Published in final edited form as:

Atherosclerosis. 2012 December ; 225(2): 341–347. doi:10.1016/j.atherosclerosis.2012.10.031.

Leptin treatment inhibits the progression of atherosclerosis by attenuating hypercholesterolemia in type 1 diabetic *Ins2^{+/Akita}:apoE^{-/-}* mice

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Abstract

Aims—The impact of leptin deficiency and its replacement in T1D remain unclear in the context of dyslipidemia and atherosclerosis. The current study has investigated the physiologic role of leptin in lipid metabolism and atherosclerosis in T1D.

Methods and results—The present study has employed *Ins2^{+/Akita}:apoE^{-/-}* mouse model that spontaneously develops T1D, hypercholesterolemia, and atherosclerosis. At age 13 weeks, diabetic *Ins2^{+/Akita}:apoE^{-/-}* mice showed leptin deficiency by ~92% compared with nondiabetic *Ins2^{+/+}:apoE^{-/-}* mice. From 13 weeks to 25 weeks of age, diabetic *Ins2^{+/Akita}:apoE^{-/-}* mice were treated with low-dose leptin (at 0.4 µg/g body weight daily). Leptin treatment diminished food intake by 22–27% in diabetic mice without affecting body weight and lean mass throughout the experiment. Importantly, leptin therapy substantially reduced plasma cholesterol concentrations by ~41%, especially in LDL fractions, in diabetic *Ins2^{+/Akita}:apoE^{-/-}* mice. Moreover, leptin therapy decreased atherosclerotic lesion in diabetic mice by ~62% comparable to that seen in nondiabetic mice. In addition, leptin restored repressed expression of hepatic sortilin-1, a receptor for LDL clearance, and reversed altered expression of several hepatic genes involved in lipogenesis and cholesterol synthesis characteristic of diabetic mice. These findings were accompanied by normalization of reduced hepatic expression of IRS1 and IRS2 mRNA as well as their protein levels, and improved hepatic insulin receptor signaling.

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Conflict of interest

No conflict of interest.

Conclusions—The present findings suggest that leptin administration may be useful to improve dyslipidemia and reduce atherosclerosis-related cardiovascular disease in human subjects with T1D.

Keywords

type 1 diabetes; leptin; dyslipidemia; atherosclerosis

1. Introduction

Despite advances in care, type 1 diabetes (T1D) continues to be associated with an increased risk of cardiovascular disease (CVD) [1]. Elevated atherogenic lipoproteins are found even in young adults and adolescents with T1D [2] but the treatment strategies to alleviate dyslipidemia and atherosclerosis in T1D remain limited.

Recent studies suggest that leptin deficiency contributes to impaired hepatic glucose and lipid metabolism in patients with T1D. To date, however, the causal relationship between hypoleptinemia and accelerated atherosclerosis in T1D has not been fully elucidated. Low plasma leptin levels are associated with increased cardiovascular events and mortality in patients with stable coronary artery disease [3]. Leptin deficiency due to leptin gene mutations or lipodystrophy in rodents and humans is characterized by dyslipidemia and ectopic lipid deposition. These defects are significantly resolved by the administration of leptin [4, 5], demonstrating the important role of leptin in regulating lipid metabolism. Studies using pharmacologic doses or overexpression of leptin in T1D animal models demonstrate beneficial effects of leptin on glucose metabolism [6, 7] and lipid metabolism [7]. Taken together, these data suggest that leptin therapy may improve dyslipidemia and alleviate atherosclerosis-associated CVD in patients with T1D. However, aforementioned T1D animal studies used supraphysiologic doses of leptin for a short period of time (12~14 days). Although the study in NOD mice [7] showed limited data wherein lower doses of leptin in combination with insulin can be used to achieve the glucose-lowering effect, this was not extended to determining whether the favorable effects on lipid metabolism are still evident with this lower dose. Moreover, no long-term studies have been conducted to directly assess the role of leptin deficiency in lipid metabolism in the context of exaggerated hypercholesterolemia and atherosclerosis associated with T1D.

