rodent models of type 1 diabetes (T1D). Direct application of leptin to the brain can lower blood glucose in diabetic rodents, and can activate autonomic efferents and non-shivering thermogenesis in brown adipose tissue (BAT). We investigated whether leptin reverses hyperglycemia through a mechanism that requires autonomic innervation, or uncoupling protein 1 (UCP1)-mediated thermogenesis.

Methods: To examine the role of parasympathetic and sympathetic efferents in the glucose-lowering action of leptin, mice with a subdiaphragmatic vagotomy or 6-hydroxydopamine induced chemical sympathectomy were injected with streptozotocin (STZ) to induce hyperglycemia, and subsequently leptin treated. To test whether the glucose-lowering action of leptin requires activation of UCP1-mediated thermogenesis in BAT, we administered leptin in STZ-diabetic *Ucp1* knockout (*Ucp1*$^{-/-}$) mice and wildtype controls.

Results: Leptin ameliorated STZ-induced hyperglycemia in both intact and vagotomised mice. Similarly, mice with a partial chemical sympathectomy did not have an attenuated response to leptin-mediated glucose lowering relative to sham controls, and showed intact leptin-induced *Ucp1* expression in BAT. Although leptin activated BAT thermogenesis in STZ-diabetic mice, the anti-diabetic effect of leptin was not blunted in *Ucp1*$^{-/-}$ mice.

Conclusions: These results suggest that leptin lowers blood glucose in insulin-deficient diabetes through a manner that does not require parasympathetic or sympathetic innervation, and thus imply that leptin lowers blood glucose through an alternative CNS-mediated mechanism or redundant target tissues. Furthermore, we conclude that the glucose lowering action of leptin is independent of UCP1-dependent thermogenesis.

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Keywords Type 1 diabetes; Glucose; Vagotomy; Sympathectomy; Brown adipose tissue; Streptozotocin

1. INTRODUCTION

The hormone leptin plays a critical role in the control of glucose metabolism [1]. Leptin can reverse hyperglycemia in rodent models of type 1 diabetes (T1D), independent of food intake, and without raising circulating insulin levels [2–8]. Central leptin administration or gene therapy can reverse hyperglycemia in rodent models of T1D in a similar manner to peripheral administration [9–16], suggesting a critical role of the central nervous system (CNS) in mediating leptin action. CNS leptin action can modulate peripheral tissues via both sympathetic and parasympathetic branches of the autonomic nervous system (ANS). Leptin-induced glucose uptake in brown adipose tissue (BAT) and skeletal muscle, and activation of hepatic 5′AMP-activated protein kinase in non-diabetic rodents have been shown to occur in a sympathetic-dependent manner [17–22]. In addition, disruption of the parasympathetic branch of the ANS with a surgical vagotomy diminishes improvements in glucose homeostasis following central leptin administration in rodent models of type 2 diabetes [23,24]. Thus, autonomic efferents play a key role in mediating leptin-induced changes to glucose metabolism in peripheral tissues and may contribute to leptin-mediated reversal of hyperglycemia.

Activated BAT thermogenesis by sympathetic stimulation causes robust glucose uptake and energy dissipation as heat [25,26], and holds therapeutic potential for obesity and metabolic disorders [27,28]. BAT thermogenesis requires mitochondrial uncoupling protein 1 (UCP1) [25,29]. Interestingly, central leptin can stimulate UCP1-dependent thermogenesis and glucose utilization in BAT in a noradrenergic-dependent manner [30]. Furthermore, central leptin

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Abbreviations: 6OHDA, 6-hydroxydopamine; ANS, autonomic nervous system; BAT, brown adipose tissue; CNS, central nervous system; CCK, cholecystokinin; iBAT, interscapular BAT; STZ, streptozotocin; T1D, type 1 diabetes; TH, tyrosine hydroxylase; UCP1, uncoupling protein 1

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therapy has been found to robustly induce *Ucp1* expression and BAT glucose uptake in rodent models of T1D [9,11,16,31]. Therefore, the activation of UCP1-dependent BAT thermogenesis may play a role in leptin-induced glucose lowering.

We aimed to elucidate the role of the ANS in the anti-diabetic effect of leptin therapy. Although leptin has been shown to improve cardiovascular function in rodent models of T1D by increasing sympathetic tone [14,15], studies using pharmacological or genetic means to partially attenuate sympathetic innervation or signaling have not found any evidence that the anti-diabetic action of leptin in rodent models of T1D requires sympathetic innervation [14,16,31]. Furthermore, the role of parasympathetic efferents in leptin-mediated glucose lowering has not been reported. Thus, we assessed whether leptin could reverse streptozotocin (STZ)-induced hyperglycemia in mice following 6-hydroxydopamine (6OHDA)-induced sympathectomy or surgical vagotomy. Subsequently, we examined the role of leptin-induced BAT thermogenesis as a possible downstream mechanism. We report that neither subdiaphragmatic vagotomy, nor partial chemical sympathectomy attenuates the glucose-lowering action of leptin. In addition, we found that although leptin induces *Ucp1* expression and thermogenesis in BAT of STZ-diabetic mice, diabetic *Ucp1*$^{-/-}$ mice are not refractory to leptin therapy.

