Leptin induces fasting hypoglycaemia in a mouse model of diabetes through the depletion of glycerol

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Abstract

Aims/hypothesis Leptin has profound glucose-lowering effects in rodent models of type 1 diabetes, and is currently being tested clinically to treat this disease. In addition to reversing hyperglycaemia, leptin therapy corrects multiple lipid, energy and neuroendocrine imbalances in rodent models of type 1 diabetes, yet the precise mechanism has not been fully defined. Thus, we performed metabolic analyses to delineate the downstream metabolic pathway mediating leptin-induced glucose lowering in diabetic mice.

Methods Mice were injected with streptozotocin (STZ) to induce insulin-deficient diabetes, and were subsequently treated with 20 µg/day recombinant murine leptin or vehicle for 5 to 14 days. Energy-yielding substrates were measured in the

liver and plasma, and endogenous glucose production was assessed by tolerance to extended fasting.

Results STZ–leptin-treated mice developed severe hypoketotic hypoglycaemia during prolonged fasting, indicative of suppressed endogenous ketone and glucose production. STZ–leptin mice displayed normal gluconeogenic and glycogenolytic capacity, but had depleted circulating glycerol and NEFA. The depletion of glycerol and NEFA correlated tightly with the kinetics of glucose lowering in response to chronic leptin administration, and was not mimicked by single leptin injection. Administration of glycerol acutely reversed fasting-induced hypoglycaemia in leptin-treated mice.

Conclusions/interpretation The findings of this study suggest that the diminution of circulating glycerol reduces endogenous glucose production, contributing to severe fasting-induced hypoglycaemia in leptin-treated rodent models of type 1 diabetes, and support that depletion of glycerol contributes to the glucose-lowering action of leptin.

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Abbreviations

HMG 3-hydroxy-3-methylglutaryl
RER Respiratory exchange ratio
STZ Streptozotocin
TAG Triacylglycerol

Introduction

Administration of the adipose-derived hormone leptin can ameliorate hyperglycaemia and hyperketonaemia in rodent models of insulin-deficient type 1 diabetes, without raising

circulating insulin levels, and independent of food intake [1–4]. Several mechanisms have been proposed for this glucose-lowering action, including suppressed glucagon and improved insulin sensitivity [1–5], and central leptin therapy was shown to inhibit hepatic glucose production [6] and enhance glucose uptake in brown adipose tissue [7]. With the plethora of metabolic changes that occur with leptin administration in rodent models of type 1 diabetes, the precise mechanism of leptin action in type 1 diabetes remains unclear.

Glycerol is critical in lipid and glucose metabolism; it is the backbone for triacylglycerol (TAG) synthesis, is released along with NEFA during lipolysis and is a substrate for gluconeogenesis. Glycerol infusion at physiological concentrations stimulates glucose output from perfused mouse livers and cultured primary hepatocytes [8], and gluconeogenesis from glycerol accounts for approximately 10% of hepatic glucose production in type 2 diabetic patients [9]. Elevated circulating glycerol levels are predictive of type 2 diabetes in men [10] and glycerol gluconeogenesis is elevated in rat models of type 1 diabetes [11]. Furthermore, suppressing glycerol gluconeogenesis is a potential therapeutic strategy for treating diabetes, as deletion of glycerol transporters in the liver lowers blood glucose in diabetic *db/db* mice [12], and pharmacological inhibition of lipolysis reduces glycaemia in rats with streptozotocin (STZ)-induced diabetes [13]. Recently, Perry et al showed that the reversal of hyperglycaemia in STZ-diabetic rats by leptin treatment is associated with a reduction in circulating glycerol and glycerol gluconeogenesis [11], suggesting that leptin may reduce glycaemia through glycerol depletion.