Based on these considerations, the current study has investigated the physiologic role of leptin in lipid metabolism and atherosclerosis using $Ins2^{+/Akita};apoE^{-/-}$ mice. Of importance, $Ins2^{+/Akita};apoE^{-/-}$ mice spontaneously develop not only T1D with typical features of reduced body weight and hyperglycemia, but also exhibit exaggerated hypercholesterolemia and atherosclerosis while avoiding both the potential adverse effects of chemicals such as streptozotocin and dietary intervention such as an atherogenic or high-fat diet [8]. We hypothesize that T1D $Ins2^{+/Akita};apoE^{-/-}$ mice characterized by reduced adiposity exhibit hypoleptinemia, which would promote exaggerated hypercholesterolemia and atherosclerosis. To this end, we injected either vehicle or leptin intraperitoneally in T1D $Ins2^{+/Akita};apoE^{-/-}$ mice on a standard chow diet.

2. Materials and methods

Full details are presented in the supplementary materials and methods.

3. Results

3.1. Leptin deficiency and leptin treatment in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice

In T1D $Ins2^{+/Akita};apoE^{-/-}$ mice at age 13 weeks, both fasting plasma insulin and leptin concentrations were significantly reduced by ~ 70% (84.2 ± 29.0 pg/mL vs. 285.2 ± 15.1 pg/mL, $n = 4-6$ /group, $P < 0.01$) and by ~ 92 % (0.28 ± 0.08 ng/mL vs. 3.30 ± 0.46 ng/mL, $n = 4-6$ /group, $P < 0.01$), respectively, compared with nondiabetic $Ins2^{+/+};apoE^{-/-}$ mice (Fig. S1-A).

Previously, leptin has been used as a replacement dose in the range of 2.4 – 9.6 μ g/day [7], which approximates to 0.1 - 0.4 μ g/g body weight/day of leptin treatment. Following intraperitoneal injection of recombinant murine leptin at 0.2 μ g/g body weight, plasma leptin concentrations were serially measured over the course of 5 hours in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice. (Fig. S1-B) The observed changes of plasma leptin concentrations followed a one compartment open model with first order absorption and elimination kinetics, as described in earlier studies [9]. The calculated mean area under the curve for 5 hours was 46.0 ng/mL•h. Accordingly, twice daily injection (92 ng/mL•h daily) was expected to expose diabetic mice to high normal range of leptin found in nondiabetic mice. Therefore, intraperitoneal injection of recombinant murine leptin (at 0.2 μ g/g body weight) was given twice daily for rational leptin treatment in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice. In order to determine whether leptin deficiency contributes to accelerated atherogenesis in T1D, we developed an experimental protocol in which a group of diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice received leptin therapy for 12 weeks beginning at 13 weeks until 25 weeks of age. As controls, a separate group of diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice and littermate nondiabetic $Ins2^{+/+};apoE^{-/-}$ mice were given PBS (Fig. S1-C).

3.2. Leptin therapy reduced food intake but did not affect body weight or body composition in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice

The well-known physiologic effect of leptin is regulation of food intake and body weight [10]. As shown in Fig. 1A, diabetic mice had significantly increased food intake by 35-40 % compared with nondiabetic mice. Leptin administration rapidly decreased food intake in diabetic mice by 22-27%. Throughout the course of the 12-week experiment, reduced food intake was maintained by leptin treatment. Diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice on PBS showed a decrease in body weight, lean and fat mass, especially after age 20~21 week (Fig. 1B & C) while nondiabetic mice gradually gained body weight and fat mass. Interestingly, despite reduced food intake, leptin-treated diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice maintained their body weight with a trend of increasing lean mass by ~9.7% and decreasing fat mass by ~ 23% by the end of the experiment compared with PBS-treated diabetic mice.