2. MATERIALS AND METHODS

2.1. Animals

Male C57Bl/6J mice for vagotomy studies, and *Ucp1*$^{+/-}$ mice [32] (Jackson Laboratories, Bar Harbor, ME), and male C57Bl/6J mice for sympathectomy studies (Centre for Disease Modeling, Vancouver, Canada) were housed with a 12/12-hour light—dark cycle with *ad libitum* access to food (2918, Harlan Laboratories, Madison, WI) and water. All animal procedures were approved by the UBC Animal Care Committee and carried out in accordance with the Canadian Council on Animal Care guidelines. *Ucp1*$^{+/-}$ mice were interbred to generate *Ucp1*$^{+/+}$, *Ucp1*$^{+/-}$ and *Ucp1*$^{-/-}$ mice, and genotyped using the *Ucp1*$^{+}$ primer (GATTTGCCTCTGAATGCCCGC), *Ucp1*$^{-}$ primer (CCTA CCCGCTTCCATTGCTCA) and, *Ucp1* common primer (GCACG GGGTGGTGTACTATCC). Experimental *Ucp1*$^{-/-}$ and *Ucp1*$^{+/+}$ controls were housed in cages maintained at thermoneutrality (29.0—30.5 °C) from 3 weeks of age to experimental endpoint.

2.2. Chemical sympathectomy

Male C57Bl/6J mice, aged 12 weeks, were injected intraperitoneally (i.p.) with 250 mg/kg 6-hydroxydopamine hydrobromide (6OHDA, Sigma—Aldrich, St. Louis, MO), prepared in sterile saline containing 0.05% L-ascorbic acid (Sigma—Aldrich, St. Louis, MO) [33], 3 weeks before STZ administration.

2.3. Vagotomy

C57Bl/6J mice received subdiaphragmatic vagotomies at 6 weeks of age, performed by Jackson Laboratories (Bar Harbor, ME) as previously described [34]. A section of both the dorsal and ventral vagal trunks, adjacent to the esophagus, were excised. In sham operated mice the vagus was exposed but not excised.

2.4. STZ administration

STZ (Sigma—Aldrich, St. Louis, MO) prepared in acetate buffer, pH 4.5, was injected i.p. at a dose of 170 mg/kg in *Ucp1*$^{-/-}$ mice and controls. For all other studies, mice received 180 mg/kg STZ. Diabetes was defined as two consecutive measures of fasting blood glucose $\geq$16.5 mmol/L.

2.5. Leptin administration via mini-osmotic pump

Alzet osmotic pumps (DURECT Corporation, Cupertino, CA) were implanted subcutaneously as previously described [2,3], delivering 10 μg/day recombinant mouse leptin (National Hormone & Peptide Program, Torrance, CA, USA), or 20 μg/day recombinant mouse leptin (Peprotech, Rocky Hill, NJ), or vehicle. The doses of leptin were adjusted between studies to account for different glucose lowering efficacies observed between the peptide sources. For the vagotomy study, mice received leptin supplied by the National Hormone & Peptide Program (Torrance, CA) prepared in PBS or PBS as vehicle. All other studies employed leptin from Peprotech (Rocky Hill, NJ) dissolved in water or water as vehicle.

2.6. Metabolic assessments

Blood glucose, plasma leptin, and insulin were measured from blood collected through the saphenous vein in conscious mice following a 4-hour fast as previously described [2,34].

2.7. Cholecystokinin (CCK)-induced satiety

CCK octapeptide (26—33) (American Peptide, Sunnyvale, CA) was prepared as previously described [34]. Following an overnight fast, mice were injected with CCK or vehicle i.p., placed individually in cages with pre-weighed food, and allowed to feed for 1 h. Food intake was measured as the difference in food weight prior to and 1-hour post-injection.

2.8. Gastric distension

Stomachs were harvested 32 days post-pump implant following a 4-hour fast. Gastric contents were removed, and stomach weights were measured.

2.9. BAT measurements

BAT was harvested 19 days post-pump implant, fixed overnight in 4% paraformaldehyde at 4 °C, incubated in 25% sucrose in PBS for 72 h, and subsequently frozen in an isopentane bath. BAT was sectioned by Wax-it Histology Services Inc. (Vancouver, Canada). Sections were immunostained for tyrosine hydroxylase (TH) (rabbit anti-TH, Millipore, Cat# AB152, 1:1000 dilution), subsequently incubated with secondary antibody, mounted and scanned as previously described [35]. Total TH positive area was expressed relative to total cell count (based on DAPI fluorescence) and total section area. RNA was isolated from BAT on day 19 and DNase treated using RNeasy Lipid Tissue Mini Kit (Qiagen, Mississauga, Canada) and RNase free DNase kit (Qiagen). RNA was converted to cDNA and RT-qPCR was performed as previously described [36] using forward primer (GGCCCTTGTAAACAACAAAATAC) and reverse primer (GGCAACAAGAGCTGACAGTAAAT). Beta-actin was selected as a reference gene by geNorm analysis as previously described [36]. Relative transcript abundance was determined by the Pfaffl method [37].