To further elucidate the mechanism of leptin-mediated glucose lowering in type 1 diabetes, we analysed the effects of leptin therapy on energy-yielding substrates in mice with STZ-induced diabetes. We found that leptin-treated mice have a severe depletion of hepatic energy-yielding substrates, and that the kinetics of blood glucose normalisation with leptin therapy closely follows the depletion of circulating glycerol, NEFA and TAG. Moreover, the depletion of these substrates is associated with the inability of mice to undergo prolonged fasting, resulting in severe hypoketotic hypoglycaemia, which can be reversed by glycerol administration.

Methods

Mice C57Bl/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) were housed at room temperature (unless otherwise indicated) with a 12/12 h light-dark cycle with ad libitum access to food (2918, Harlan Laboratories, Madison, WI, USA) and water. All studies used littermate controls. All animal procedures were approved by the UBC Animal Care Committee and performed in accordance with the Canadian Council on Animal Care guidelines.

STZ administration Mice aged 8–10 weeks were injected i.p. with 180 mg/kg STZ (Sigma-Aldrich, St Louis, MO, USA) dissolved in sodium acetate buffer, pH 4.5, 5–7 days before pump implantation.

Leptin therapy Recombinant murine leptin (Peprotech, Rocky Hill, NJ, USA) was dissolved in water and administered by s.c. implantation of osmotic pumps (DURECT Corporation, Cupertino, CA, USA) at a dose of 20 µg/day, unless otherwise specified. For leptin injections, leptin was solubilised in water and further diluted to 0.1 mg/ml in saline (154 mmol/l NaCl).

Metabolic assays Measurement of blood glucose and plasma analytes are described in the Electronic Supplementary Material (ESM) [Methods](#). Livers were collected from mice under isoflurane anaesthesia, clamp frozen with liquid N₂ and stored at −80°C. Hepatic glycogen, glucose, glycerol, TAG and cholesterol measurements are described in the ESM [Methods](#). Hepatic CoA esters were measured by reversed phase HPLC, as described previously [14]. RT-qPCR of hepatic transcripts was performed as previously described [15], with *Ppia* as a reference gene selected by geNorm analysis, and analysed using the Pfaffl method [16]. Primer sequences are in ESM Table 1.

Body composition and indirect calorimetry Indirect calorimetry, activity and food intake were measured using PhenoMaster metabolic cages (TSE Systems, Chesterfield, MO, USA) at 29°C. Mice were acclimated to metabolic cages for 5 days before pump implantation and the metabolic run was initiated on day 3 of treatment for ~72 h. Body composition was measured immediately after by dual energy x-ray absorbance (DEXA), using a Lunar PIXImus 2.0 Densitometer (Inside Outside Sales, Madison, WI, USA). Analysis and normalisation procedures are described in the ESM [Methods](#).

Prolonged fasting test Blood glucose/samples were collected immediately before fasting and at indicated times following food removal.

Glycerol and pyruvate tolerance tests

Mice were fasted for 7 h, and received glycerol (2.5 g/kg dissolved in water), pyruvate (2 g/kg dissolved in saline) or the corresponding vehicle injection i.p. at time 0, and blood glucose was measured by glucometer.

Data analysis

Data are expressed as means ± SEM and were analysed in GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Statistical analyses between two groups were performed using a Student's *t* test, while analyses among three or more groups were performed by one- or two-way ANOVA with a Tukey post hoc test, unless otherwise stated.

Results

Leptin therapy attenuates metabolic disturbances in STZ-diabetic mice STZ-diabetic mice were implanted with s.c. osmotic pumps delivering 20 μ g/day leptin (STZ–leptin) or vehicle (STZ–vehicle). STZ–vehicle mice were hyperglycaemic, had significantly reduced plasma leptin and insulin levels, and showed a steady decline in body weight relative to non-diabetic controls (Fig. 1a–d). Leptin treatment raised plasma leptin to supraphysiological levels and normalised 4 h fasting blood glucose over the course of several days,