3.3. Leptin therapy attenuated hypercholesterolemia in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice

A time-course study demonstrated that leptin treatment did not significantly affect fasting plasma glucose, insulin, or triglyceride concentrations in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice (Fig. 2A-C). On the other hand, leptin administration resulted in a substantial reduction in plasma cholesterol concentrations in diabetic mice by ~25 % after 4 weeks and by ~41% after 12 weeks of treatment compared with PBS-treated diabetic mice (Fig. 2D). Notably, while PBS-treated diabetic mice showed progressively increasing cholesterol concentrations as they aged, leptin administration prevented such progression of hypercholesterolemia and reduced plasma cholesterol concentrations to the range seen in nondiabetic mice by the end of the experiment. FPLC analysis revealed a significant reduction of cholesterol in atherogenic LDL fractions by ~33% in leptin-treated diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice (LDL-cholesterol: 385 mg/dL vs. 575 mg/dL) compared with PBS-treated diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice (Fig. 2E).

In order to determine whether reduced food intake associated with leptin administration contributes to attenuation of hypercholesterolemia, we performed pair-feeding experiments. Diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice were pair fed to achieve the ad libitum food intake of leptin-treated diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice for 4 weeks from 13 weeks to 17 weeks of age. While pair feeding further reduced the body weight of diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice, it did not affect the plasma cholesterol concentrations in these mice (Fig. S2). These findings suggest that cholesterol-lowering effects of leptin in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice occur by mechanisms independent of the changes in food intake.

3.4. Leptin therapy decreased atherosclerotic lesion in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice

At age 25 weeks, mice were euthanized and subjected to the analysis of atherosclerotic lesion area in the aorta. As previously reported [8], the atherosclerotic lesion was clustered in the aortic arch (Fig. 3A), and hence, this region was used for lesion quantification. As shown in Fig. 3B & C, the atherosclerotic lesion areas of the aortic arch in nondiabetic $Ins2^{+/+};apoE^{-/-}$ mice on PBS, diabetic $Ins2^{+/Akita};apoE^{-/-}$ on PBS and diabetic $Ins2^{+/Akita};apoE^{-/-}$ treated with leptin were $2.34 \pm 1.05\%$, $10.89 \pm 1.16\%$ and $4.19 \pm 1.29\%$, respectively ($n = 4-6$) as revealed by *en face* analysis. Thus, 12-week leptin treatment reduced the atherosclerotic lesion area by ~62 % in diabetic $Ins2^{+/Akita};apoE^{-/-}$ close to the levels seen in nondiabetic mice.

3.5. Leptin therapy restored hepatic sortilin-1 expression in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice

Impaired clearance of lipoprotein contributes to exaggerated hypercholesterolemia in T1D [11]. ApoE-mediated lipoprotein clearance occurs through heparan sulfate proteoglycans (HSPGs), lipoprotein receptor-related protein (LRP), and LDL receptor, while apoB-mediated lipoprotein clearance occurs through LDL receptor [12] and lipolysis-stimulated lipoprotein receptor (LSR) [13]. Recently, hepatic sortilin-1 was also shown to serve as a cell-surface receptor for LDL catabolism [14]. In apoE-deficient mice, HSPG and LRP-mediated processes are impaired, and thus apoB-mediated pathways are mainly responsible for lipoprotein clearance. Leptin treatment was shown to increase hepatic LSR expression in wild-type mice [15] and leptin-deficient *ob/ob* mice exhibit marked suppression of sortilin-1 expression [16]. Therefore, in order to examine whether altered expression of hepatic lipoprotein receptors contributes to improved hypercholesterolemia in leptin-treated diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice, we performed immunoblot analysis using liver tissue extracts. As shown in Fig. 4, there was no significant change in hepatic LDL receptor expression between groups. While diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice showed mildly reduced LSR expression, leptin treatment did not affect its expression. On the contrary, leptin treatment fully restored markedly reduced expression of sortilin-1 in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice.

3.6. Altered hepatic gene expression in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice after leptin therapy

To investigate the possible molecular mechanisms through which leptin regulates lipid metabolism in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice, we examined hepatic gene expression using quantitative real-time PCR (Fig. 5A). As expected, hepatic expression of the key gluconeogenic gene *Pepck* was markedly increased by approximately twofold in the liver of diabetic mice relative to nondiabetic controls. Leptin treatment at physiologic dose did not significantly affect the expression of *Pepck* gene, providing an explanation for persistent hyperglycemia in leptin-treated mice. Expression of genes related to insulin-receptor signaling *Irs1* and *Irs2* were significantly decreased in diabetic mice while the expression of *Foxo1* was not changed. Interestingly, leptin treatment considerably restored the expression levels of *Irs1* and *Irs2*. Leptin partially or fully restored altered expression of several key genes involved in fatty acid metabolism/lipogenesis in diabetic mice. The expression of gene *Cd36*, fatty acid transporter, was significantly increased by ~2 fold in diabetic mice but lowered to near-normal levels with leptin treatment. Similar changes in hepatic mRNA