2.10. Interscapular BAT (iBAT) thermogenesis

Temperature transponders (Implantable Programmable Temperature Transponder IPTT-300; Bio Medic Data Systems Inc, Seaford, USA) were implanted interscapularly at the same time as osmotic pumps. Non-fasted temperatures were recorded using a hand held Pocket Scanner (DAS-5007; Bio Medice Data Systems Inc).

2.11. Statistical analyses

Data are presented as mean ± SEM. Data and statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla,

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USA). Unless otherwise specified, statistical analyses were performed by two-way ANOVA with a Tukey post-hoc test.

3. RESULTS

3.1. Subdiaphragmatic vagotomy does not block leptin action in STZ-diabetes

To assess whether parasympathetic efferents are necessary for glucose lowering by leptin therapy, subdiaphragmatic vagotomies or sham surgeries were performed in 6 week old C57Bl/6J male mice. Vagotomised and sham-operated mice were injected with STZ to induce insulin-deficient diabetes, and subsequently implanted with osmotic pumps delivering 10 μg/day leptin (sham-leptin and vagotomy-leptin groups) or vehicle (sham-vehicle group). Following STZ injection, vagotomised mice displayed heightened sensitivity to STZ effects compared to controls; of 14 STZ-injected vagotomised mice, 10 reached humane endpoint (decreased activity, weight loss, dehydration) within several days of STZ injection, whereas no sham mice reached humane endpoint. We suspect that this heightened sensitivity is due to the combined gastroparesis effect of vagotomy and STZ. The 4 surviving vagotomised mice developed STZ-induced hyperglycemia, that was slightly lower but not statistically different compared to STZ-injected sham controls (Figure 1A). Following the implantation of osmotic pumps, sham-vehicle controls remained severely diabetic for the duration of the study, whereas sham-leptin mice showed robust blood glucose lowering in response to leptin. Interestingly, blood glucose levels were significantly lower in vagotomy-leptin mice compared to sham-leptin mice at most time points during leptin therapy. Leptin treatment did not decrease body weight relative to vehicle treatment in sham controls (Figure 1B); the lack of leptin-induced weight loss in type 1 diabetic models has been reported in multiple studies [2,4–7,9–13,16]. Plasma leptin was increased to similar levels in sham-leptin and vagotomy-leptin groups compared to pre-STZ values ($P \leq 0.0003$) (Figure 1C). Insulin levels (Figure 1D) were significantly decreased by STZ in all groups ($P \leq 0.008$) and remained low for the duration of the study. To confirm that the reduction of glycemia was due to leptin therapy, we continued to track blood glucose in mice after leptin release from the osmotic pumps ceased (~17.7 days post-surgery), at which point hyperglycemia returned to pre-treatment levels in both vagotomised and sham-operated mice, concomitant with a drop in plasma leptin to sham-vehicle levels. All vagotomy-leptin mice had substantially enlarged stomachs compared to sham-vehicle and sham-leptin groups, consistent with gastric distension caused by vagal denervation [34,38] (Figure 1E). In addition, 1 week before STZ-injection, we examined CCK-induced satiety, an effect that is partly mediated by vagal afferents [34,38]. While sham-operated control mice displayed the expected decrease in food intake in response to CCK injection, this response was blunted in mice that underwent vagotomy (Figure 1F). These data reveal that subdiaphragmatic vagal efferents are not required to mediate the glucose lowering action of leptin in STZ-induced diabetes.

3.2. Partial chemical sympathectomy does not block leptin action in STZ-diabetes

We next examined whether sympathetic efferents are a requisite for leptin-mediated glucose lowering. We chemically sympathectomised 12 week old C57Bl/6J male mice by injecting 6OHDA, which when administered peripherally in rodents, specifically causes destruction of noradrenergic neurons [39,40]. Control (sham) mice received injections of buffer instead of 6OHDA. Administration of 6OHDA produced symptoms in mice consistent with decreased sympathetic function, including ptosis and piloerection [41,42]. Subsequently, mice were injected with STZ to induce insulin-deficient diabetes, and implanted with osmotic pumps delivering 20 μg/day leptin (sham-leptin or 6OHDA-leptin groups) or vehicle (sham-vehicle group). In response to STZ-injection, sham and 6OHDA groups displayed similar degrees of hyperglycemia (Figure 2A) and weight loss (Figure 2B). Following the implantation of osmotic pumps, leptin administration reversed hyperglycemia in both sham-leptin and 6OHDA-leptin mice. As expected, leptin therapy had no impact on body weight (Figure 2B). Leptin treatment produced similar supraphysiological elevations in plasma leptin in 6OHDA-leptin and sham-leptin mice compared to pre-STZ levels ($P < 0.0001$ for both groups), whereas plasma leptin levels were significantly reduced in sham-vehicle mice by STZ injection ($P = 0.04$; Figure 2C). After leptin release from the osmotic pumps had ceased (~9.2 days after pump implantation), hyperglycemia rapidly returned to pre-treatment levels in 6OHDA-leptin and sham-leptin groups, concomitant with the reduction of plasma leptin levels (Figure 3A,C). Expectedly, following STZ administration, plasma insulin dropped substantially in all groups, and remained low for the duration of the study (Figure 2D).

