BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.01056.x
www.brjpharmacol.org

RESEARCH PAPER

Thyroid hormone potentiates insulin signaling and attenuates hyperglycemia and insulin resistance in a mouse model of type 2 diabetes

Yi Lin and Zhongjie Sun

Department of Physiology, College of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

Correspondence
Zhongjie Sun, The Robert & Mary Cade Laboratory, BMSB 662A, Box 26901, Department of Physiology, BMSB 662A, College of Medicine, University of Oklahoma Health Sciences Center (OUHSC), 940 S.L. Young Blvd., Oklahoma City, OK 73126-0901, USA. E-mail: zhongjie-sun@ouhsc.edu

Keywords
T3; TRα1; IRS-1; PI3 kinase; insulin sensitivity

Received
6 July 2010
Revised
19 August 2010
Accepted
14 September 2010

BACKGROUND AND PURPOSE
The thyroid hormone, triiodothyronine (T3) has many metabolic functions. Unexpectedly, exogenous T3 lowered blood glucose in *db/db* mice, a model of type 2 diabetes. Here, we have explored this finding and its possible mechanisms further.

EXPERIMENTAL APPROACH
db/db and lean mice were treated with T3, the phosphoinositide 3- kinase (PI3-kinase) inhibitor, LY294002, plus T3, or vehicles. Blood glucose, insulin sensitivity, levels and synthesis were measured. Effects of T3 on intracellular insulin signaling were analyzed in 3T3-L1 pre-adipocytes with Western blotting. Knock-down of the thyroid hormone receptor α1 (TRα1) in 3T3-L1 cells was achieved with an appropriate silencing RNA (siRNA).

KEY RESULTS
Single injections of T3 (7 ng·g^{-1} i.p.) rapidly and markedly attenuated hyperglycemia. Treatment with T3 (14 ng·g^{-1}·day^{-1}, 18 days) dose-dependently attenuated blood glucose and increased insulin sensitivity in *db/db* mice. Higher doses of T3 (28 ng·g^{-1}·day^{-1}) reversed insulin resistance in *db/db* mice. T3 also increased insulin levels in plasma and the neurogenic differentiation factor (an insulin synthesis transcription factor) and insulin storage in pancreatic islets in *db/db* mice. These anti-diabetic effects of T3 were abolished by the PI3-kinase inhibitor (LY294002). In 3T3-L1 preadipocytes, T3 enhanced insulin-induced tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and activation of PI3-kinase, effects blocked by siRNA for TRα1.

CONCLUSIONS AND IMPLICATIONS
T3 potentiated insulin signaling, improved insulin sensitivity, and increased insulin synthesis, which may contribute to its anti-diabetic effects. These findings may provide new approaches to the treatment of type 2 diabetes.

Abbreviations
IRS, insulin receptor substrate; siRNA, silencing RNA; T2DM, type 2 diabetes; TRα1, thyroid hormone receptor α1

Introduction

Diabetes affects about 150 million people worldwide and this figure is expected to be doubled in the next 20 years (Zimmet *et al.*, 2001). About 90–95% of all North American cases of diabetes are type 2 diabetes mellitus (T2DM), and about 20% of the population over the age of 65 has T2DM (Zimmet *et al.*, 2001). T2DM may result in severe complications, including renal failure, blindness, slow wound healings and cardiovascular diseases.

T2DM is characterized by increased levels of blood glucose due to impaired insulin sensitivity (insulin resistance)

and disrupted insulin secretion (β cell dysfunction) (Das and Elbein, 2006). Insulin resistance refers to a reduced ability of insulin to activate its signaling pathways (Das and Elbein, 2006). Insulin binds to the α-subunits of the heterotetrameric insulin receptor (α,α,β,β), increases flexibility of the activation loop to allow ATP to enter the catalytic site, and stabilizes the activation loop in the active conformation by autophosphorylation (Hubbard *et al.*, 1994). The activated insulin receptor recruits specific substrates such as insulin receptor substrate 1 (IRS-1) and phosphorylates tyrosine residue in IRS-1. Tyrosine phosphorylation of IRS-1 coordinates downstream signaling cascades by binding to the SH2 domains in proteins – including phosphoinositide 3-kinase (PI3-kinase). Many mechanisms contributing to T2DM may also trigger β-cell apoptosis and reduce β-cell ability to compensate for insulin resistance (Rhodes, 2005). Early failure of insulin secretion has been found in the pathogenesis of T2DM (Ferrannini, 1998; Kahn, 2003).