expression was noted for gene *Pgc1a*, which is an important coactivator for genes involved in hepatic fatty acid oxidation and whose overexpression is implicated in hepatic insulin resistance [17]. Leptin treatment in diabetic mice normalized decreased expression of *Gpat* (an enzyme for triglycerides synthesis) and *Ppara* while it did not change reduced expression of *Fas*, *Scd1*, *Sirt1* and *Srebp1c*. On the other hand, while leptin treatment in diabetic mice restored the expression levels of *Srebp1a* and *Srebp2* (transcription factors involved in cholesterol synthesis), it did not affect the expression of *Acat1*, *Acat2*, and *Hmgcr* (enzymes for cholesterol synthesis).

3.7. Leptin therapy restored the expression of IRS-1 and -2 in diabetic *Ins2^{+/Akita}:apoE^{-/-}* mice

Insulin and leptin can both stimulate IRS-mediated PI 3-kinase activity via insulin receptor tyrosine kinase and JAK2-dependent pathway [18], respectively. At the same time, leptin receptor signaling exerts its major metabolic functions through STAT-3 and also mediates signaling by ERK [18]. Since leptin restored the expression of gene *Irs1* and *Irs2*, we investigated the effects of leptin treatment on hepatic insulin receptor signaling cascade. As shown in Fig. 5B, diabetic mice had considerably reduced phosphorylation of both STAT-3 and ERK in the liver. Leptin treatment completely restored their phosphorylation in diabetic mice. These results suggest that hepatic leptin receptor signaling remains largely intact in diabetic mice. Consistent with quantitative real-time PCR data, leptin treatment not only reversed reduced expression of IRS-1 and -2 proteins but also resulted in improved phosphorylation of Akt in diabetic mice. Furthermore, insulin tolerance tests were performed to determine whether leptin treatment improves insulin sensitivity and insulin-stimulated phosphorylation of hepatic Akt in diabetic mice (Fig. S3). After a 4-week treatment from 13 weeks to 17 weeks of age, leptin treatment significantly improved the glucose-lowering effect of insulin in diabetic mice. These findings were associated with restored phosphorylation of hepatic Akt in leptin-treated diabetic mice. Taken together, these data support the notion that leptin may exert its biologic actions by improving hepatic insulin receptor signaling cascades in the setting of T1D.

4. Discussion

The current study reveals several important and novel observations on the metabolic action of leptin in the context of dyslipidemia and accelerated atherosclerosis associated with T1D. First, the sensitivity to the biologic actions of leptin is preserved for an extended period of time in lean T1D mice. Second, leptin treatment alone inhibits the progression of atherosclerotic lesion in T1D mice despite persistent hyperglycemia. Third, leptin treatment exerts cholesterol-lowering effect without an impact on glucose and triglyceride concentrations in T1D. Finally, leptin therapy restores decreased expression of hepatic IRS-1 and -2 and in turn improves proximal hepatic insulin receptor signaling in T1D. To our knowledge, the current study is the first to demonstrate the therapeutic potential of long-term treatment with low-dose leptin toward limiting atherosclerosis and the associated lipid changes in a T1D model.

The clinical usage of leptin has been limited due to the development of leptin resistance in the setting of obesity. In addition, the resultant hyperleptinemia often coexist with insulin resistance and are correlated with increased cardiovascular complications in people with metabolic syndrome and type 2 diabetes [19]. On the other hand, low leptin levels predict cardiovascular mortality in patients with stable coronary artery disease [3] and in middle-aged women with normal or impaired glucose tolerance [20]. Thus, the impact of leptin on cardiovascular conditions is complex.