To examine the extent of 6OHDA-mediated sympathectomy we quantified TH immunoreactivity, a sympathetic neuron marker, in BAT which is densely innervated by sympathetic neurons, and measured BAT *Ucp1* expression, which is induced by the release of noradrenaline from sympathetic neurons [25]. Mice injected with 6OHDA displayed decreased BAT TH immunoreactivity (Figure 2E). TH positive area relative to whole section area (Figure 2F), and TH positive area relative to total cell number (Figure 2G), revealed a ~50% decrease in TH immunoreactivity in 6OHDA injected mice compared to sham controls, revealing that partial sympathectomy was achieved by 6OHDA injection. Interestingly, although leptin therapy had ceased 10 days prior to measurement, sham-leptin mice had increased *Ucp1* transcript levels relative to sham-vehicle controls, and 6OHDA-leptin mice showed a similar elevation of *Ucp1* (Figure 2H). This demonstrated two points: firstly, leptin has long lasting effects on *Ucp1* expression in STZ-diabetic mice, consistent with the increase in *Ucp1* during ICV-leptin treatment in STZ-diabetic rats [9,31]; secondly, leptin-induced *Ucp1* expression is not blunted despite partial sympathectomy in this model. Collectively, these data suggest that partial ablation of sympathetic neurons is insufficient to blunt leptin's therapeutic actions in diabetic mice. This may have been due to the preservation of BAT *Ucp1* induction by leptin.

3.3. Global *Ucp1* deficiency does not block leptin action in STZ-diabetes

Observing the induction of *Ucp1* transcript in BAT of leptin-treated mice, we examined whether leptin therapy activated BAT thermogenesis, and whether this may contribute to the reversal of hyperglycemia. Wildtype C57Bl/6J male mice were injected with STZ, and subsequently treated with 20 μg/day leptin (STZ-leptin) or vehicle (STZ-vehicle), and compared to non-diabetic controls. Since the iBAT thermal response reflects BAT thermogenic capacity [43,44], we measured the interscapular temperature in leptin treated STZ-mice. The interscapular temperature was decreased in STZ-vehicle mice, and leptin treatment increased the interscapular temperature to non-diabetic levels (Figure 3A). Furthermore, iBAT weight was decreased in STZ-leptin mice relative to non-diabetic and STZ-vehicle controls (Figure 3B), consistent with induction of lipolysis and thermogenesis in BAT. Next, we directly examined whether UCP1-dependent thermogenesis is required for the glucose lowering effect of leptin in STZ-

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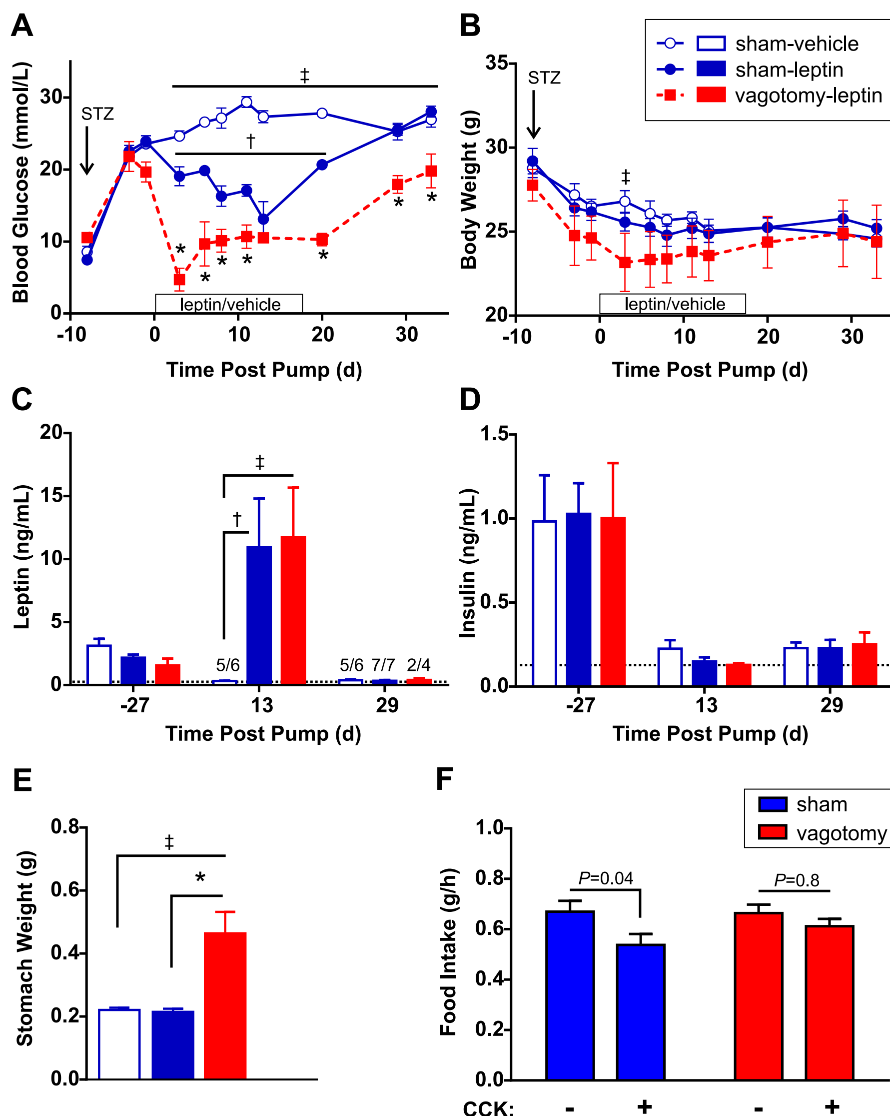