without restoring plasma insulin levels or affecting body weight (Fig. 1a–d). At this dose, leptin fully normalised 4 h fasted glucose levels, but fed blood glucose levels were only partially reduced (Fig. 1e–f). STZ–vehicle mice displayed polyuria, polydipsia, hyperphagia and decreased locomotor activity, relative to non-diabetic controls, all of which were largely corrected by leptin treatment (Fig. 1g–k). Despite the normalisation of food intake in STZ–leptin mice, pair-feeding studies demonstrate that the metabolic action of leptin therapy is not mimicked by caloric restriction [1–3]. Respiratory exchange ratios (RERs) were downward shifted in STZ–vehicle mice, indicative of enhanced lipid over glucose oxidation (Fig. 1l). Interestingly, RERs were partially corrected in STZ–leptin mice. Body composition was altered by STZ administration, and lean mass was modestly reduced by leptin therapy (Fig. 1m), although not when corrected for body weight (data not shown). Collectively, these results show that

Fig. 1 Leptin therapy reverses the disturbances of insulin-deficient diabetes. Four hour fasted blood glucose (a), day 8 plasma leptin and insulin (b, c), and body weight (d) were measured in STZ–leptin (grey circles/bars), STZ–vehicle (white circles/bars) and non-diabetic (black circles/bars) mice, $n=5-8$. Four hour fasted and fed blood glucose levels on days 6 and 8 in a separate cohort, respectively (e, f). Representative cages showing bedding from singly housed mice (g). Cumulative water intake (h), food intake (i), average energy expenditure (j), average locomotor activity (k), RER (l) and body composition (m). * $p<0.05$, STZ–leptin vs STZ–vehicle; † $p<0.05$, STZ–vehicle vs non-diabetic; ‡ $p<0.05$, STZ–leptin vs non-diabetic mice

leptin therapy corrects many of the metabolic disturbances of uncontrolled diabetes.

Leptin depletes hepatic energy-yielding stores in STZ-diabetic mice Several studies have shown that leptin reduces endogenous glucose production in rodent models of type 1 diabetes [1, 6, 11], suggesting that this may drive the glucose-lowering effect of leptin. Therefore, we assessed hepatic energy-yielding substrates and gluconeogenic capacity in leptin-treated STZ-diabetic mice. Expression of *Pck1*, the gene encoding phosphoenol pyruvate carboxykinase (Pepck), was elevated in STZ-diabetic mice, but was not reduced by leptin therapy (Fig. 2a). Interestingly, leptin therapy reduced transcript levels of *Slc2a2*, which encodes the bidirectional glucose transporter Glut2 (Fig. 2a). To elucidate whether gluconeogenesis is inhibited by leptin, we performed pyruvate tolerance tests. Surprisingly, STZ–leptin mice did not have a blunted gluconeogenic response to pyruvate relative to STZ–vehicle controls (Fig. 2b), suggesting that leptin therapy does not inhibit gluconeogenic capacity. Hepatic glycogen was depleted ~100 fold in STZ–leptin mice, along with a robust reduction in glucose and TAG content, while hepatic cholesterol was unaltered (Fig. 2c–f). Elevated acetyl-CoA levels in

uncontrolled diabetes were reduced by leptin therapy (Fig. 2g) with no changes in free CoA levels (Fig. 2h), indicative of increased hepatic glucose oxidation relative to lipid oxidation, consistent with RER data (Fig. 1l) and previous findings [11]. Acetyl-CoA:free CoA ratios (Fig. 2i) were not significantly altered. Hepatic 3-hydroxy-3-methylglutaryl (HMG)-CoA levels were reduced ($p=0.08$) in STZ–leptin compared with STZ–vehicle mice (Fig. 2j), reflecting a reduction in ketogenesis. Collectively, these data suggest that while inhibition of the gluconeogenic pathway cannot account for the glucose-lowering effect of leptin, leptin therapy robustly diminishes hepatic energy-yielding substrates, and normalises ketogenesis and lipid oxidation.