In the present study, in $Ins2^{+/Akita}:apoE^{-/-}$ mice, an animal model of spontaneous T1D, hypercholesterolemia and atherosclerosis [8], leptin-induced reduction in food intake persisted throughout the 12-week time frame. Additionally, leptin treatment resulted in partial reversal of hyperphagia without affecting hyperglycemia, probably due to profound insulin deficiency in these mice. Remarkably, despite reduced food intake and persistent hyperglycemia, leptin-treated diabetic mice maintained their body weight and lean mass with modestly reduced fat mass, suggesting that leptin alone alleviates the wasting effects of uncontrolled T1D. These effects may be attributable to leptin-induced attenuation of increased energy expenditure caused by T1D [7, 21]. Together, these findings indicate that in T1D, leptin sensitivity is preserved and leptin therapy partially reverses its catabolic state despite persistent hyperglycemia, providing a unique metabolic environment to investigate the long-term therapeutic potential of leptin.

Previous studies examining the role of leptin in atherogenesis have yielded conflicting findings. Leptin deficiency was shown to attenuate atherosclerotic lesion formation when plasma cholesterol concentrations in leptin-deficient and littermate control groups were matched by a means of dietary intervention [22, 23]. In contrast, leptin-deficient *ob/ob* mice under *ldlr*^{-/-} background [24] and leptin-receptor defective *db/db* mice under *apoE*^{-/-} background [25] exhibited severe hypercholesterolemia and marked acceleration of atherosclerosis on a standard chow diet. Of note, the above strategies involving deletion of leptin gene or leptin receptor gene result in significant obesity, hyperinsulinemia and insulin resistance in animals. In our current study, however, leptin deficiency was observed in lean and insulin-deficient diabetic $Ins2^{+/Akita}:apoE^{-/-}$ mice and was associated significant hypercholesterolemia and accelerated atherosclerosis on a standard chow diet. With regard to exogenously administered leptin, Bodary et al. [26] have shown that intraperitoneal delivery of pharmacologic dose of leptin (125 µg/day) to high-fat fed nondiabetic *apoE*^{-/-} mice enhanced atherosclerosis without affecting plasma cholesterol levels. In the present study, low-dose (~10 µg/day) leptin administration to T1D $Ins2^{+/Akita}:apoE^{-/-}$ mice on a standard chow diet attenuated hypercholesterolemia and atherosclerosis. Together, these findings suggest that the extent of beneficial or detrimental effects of leptin toward limiting atherosclerotic lesion progression may depend on several factors, including lean vs. obese phenotype, insulin deficiency vs. hyperinsulinemia, and normal vs. atherogenic diet.

The mechanisms by which leptin regulates lipid metabolism in T1D have not been fully elucidated. Previous studies suggest suppressive effects of leptin on lipogenesis [4] and VLDL production/secretion [27]. Wang et al. showed limited data that a pharmacologic dose of leptin had the favorable effects on lipid metabolism in T1D NOD mice [7]. However, it was a short-term study (12 days) and did not examine whether a lower dose of leptin would maintain favorable effects on lipid metabolism. Our current study provides direct evidence that leptin treatment not only lowered plasma cholesterol concentrations but also suppressed atherogenesis in a T1D mouse model with human-like lipid profile. However, the cholesterol-lowering effects of leptin cannot be fully explained by altered expression of hepatic genes. While leptin normalized enhanced expression of genes (*Cd36* and *Pgc1a*) and reduced expression of genes (*Gpat*, *Ppara*, *Srebp1a*, and *Srebp2*) in T1D, it had little impact on other genes involved in lipogenesis and cholesterolgenesis, in particular, *Hmgcr*, a rate-limiting enzyme in cholesterol synthesis. On the other hand, impaired lipoprotein clearance is likely responsible for exaggerated hypercholesterolemia in T1D [8, 11]. Diabetic $Ins2^{+/Akita}:apoE^{-/-}$ mice did not exhibit apparently reduced expression of hepatic LDL receptor but showed a decrease in lipolysis-stimulated lipoprotein receptor (LSR) levels [8]. Although leptin has been shown to be an important regulator of hepatic LSR expression [15], leptin treatment did not restore the diminished LSR expression in diabetic $Ins2^{+/Akita}:apoE^{-/-}$ mice. On the other hand, leptin treatment effectively restored reduced expression of sortilin-1 which may enhance LDL clearance [14]. It is also possible that

leptin may alter factors that improve the affinity of the lipoprotein particles for the corresponding receptors in liver [28]. Collectively, leptin exerts cholesterol-lowering effects in the setting of T1D likely by improving lipoprotein clearance.