Figure 1: Subdiaphragmatic vagotomy does not attenuate leptin action in STZ-diabetic mice. C57Bl/6J male mice received vagotomy or sham operations at 6 weeks of age, and were injected with STZ to induce diabetes at 14 weeks of age, 7 days prior to pump implantation. On day 0, osmotic pumps were implanted delivering either 10 μg/day leptin or vehicle for a calculated delivery lasting 17.7 days. Four-hour fasted parameters in sham-vehicle (open blue symbols, n = 6), sham-leptin (filled blue symbols, n = 7), and vagotomy-leptin (filled red symbols, n = 4) mice. Blood glucose (A) and body weight (B) were tracked throughout the study. Statistical analyses were performed by repeated measures two-way ANOVA with a Tukey post-hoc test. Plasma leptin (C) and insulin (D) were measured 3 weeks prior to STZ administration, and on day 13 (during leptin therapy), and day 29 (after leptin therapy had ceased). Limits of detection are shown by the broken horizontal line, and in groups where some samples were undetectable, the numbers above indicate the proportion of samples which had detectable values. Statistical analyses were performed by two-way ANOVA with a Tukey post-hoc test. (E) Empty stomach weight was measured following sacrifice on day 32 and analyzed by one-way ANOVA with a Tukey post-hoc test, $n \geq 4$. (F) Food intake 1 h following cholecystokinin (CCK) or vehicle injection was measured on day −15 prior to pump implantation, $n \geq 6$ per group (sham in blue bars, vagotomy in red bars), and analyzed by two-way ANOVA with a Bonferroni post-hoc test. Data are presented as mean ± SEM. *$P < 0.05$ vagotomy-leptin vs sham-leptin mice; †$P < 0.05$ sham-leptin vs sham-vehicle mice; ‡$P < 0.05$ vagotomy-leptin vs sham-vehicle mice.

diabetes. Mice with a global ablation of the *Ucp1* gene (*Ucp1*$^{-/-}$), which lack thermogenic potential in BAT [25,26,29,32,45], and wild-type controls (*Ucp1*$^{+/+}$) were injected with STZ to induce insulin-deficient diabetes. Following the development of diabetes, these mice were implanted with osmotic pumps delivering 20 μg/day leptin (*Ucp1*$^{-/-}$-leptin and *Ucp1*$^{+/+}$-leptin groups) or vehicle (*Ucp1*$^{-/-}$-vehicle and *Ucp1*$^{+/+}$-vehicle groups). *Ucp1*$^{+/+}$ and *Ucp1*$^{-/-}$ mice displayed a similar degree of hyperglycemia (Figure 3C) and weight loss in response to STZ (Figure 3D). Although leptin did not lower blood glucose as substantially in *Ucp1*$^{-/-}$-leptin vs *Ucp1*$^{+/+}$-leptin mice on day 2 post-pump implant ($P = 0.03$), afterward both groups showed a similar glucose lowering response to leptin therapy. *Ucp1*$^{-/-}$-vehicle and *Ucp1*$^{+/+}$-vehicle mice remained similarly hyperglycemic over the course of treatment (Figure 3C). Collectively, these data reveal that UCP1-dependent thermogenesis is not required to mediate the glucose lowering effect of leptin in rodent modeled T1D.

4. DISCUSSION

Here we examined whether peripherally administered leptin therapy requires parasympathetic or sympathetic efferents to lower blood glucose through the use of subdiaphragmatic vagotomy and partial