Leptin therapy depletes circulating glycerol and NEFA, concomitant with glucose lowering Observing the depletion of hepatic energy-yielding substrates, we examined whether this corresponded to reduced circulating energy-yielding substrates, and whether this may contribute to the reversal of hyperglycaemia. Leptin therapy gradually normalised blood glucose levels over the course of several days in STZ-diabetic mice treated with leptin; initially, there was a modest decrease in blood glucose levels, and subsequently blood glucose

Fig. 2 Leptin therapy depletes hepatic energy-yielding substrates. Livers were harvested following a 4 h fast on day 8 in STZ–leptin (grey circles/bars), STZ–vehicle (white circles/bars) and non-diabetic (black circles/bars) mice (**a, c–k**). (**a**) Abundance of hepatic *Pck1* and *Slc2a2* transcripts measured by RT-qPCR, $n=4–6$. (**b**) Pyruvate tolerance test on day 5 in a separate cohort (pyruvate injection, solid line; vehicle injection, dotted line). Hepatic glycogen (**c**), glucose (**d**), TAG (**e**) and cholesterol (**f**)

content, acetyl-CoA and free CoA levels (**g, h**), acetyl-CoA:free CoA ratio (**i**), HMG-CoA (**j**) and succinyl-CoA (**k**), $n=6–8$. * $p<0.05$, STZ–leptin vs STZ–vehicle; † $p<0.05$, STZ–vehicle vs non-diabetic; ‡ $p<0.05$, STZ–leptin vs non-diabetic; § $p<0.05$, pyruvate vs vehicle injection in the STZ–leptin group. ¶ $p<0.05$ pyruvate vs vehicle injection in the STZ–vehicle group

Fig. 3 The kinetics of hyperglycaemia reversal by leptin correlates with reduced plasma glycerol. Four hour fasted blood glucose (**a**) and plasma levels of leptin (**b**), lactate (**d**), alanine (**e**), glycerol (**f**), NEFA (**g**), β -hydroxybutyrate (β OH, **h**), TAG (**i**) and cholesterol (**j**), measured daily in STZ–leptin (grey circles), STZ–vehicle (white circles) and non-diabetic (black circles) mice, $n=5$ –10. Hepatic glycogen in a matched cohort on day 1 post pump implant, $n=5$ (**c**). * $p<0.05$, STZ–leptin vs STZ–vehicle; † $p<0.05$, STZ–vehicle vs non-diabetic; ‡ $p<0.05$, STZ–leptin vs non-diabetic mice

rapidly fell, reaching non-diabetic levels by day 4 (Fig. 3a). This slow normalisation of glucose occurred despite maximal leptin levels being achieved by the first day of leptin therapy (Fig. 3b). Remarkably, 4 h fasted hepatic glycogen levels were depleted after 24 h of leptin therapy relative to diabetic and non-diabetic controls (Fig. 3c); however, STZ–leptin mice remained hyperglycaemic until day 4. We next examined the levels of three circulating gluconeogenic substrates; while there was no lasting effect on plasma lactate and alanine levels (Fig. 3d, e), plasma glycerol levels gradually reduced to ~65% relative to both controls and initial levels in STZ–leptin mice (Fig. 3f), mirroring the kinetics of glucose lowering. Plasma β -hydroxybutyrate, NEFA and TAG levels decreased following similar kinetics (Fig. 3g–i). Injection of leptin to induce acute hyperleptinaemia similar to the level achieved by pump implants did not reduce circulating glucose, NEFA, TAG, β -hydroxybutyrate or glycerol levels compared with vehicle-injected STZ-diabetic controls (ESM Fig. 1). As the regulation of glycerol metabolism by leptin has not been thoroughly described, we also examined glycerol levels in leptin deficient (*ob/ob*) and leptin receptor deficient (*db/db*) mice (ESM Fig. 2); both models had increased plasma glycerol relative to lean controls, suggesting a role for leptin in glycerol metabolism. Collectively, these findings reveal that the reversal of hyperglycaemia and hyperketonaemia by chronic leptin administration is associated with the gradual reduction of energy-yielding substrates, namely glycerol, NEFA and TAG.