Growing body of evidence suggests that impaired hepatic insulin receptor signaling plays a central role in dyslipidemia seen in T1D. In particular, it is associated with impaired LDL-receptor-mediated uptake, decreasing the clearance of LDL. German et al. showed that leptin deficiency causes insulin resistance in a T1D rat model and that leptin replacement restores insulin receptor signaling in liver [21]. Our present study using diabetic *Ins2^{+/Akita};apoE^{-/-}* mice suggest that impaired hepatic insulin receptor signaling associated with leptin deficiency in T1D is in part due to decreased expression of IRS-1 and IRS-2. Importantly, leptin treatment effectively restored the expression levels of IRS-1 and IRS2 and improved insulin receptor signaling as manifested by enhanced Akt phosphorylation in diabetic mice. IRS-1 and IRS-2 exert distinct actions in different conditions and their dynamic interaction for hepatic lipid metabolism is complex. For instance, short-term downregulation of hepatic IRS-1 and IRS-2 results in increased lipogenesis, plasma triglyceride concentrations and hepatic steatosis [29], whereas their liver-specific ablation does not affect hepatic triglyceride content but reduces triglyceride secretion, plasma triglyceride and cholesterol concentrations [30]. Thus, further studies are warranted to investigate how leptin regulates hepatic insulin receptor signaling and exerts functional impact on lipid metabolism in T1D.

In summary, the current study demonstrates that leptin deficiency plays a critical role in the pathogenesis of dyslipidemia and atherogenesis in T1D. Our findings point toward the therapeutic potential of leptin to address the increased burden of CVD in people with T1D beyond glycemic control. While insulin injection remains the mainstay of therapy for T1D, it is presented with the problem of hypoglycemic episodes. Moreover, sustained hyperinsulinemia associated with intensive insulin regimen may also promote lipogenesis and cholesterol synthesis. In conjunction with the present observations, future studies should be aimed at combined therapy with low-dose insulin and leptin to determine the extent of beneficial outcomes with regard to glycemic control and reversal of dyslipidemia, which would limit the progression of atherosclerotic plaque and acute coronary syndrome in vulnerable subjects with T1D.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the Pennsylvania State University College of Medicine and WW Smith Charitable Trust to J. Y. Jun, and National Heart, Lung, and Blood Institute/National Institutes of Health Grant R01-HL-097090 to L. Segar. FPLC analysis was provided by the University of Cincinnati Mouse Metabolic Phenotyping Center (Cincinnati, OH; Grant U24-DK-059630).

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HIGHLIGHTS

1. The leptin sensitivity is preserved for an extended period of time in lean T1D
2. Leptin replacement alone inhibits the progression of atherosclerosis in T1D
3. Physiologic leptin replacement exerts cholesterol-lowering effect in T1D.
4. Leptin therapy restores the decreased expression of hepatic IRS-1 and -2 in T1D

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Fig. 1.

Changes in food intake, body weight, and body composition in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice treated with leptin. Food intake (A), body weight (B), and lean and fat mass (C) were determined in nondiabetic $Ins2^{+/+};apoE^{-/-}$ mice on PBS (non-DM-PBS), diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice on PBS (DM-PBS) and diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice on leptin (DM-Leptin). ($n = 4$ to 6 /group) The data shown are means $\pm$ SE. * $P < 0.05$ compared with non-DM-PBS. # $P < 0.05$ compared with DM-PBS

C

Fig. 2.