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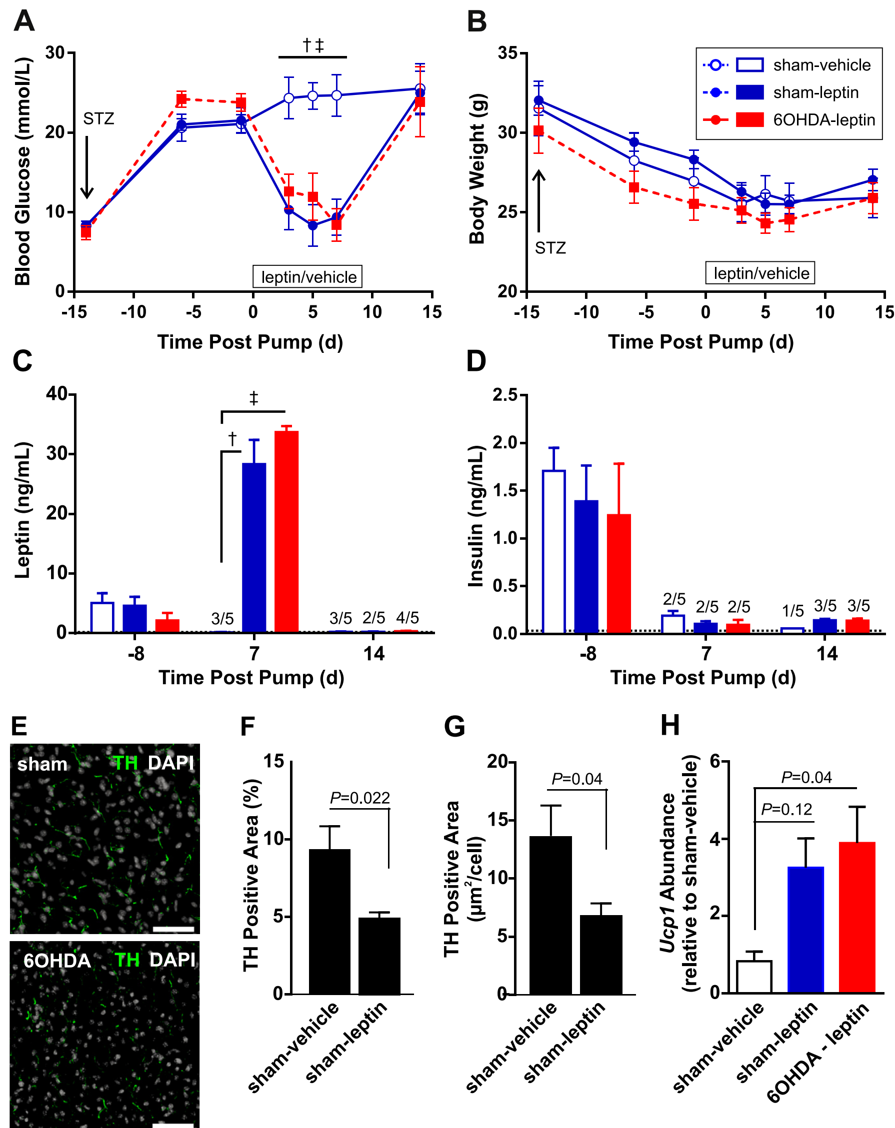

Figure 2: Injection of 6OHDA to chemically sympathectomised mice does not attenuate therapeutic leptin action in STZ-diabetes. Sympathectomy was induced in male C57Bl/6J mice by injection of 6OHDA at 12 weeks of age. Sham controls received buffer only injections instead of 6OHDA. Mice were injected with STZ to induce diabetes 14 days prior to implantation of osmotic pumps delivering 20 μg/day leptin or vehicle on day 0, for a calculated delivery period of 9.2 days. Four-hour fasted parameters in sham-vehicle (open blue circles/bars, n = 5), sham-leptin (filled blue circles/bars, n = 5), and 6OHDA-leptin (filled red squares/bars, n = 5) mice. Blood glucose (A) and body weight (B) were tracked over the course of the study, and statistical analyses were performed by repeated measures two-way ANOVA with a Tukey post-hoc test. Plasma leptin (C) and insulin (D) levels were measured on indicated days relative to pump implantation. Statistical analyses for leptin were performed by two way ANOVA with a Tukey post-hoc test. Statistical analyses for insulin were performed by one way ANOVA with a Tukey post-hoc test on day −8 values only. Limits of detection are shown by the broken horizontal line, and in groups where some samples were undetectable, the numbers above indicate the proportion of samples which had detectable values. Brown adipose tissue was harvested on day 19 at the end of the study. Representative images of tyrosine hydroxylase (TH) immunofluorescence are shown in (E), scale bar is 50 μm. Quantification of TH immunoreactive area was expressed as percent total section area (F) and relative to total cell number (G). (H) BAT Ucp1 transcript was measured by RT-qPCR relative to sham-vehicle controls. Data are presented as mean ± SEM. †$P < 0.05$ sham-leptin vs sham-vehicle mice; ‡$P < 0.05$ 6OHDA-leptin vs sham-vehicle mice.

chemical sympathectomy, respectively. STZ-diabetic vagotomised mice responded fully to the anti-diabetic action of leptin, even displaying lower blood glucose levels than those of leptin-treated intact controls. The apparent potentiation of leptin's anti-diabetic action by subdiaphragmatic vagotomy was not investigated further, but suggests that vagal relays may partly counter leptin's anti-diabetic action. Similarly, injection of 6OHDA, which resulted in ∼50% reduction in sympathetic nerve terminals, did not block the glucose lowering action of leptin in STZ-diabetic mice. We observed that, despite partial sympathectomy, leptin induced BAT *Ucp1* expression that lasted long after leptin therapy was ceased. Thus, to examine whether the induction of *Ucp1*, and UCP1-dependent BAT thermogenesis, was required for the anti-diabetic actions of leptin, we tested whether leptin could lower blood glucose in STZ-diabetic mice with a global ablation of *Ucp1*. This study revealed that although leptin did activate thermogenesis, as indicated by increased iBAT temperature, UCP1 deficiency did not blunt the glucose lowering action of leptin. Collectively, these results suggest that the glucose lowering effect of chronic leptin administration does not depend on communication between the CNS and