Leptin-treated STZ-diabetic mice are intolerant to fasting We postulated that the depletion of key energy-yielding substrates by leptin therapy might critically limit endogenous glucose production during fasting. Therefore, we assessed whether STZ–leptin mice could maintain blood glucose levels during a prolonged fast. Following food removal, glycaemia rapidly declined in STZ–leptin mice, reaching those of non-diabetic mice in 2 h, and subsequently progressing to overt hypoglycaemia (Fig. 4a, ESM Table 2). In contrast, non-diabetic and STZ–vehicle controls maintained stable glycaemic levels. In the non-fasted state, plasma glucagon was normalised in STZ–leptin mice (Fig. 4b), consistent with previous reports [2]. In response to fasting, hyperglucagonaemia was diminished in STZ–vehicle mice, potentially indicative of an impaired stimulated-glucagon response as has been shown for STZ-diabetic animals [17, 18],

whereas STZ–leptin mice had a non-significant trend towards increased glucagon levels compared with non-fasted levels. As corticosterone is implicated in the glucose-lowering action

Fig. 4 Leptin-treated STZ-diabetic mice are sensitive to prolonged fasting. STZ-diabetic mice were treated with leptin (grey bars/grey dotted line) at a dose of 20 µg/day (a–c) or 10 µg/day (d–f) and compared with STZ-vehicle (white bars/black dotted line) and non-diabetic controls (black bars/solid black line) on day 7 or 11 of treatment, respectively, $n=4$ –7. One STZ-leptin mouse was rescued at 8 h (a) and 14 h (d)

because of hypoglycaemia (blood glucose <2 mmol/l). Plasma glucagon (b), corticosterone (c), β -hydroxybutyrate (β OH, e) and leptin (f) were measured at the indicated times. * $p<0.05$, STZ-leptin vs STZ-vehicle; † $p<0.05$, STZ-vehicle vs non-diabetic; ‡ $p<0.05$, STZ-leptin vs non-diabetic; § $p<0.05$, between time points

of leptin [11], we examined whether suppressed corticosterone levels could account for fasting-induced hypoglycaemia in STZ-leptin mice (Fig. 4c). In the non-fasted state, hypercorticonaeemia was reversed in STZ-leptin mice, consistent with previously published observations [11]. Interestingly, however, STZ-leptin mice displayed a robust counter-regulatory corticosterone response during prolonged fasting. These data suggest that the hypoglycaemic effect of leptin therapy is independent of plasma corticosterone levels. We repeated the prolonged fast in mice treated with a lower dose of leptin (10 µg/day), in which hyperglycaemia is more severe in the fed state (Fig. 4d, ESM Table 3). Again, glycaemic levels rapidly declined during fasting in the STZ-leptin group, and three STZ-leptin mice exhibited hypoglycaemia by 15 h. Expectedly, 4 h fasted β -hydroxybutyrate levels were normalised in STZ-leptin mice (Fig. 4e), yet, while non-diabetic controls displayed significantly increased 15 h fasted β -hydroxybutyrate levels compared with 4 h fasted levels, this was completely abrogated in STZ-leptin-treated mice. These data are consistent with a robust suppression of endogenous glucose and ketone production. Unlike non-diabetic and STZ-vehicle controls, leptin levels were not reduced after 15 h of fasting in STZ-leptin mice due to exogenous leptin infusion (Fig. 4f), suggesting that the inability to suppress leptin could be deleterious in prolonged fasting conditions.