Changes of plasma glucose, insulin, triglyceride, and cholesterol concentrations in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice treated with leptin. Plasma glucose (A), insulin (B), triglyceride (C) and cholesterol (D) concentrations were determined in nondiabetic $Ins2^{+/+};apoE^{-/-}$ mice on PBS (non-DM-PBS), diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice on PBS (DM-PBS) and $Ins2^{+/Akita};apoE^{-/-}$ mice on leptin (DM-Leptin) at 13, 17, and 25 weeks of age. Plasma samples were obtained from mice after a 5-h fast. ($n = 4$ to 6/group) The data are means $\pm$ SE. * $P < 0.05$ and ** $P < 0.01$ compared with non-DM-PBS. # $P < 0.05$ and ## $P < 0.01$ compared with DM-PBS. (E) Plasma samples from mice at 25 weeks of age were subjected to fast-performance liquid chromatography analysis, and cholesterol concentration was

determined in each of the eluted fractions. VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

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A **non-DM
-PBS****DM-PBS****DM-Leptin**

B **C** **Fig. 3.**

En face analysis of atherosclerotic lesion area in the aortic arch using Oil-red O stain. Non-diabetic $Ins2^{+/+};apoE^{-/-}$ received *i.p.* PBS (non-DM-PBS) while two groups of diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice received *i.p.* PBS (DM-PBS) or leptin ($0.2 \mu\text{g/g}$) (DM-Leptin) twice daily from 13 to 25 weeks of age ($n = 4$ to $6/\text{group}$). (A and B) Representative photomicrographs of the entire aorta and aortic arch. (C) Atherosclerotic lesion area is expressed as the percentage of the total luminal surface area of the aortic arch. Each closed circle represents atherosclerotic lesion area in one animal and the horizontal line is the mean of the group. ** $P < 0.01$ compared with non-DM-PBS. # $P < 0.05$ compared with DM-PBS. NS, not significant.

A

B

Fig. 4.

Expression of hepatic lipoprotein receptors in diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice with and without leptin treatment. (A) Immunoblot analysis of LDLr, LSR and sortilin-1 expression in the liver extracts from 5-h-fasted 25-week-old mice. Diabetic $Ins2^{+/Akita};apoE^{-/-}$ mice received *i.p.* PBS (DM-PBS) or leptin (0.2 μ g/g) (DM-Leptin) twice daily from 13 to 25 weeks of age and age-matched nondiabetic $Ins2^{+/+};apoE^{-/-}$ control mice treated with PBS (non-DM-PBS) ($n = 4$ to 6/each group). To normalize protein level, superoxide dismutase-1 (SOD-1) was used as an internal control. (B) The relative expression of hepatic lipoprotein receptors is shown in the bar graph. The data are means $\pm$ SE. * $P < 0.05$, ** $P < 0.01$ compared with non-DM-PBS. # $P < 0.05$ compared with DM-PBS.

A

Fig. 5. Changes in hepatic mRNA and protein expression levels in diabetic *Ins2^{+/-}Akita⁺*:*apoE^{-/-}* mice with leptin treatment. (A) Quantitative real-time PCR analysis of mRNAs was performed using liver samples obtained from 25-week old diabetic *Ins2^{+/-}Akita⁺*:*apoE^{-/-}* mice treated with PBS (DM-PBS), and leptin (DM-Leptin) as well as nondiabetic controls (non-DM-PBS) after 12 weeks of treatment. ($n = 4-6$ /each group) Abbreviations are provided in Table S1. (B) Immunoblot analysis of leptin and insulin receptor signaling components was performed using liver samples obtained from 25-wk old diabetic *Ins2^{+/-}Akita⁺*:*apoE^{-/-}* mice treated with PBS (DM-PBS), and leptin (DM-Leptin) as well as nondiabetic controls (non-DM-PBS) after 12 weeks of treatment. ($n = 4-6$ /each group) Representative immunoblots were

obtained using primary antibodies specific for phospho-STAT-3 at tyrosine 705, STAT-3, phospho-ERK1/2, ERK1/2, IRS-1, IRS-2, SOD-1 (used as internal control for IRS-1 and IRS-2), phospho-Akt (p-Akt) at serine 473, and Akt. The relative expression levels of proteins are shown in the bar graph. The data are means $\pm$ SE. * $P < 0.05$ compared with non-DM-PBS. # $P < 0.05$ compared with DM-PBS.

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