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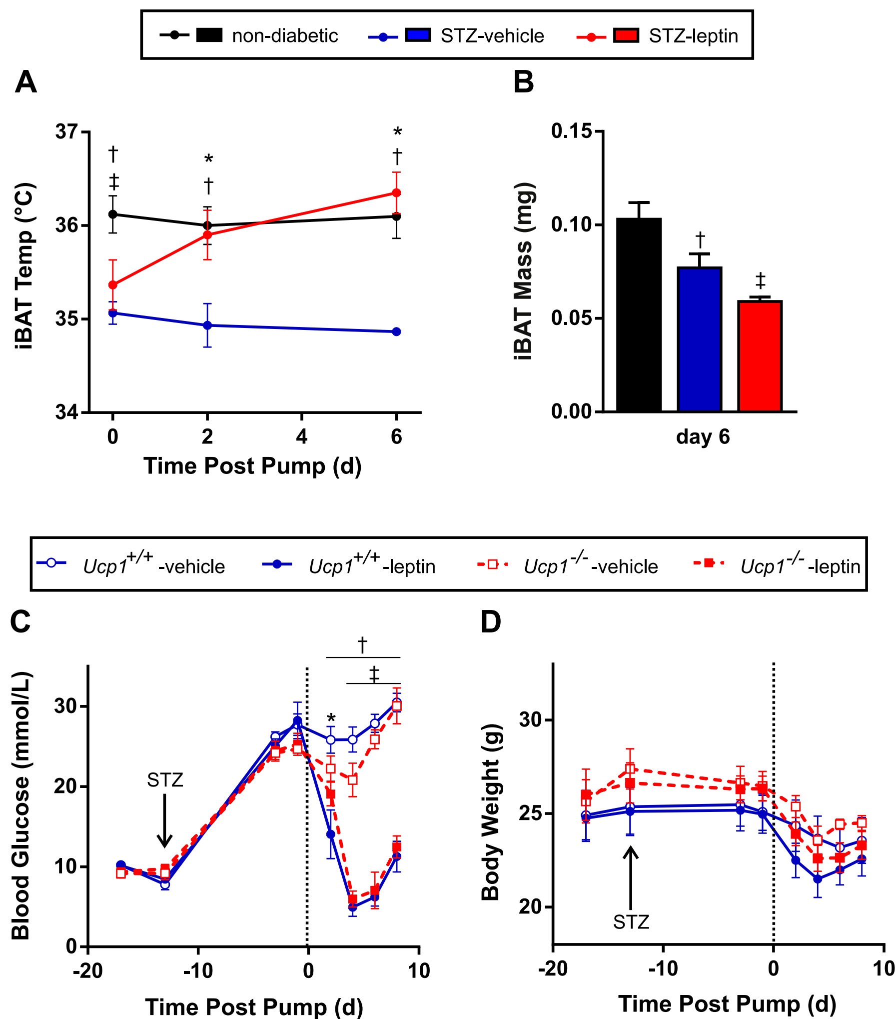

Figure 3: Leptin-induced glucose lowering is independent of UCP1-dependent BAT thermogenesis. Interscapular brown adipose tissue (iBAT) temperature (A), and iBAT mass (B, day 6 of treatment) were measured in STZ-diabetic C57Bl/6J male mice treated with 20 μg/day leptin (STZ-leptin, red) or vehicle (STZ-vehicle, blue) and non-diabetic controls (black), n = 4–6. *P < 0.05 STZ-leptin vs STZ-vehicle. †P < 0.05 STZ-vehicle vs non-diabetic. ‡P < 0.05 STZ-leptin vs non-diabetic. Blood glucose (C) and body weight (D) were measured following a 4-hour fast on the indicated days in *Ucp1*$^{-/-}$ and *Ucp1*$^{+/+}$ mice injected with STZ and treated with 20 μg/day leptin (*Ucp1*$^{-/-}$leptin, filled red squares; *Ucp1*$^{+/+}$-leptin, filled blue circles) or vehicle (*Ucp1*$^{-/-}$-vehicle, open red squares, *Ucp1*$^{+/+}$-vehicle, open blue circles) for 8 days, n = 5–6. *P < 0.05 *Ucp1*$^{+/+}$-leptin vs *Ucp1*$^{-/-}$-leptin; ‡P < 0.05 leptin vs vehicle treated *Ucp1*$^{-/-}$ mice. †P < 0.05 leptin vs vehicle treated *Ucp1*$^{+/+}$ mice.

peripheral tissues through parasympathetic or sympathetic efferents, nor does it require UCP1-mediated thermogenesis.