Next, we examined the dynamics of energy-yielding substrates between the fed and prolonged fasted (9 h) states. As

above (Fig. 4e), STZ-leptin mice displayed the expected hypoketonaemia during prolonged fasting (Fig. 5a). Non-fasted glycogen stores were ~60% lower in STZ-leptin mice compared with STZ-vehicle controls (Fig. 5b), but not depleted to the same extent as seen with 4 h fasting (Figs. 2c, 3c). In response to prolonged fasting, hepatic glycogen was decreased in all groups including STZ-leptin mice relative to non-fasted levels, revealing that STZ-leptin mice are capable of mobilising hepatic glycogen stores. Plasma lactate (Fig. 5c) and alanine (Fig. 5d) levels were significantly reduced by fasting in all treatment groups, but were not significantly altered by leptin treatment relative to controls. In contrast, fed and prolonged fasted plasma glycerol levels were respectively reduced by 54% ($p=0.092$) and 72% ($p=0.088$) in STZ-leptin mice, respectively relative to STZ-vehicle mice, although this did not reach statistical significance (Fig. 5e). Correlating with β -hydroxybutyrate levels, there was a trend towards increased NEFA in non-diabetic mice in response to fasting, whereas STZ-leptin mice had diminished NEFA after extended fasting (Fig. 5f). Fed and fasted plasma TAG levels were also diminished by leptin therapy (Fig. 5g), and plasma cholesterol was modestly decreased (Fig. 5h). These data indicate that fasting-induced hypoglycaemia and hypoketonaemia in leptin-treated mice may be due to insufficient availability of glycerol and NEFA. Interestingly, when leptin was injected to achieve a beneficial effect on glycaemia, and plasma leptin allowed to return to baseline levels following injection, this resulted in rapid repletion of plasma

Fig. 5 Gluconeogenic substrate availability is altered during prolonged fasting in leptin-treated mice. On day 7 of leptin therapy, STZ–leptin (grey bars), STZ–vehicle (white bars) and non-diabetic (black bars) mice were killed after 0 or 9 h of fasting (blood glucose for 9 h fasted mice in Fig. 4a), and plasma β -hydroxybutyrate (β OH, **a**), hepatic glycogen (**b**),

plasma lactate (**c**), alanine (**d**), glycerol (**e**), NEFA (**f**), TAG (**g**) and cholesterol (**h**) were measured, $n=5$. * $p<0.05$, STZ–leptin vs STZ–vehicle; † $p<0.05$, STZ–vehicle vs non-diabetic; ‡ $p<0.05$, STZ–leptin vs non-diabetic; § $p<0.05$, for same group 0 h vs 9 h fasted

glycerol; subsequently upon prolonged fasting, STZ–leptin mice were able to maintain steady blood glucose levels (ESM Fig. 3).

Acute glycerol infusion prevents fasting hypoglycaemia in leptin-treated mice To test whether the depletion of glycerol may mediate the hypoglycaemic effect of leptin in the mouse model of type 1 diabetes, we examined whether acute glycerol administration could stimulate gluconeogenesis in STZ–leptin mice and prevent fasting hypoglycaemia. Notably, 4 h fasted hepatic glycerol content was reduced in STZ–leptin mice relative to controls, consistent with decreased glycerol delivery to the liver (Fig. 6a). Hepatic transcript abundance of *Aqp9* (aquaglyceroporin9), which facilitates hepatic glycerol uptake, was also reduced (Fig. 6b). To examine the capacity for glycerol gluconeogenesis in STZ–leptin mice, 7 h fasted mice were injected with vehicle or glycerol. Expectedly, the STZ–leptin mice injected with vehicle continued progressing towards hypoglycaemia (Fig. 6c). In contrast, glycerol injection acutely raised blood glucose levels, temporarily ameliorating fasting hypoglycaemia in STZ–leptin mice. The glycaemic response to glycerol in STZ–leptin mice is consistent with intact glycerol-fuelled gluconeogenesis. STZ–vehicle and STZ–leptin mice had significantly increased plasma corticosterone and similar plasma glucagon levels following the injection of glycerol (ESM Fig. 4). Taken together with the reduced glycerol gluconeogenic flux reported by Perry et al [11], these data suggest that glycerol depletion contributes to

leptin-induced hypoglycaemia during fasting in this mouse model of type 1 diabetes.