The current study suggests that leptin lowers blood glucose through a mechanism that is independent of the ANS and UCP1; however, some limitations should be considered. Firstly, subdiaphragmatic vagotomy leaves parasympathetic innervation to tissues above the diaphragm intact, thus a role for parasympathetic innervation of supradiagphragmatic tissues cannot be ruled out. Secondly, as most of the vagotomised mice did not tolerate STZ well, it is possible that the mice that tolerated STZ have specific attributes that impair comparability to controls. While we were unable to include a vagotomised control group (non-leptin treated) due to low numbers, the recovery of hyperglycemia following cessation of leptin treatment in vagotomised mice supports that glucose lowering was leptin induced. In regard to the sympathetic nervous system, since only a partial sympathectomy was achieved, we cannot rule out that the remainder of intact sympathetic neurons could be responsible for leptin's anti-diabetic action. The similar levels of BAT *Ucp1* expression in sham-leptin and 6OHDA-leptin controls could indicate an intact sympathetic response, although BAT *Ucp1 expression* can be induced by catecholamines produced by alternatively activated macrophages [46], and thyroid hormone [31]. It should also be noted that despite only a 50% reduction in TH positive BAT area, we observed overt symptoms of sympathectomy in 6OHDA-injected mice, including ptosis and piloerection, suggesting that sympathetic tone had been substantially decreased, and yet leptin still reversed hyperglycemia. Several other studies support a sympathetic-independent mode of leptin action. ICV leptin is able to reverse diabetes in STZ-diabetic mice with a global deletion of beta-adrenergic receptors [16], and in STZ-diabetic rats treated with a combination of adrenergic receptor antagonists [14]. Moreover, the anti-diabetic action of leptin is not mimicked by administration of pharmacological β3 agonists [14,31]. Several studies have shown that in addition to reversing hyperglycemia, ICV leptin administration in rodent models of T1D robustly induces *Ucp1* expression [9,31] and glucose uptake in BAT [11,16]. Our study

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supports that leptin has a similar effect on BAT thermogenesis when administered peripherally, evidenced by increased iBAT temperature and *Ucp1* expression, and decreased BAT mass. As BAT thermogenesis requires UCP1 [25,26,29], and thermogenesis is associated with robust glucose uptake in BAT, it was unexpected that the glucose lowering effect of leptin is intact in UCP1-deficient mice. It is possible that *Ucp1* induction and BAT glucose uptake may be uncoupled, as was suggested by Matsen and colleagues [31]. Therefore, while the current study rules out a requirement for any UCP1-dependent processes, it remains possible that the stimulation of glucose utilization in BAT through other processes may contribute to leptin-induced glucose lowering. Interestingly however, the initial delay in leptin-induced glucose lowering in $Ucp1^{-/-}$ mice relative to $Ucp1^{-/-}$-vehicle and $Ucp1^{+/+}$-leptin groups, may indicate that UCP1 has a role in the initial but not the prolonged anti-diabetic response to leptin therapy.

Although the ANS does not appear to be critical for leptin action in insulin-deficient diabetes, by accessing the CNS peripherally administered leptin may lower blood glucose through a neuroendocrine mechanism. Suppression of hypothalamic pituitary adrenal (HPA) axis activity has been implicated as a potential mechanism of leptin-mediated glucose-lowering in uncontrolled diabetes [47], although recent evidence suggests the lowering of corticosterone is neither necessary nor sufficient for leptin's anti-diabetic effect [48,49]. The hypothalamic pituitary thyroid axis is also a candidate mechanism, although administration of thyroid hormone does not mimic the action of leptin in diabetic rats [31]. Peripheral leptin could also induce neuroendocrine changes through leptin-sensitive spinal afferents which have been recently identified in adipose tissue [50]. An alternative interpretation of the data presented here is that although we expect peripherally administered leptin to access and activate pathways initiated in the CNS, perhaps central leptin action is sufficient, but not required to reverse hyperglycemia due to the presence of redundant peripheral pathways. Indeed, leptin receptors are expressed in multiple peripheral tissues [51]. We previously found that hepatic leptin signaling is not required for the amelioration of hyperglycemia by leptin [2]; however, mechanisms involving direct leptin action in other peripheral tissues have not yet been ruled out. The glucose-lowering mechanism of leptin was previously suggested to involve the reversal of hyperglucagonemia [4,5], through either centrally-mediated [10,11,16], or direct [52] leptin action on alpha-cells. However, there are examples where leptin therapy has reversed hyperglucagonemia in diabetic rodents without lowering glucose levels [53,54], and acute leptin administration can lower blood glucose prior to glucagon suppression [47]. We previously found that the glucose- and glucagon-lowering actions of leptin were intact in mice with partially disrupted leptin signaling in alpha-cells [55]. Further dissection of central and peripheral pathways through which leptin lowers blood glucose is crucial to elucidating the mechanism of leptin action, and may reveal new strategies to improve glycemic control in diabetes.

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CONFLICT OF INTEREST

HCD, MMK, MMG, ET, MP, WLQ, and TJK have nothing to disclose.

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