Discussion

In the current study, we found that glucose normalisation following leptin therapy in STZ-diabetic mice correlated with the gradual depletion of circulating glycerol, NEFA and TAG. Leptin-treated STZ-diabetic mice rapidly developed hypoketotic hypoglycaemia during prolonged fasting, consistent with reduced gluconeogenesis and ketogenesis. However, STZ–leptin mice were capable of mobilising hepatic glycogen stores (as shown by the reduction of hepatic glycogen before and after fasting), and did not have a diminished gluconeogenic response to pyruvate, relative to controls, indicating that inhibition of glycogenolysis or gluconeogenesis pathways cannot account for the glucose-lowering action of leptin. Leptin-induced hypoketotic hypoglycaemia correlated with depleted plasma glycerol, NEFA and TAG levels, indicative of insufficient substrate availability. Injection of glycerol during prolonged fasting and acute repletion of endogenous glycerol following clearance of injected leptin, ameliorated hypoglycaemia in STZ–leptin mice, suggesting that the gluconeogenic response to glycerol is intact, and that glycerol depletion plays a key role in hypoglycaemia. In parallel, our data are consistent with insufficient NEFA availability for

Fig. 6 Glycerol injection prevents hypoglycaemia in STZ-leptin-treated mice. Hepatic glycerol content (**a**) and transcript abundance of *Aqp9* (**b**) in livers of 4 h fasted STZ-leptin (grey bars), STZ-vehicle (white bars) and non-diabetic mice (black bars), on day 8 of treatment, $n=6-7$. (**c**) Mice were injected with either glycerol (solid lines) or vehicle (dotted lines), on day 5 of treatment, $n=4-8$. Statistical analyses in (**c**) were

performed by two-way ANOVA with a Sidak post hoc test. $*p<0.05$, STZ-leptin vs STZ-vehicle; $^{\dagger}p<0.05$, STZ-vehicle vs non-diabetic; $^{\ddagger}p<0.05$, STZ-leptin vs non-diabetic. $^{\S}p<0.05$, glycerol vs vehicle injection in the STZ-leptin group. $^{\P}p<0.05$ pyruvate vs vehicle injection in the non-diabetic group

oxidation causing fasting hypoketonaemia. Collectively, these data reveal that leptin treatment limits energy substrate availability in STZ-diabetic mice, and that the depletion of glycerol in particular may limit gluconeogenesis resulting in hypoglycaemia during extended fasting.

The inability of leptin-treated STZ-mice to tolerate prolonged fasting is consistent with several other studies showing that leptin therapy decreases hepatic or endogenous glucose production in rodent models of type 1 diabetes [1, 6, 11]. While leptin therapy suppresses glucagon levels in STZ-diabetic mice [2–5, 7, 19], leptin therapy can reverse hyperglycaemia prior to glucagon suppression [11], and can reverse hyperglucagonaemia without normalising glycaemia in STZ-diabetic rats [5]; this suggests that glucagon suppression alone cannot sufficiently account for the glucose-lowering action of leptin. Our finding that, during prolonged fasting, STZ-leptin mice showed a trend towards elevated plasma glucagon, but were hypoglycaemic, supports this notion. Interestingly, although the Perry et al study implicated the suppression of corticosterone in the glucose-lowering effect of leptin [11], we observed a robust elevation of plasma corticosterone during fasting hypoglycaemia in STZ-leptin mice, indicating that the hypoglycaemic effect of leptin during fasting is at least partly independent of corticosterone suppression. Alternatively, leptin can stimulate glucose uptake in the peripheral tissues of rodent models of type 1 diabetes [6, 7]. However, glucose utilisation in white adipose and some skeletal muscle groups is unaltered in response to leptin [6, 7], and other studies report that glucose uptake or disposal is not substantially augmented by leptin therapy [1, 4]. Considered with the profound fasting intolerance in the current study, these findings suggest that energy-yielding substrate depletion plays an important role in the glucose-lowering action of leptin.

While this study suggests that glycerol depletion contributes to fasting-induced hypoglycaemia in leptin-treated STZ-diabetic rodents, its role in the reversal of hyperglycaemia by leptin is less clear. Perry et al showed that leptin infusion in rat models of type 1 diabetes reduced glycerol gluconeogenesis

by ~90% [11]. Although this could be achieved by inhibition of the glycerol gluconeogenic pathway by leptin, our data reveal that STZ-leptin mice have reduced hepatic glycerol concentrations (consistent with substrate depletion), and a robust gluconeogenic response to glycerol injection. Moreover, it has been widely reported that leptin therapy takes several days to normalise glycaemia in diabetic rodents [2–4, 6, 7, 19–26], and the current study shows this correlates with the gradual diminution of glycerol levels. Perry et al found that infusion of intralipid with heparin (raising both NEFA and glycerol levels) prevents the glucose-lowering effects of leptin in STZ-diabetic rats [11]. While the Perry study did not test the individual contribution of glycerol and NEFA replacement, our study suggests that glycerol depletion could at least contribute to the hypoglycaemic action of leptin. However, our study also revealed that neither acute glycerol injection nor acute replenishment of endogenous glycerol, fully restored glycaemia to STZ-vehicle levels, indicating that depletion of other energy-yielding substrates (i.e. glycogen, NEFA, TAG), or additional mechanisms, contribute to leptin-induced glucose lowering. Collectively, these observations suggest that glycerol depletion is critical for the hypoglycaemic action of leptin, and that its role in the beneficial glucose-lowering action of leptin in type 1 diabetes warrants further investigation.

Several important concepts emerge in light of the fasting intolerance induced by leptin therapy in STZ-diabetic mice. First, this demonstrates that the level of glycaemic normalisation achieved by leptin is dependent on fasting duration, which should be taken into account when comparing results across studies. Differences in fasting duration could account for discrepancies between studies as to whether physiological leptin doses reverse hyperglycaemia in STZ-diabetic rodents [5, 11, 20], and why studies examining free-feeding or 2 h fasted rodents have not reported leptin-induced hypoglycaemia [2, 3, 7, 19]. Second, the hypoketotic hypoglycaemia observed during prolonged fasting suggests that leptin therapy may carry hypoglycaemic risk in type 1 diabetes. We have reported similar profound hypoglycaemic

effects of leptin in STZ-diabetic mice during insulin tolerance tests [4] and in the recovery phase of glucose tolerance tests [20]. Importantly, when leptin levels were allowed to fall to baseline levels prior to fasting, hypoglycaemia was not observed, corresponding to a lack of glycerol depletion. This suggests that hyperleptinaemia and glycerol depletion are necessary for hypoglycaemia to occur in STZ-diabetes, and that diabetic patients given leptin by injection may suffer hypoglycaemia, depending upon when fasting occurs in relation to leptin injection. Taken together, our study suggests that hyperleptinaemia may cause hypoglycaemia during prolonged fasting due to glycerol depletion, and highlights that additional studies are warranted to fully assess the hypoglycaemic risk of leptin for type 1 diabetes treatment.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement HCD, SDC and TJK conceived and designed experiments. HCD acquired and analysed data, and wrote the manuscript. MMK, WLQ and UHN acquired and analysed data. JEK and RWB designed experiments, acquired and analysed data for all HPLC experiments. SK and SMC designed experiments, acquired and analysed data for all metabolic cage experiments. All authors were involved in the discussion and revision of the manuscript, and approved the final version to be published. TJK is the guarantor of this work.

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