The thyroid hormone triiodothyronine (T3) exhibits an extensive range of physiological functions, which are related to the regulation of thermogenesis, metabolism, systemic vascular resistance, heart rate, renal sodium reabsorption and blood volume (Klein and Danzi, 2007; Crunkhorn and Patti, 2008). T3 exerts its functions by binding to the thyroid hormone receptors, TRα1 and TRβ1 (receptor nomenclature follows Alexander *et al.*, 2009). TRα1 is expressed in heart, brain, skeletal muscle and adipose tissue, whereas TRβ1 is expressed at higher levels in liver and kidney (Cheng, 2005; Bryzgalova *et al.*, 2008). TR is a nuclear hormone receptor, which heterodimerizes with retinoid X receptor, or in some cases, with itself. The dimers bind to the thyroid response elements (TREs) in the absence of ligand and act as transcriptional repressors. Binding of T3 to TR de-represses TRE-dependent genes and induces the expression of various target genes. It is not known, however, if thyroid hormone has any effects on insulin resistance and hyperglycemia in T2DM.

In a preliminary project, we found that, in *db/db* mice, a strain characterized by mutated leptin receptors, obesity and hyperglycaemia, the body temperature (34.8 ± 0.1°C) was significantly lower than that of wild-type mice (37.2 ± 0.1°C), indicating that *db/db* mice could not maintain their body temperatures. We therefore injected T3 to increase thermogenesis in these mice. Unexpectedly, injection of T3 rapidly decreased blood glucose levels in *db/db* mice, which led to the inception of this study. The purpose of this study was to test the effects of T3 in a mouse model of T2DM. The *db/db* mice are generated by genetic mutation of leptin receptors and have been widely used as a model of T2DM.

Methods

Animals

All animal care and experimental procedures complied with the guidelines of the National Institutes of Health on the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center. *BKS.Cg -+ Lepr^{db}/+ Lepr^{db}/OlaHsd* mice (*db/db* mice) and *BKS.Cg-m +/+ Lepr^{db}/OlaHsd* (lean mice) (all males, 16 weeks) were purchased from Harlan (Indianapolis, USA). Three groups of db/db and three groups of lean mice were used (five mice per group). Body weight was monitored daily. All mice were housed individually in wire-mesh cages at room temperatures (25 ± 1°C) and were provided with Purina laboratory chow (No. 5001) and tap water *ad libitum* throughout the experiment.

Effects of T3 on blood glucose in db/db mice

Blood glucose was measured twice from the tail vein blood during the control period using a *Reli On* Ultima glucose reader (Solartek Products, Inc; Alameda, CA). Animals were fasted for 17 hours before glucose measurement. For testing the acute effect of T3 on blood glucose, three groups of each strain received intraperitoneal injections of vehicles (35% DMSO and PBS), T3 (7 ng·g^{-1} b.w. in PBS; Sigma, Saint Louis, MO) and LY294002 (3 μg·g^{-1} in 35% DMSO; Sigma) plus T3, respectively. DMSO was the solvent for LY294002. Glucose levels were monitored before injections and at 60, 120, 180 and 240 min after the injections. For testing the effect of chronic treatment with T3, the same doses of T3 and LY294002 were given twice a day (9:00 a.m. and 5:00 p.m.) for 18 days (14 ng·g^{-1}·day^{-1} for T3 and 6 μg·g^{-1}·day^{-1} for LY294002). Animals were further treated with higher doses of T3 (28 ng·g^{-1}·day^{-1}) and LY294002 (9 μg·g^{-1}·day^{-1}) for additional 10 days. Glucose levels were measured twice a week. A group treated with LY294002 alone was not included because the *db/db* mice would die after such treatment due to severe hyperglycemia.

Insulin sensitivity

Insulin sensitivity test (IST) was performed during weeks 1, 2 and 3 after treatments with T3. Briefly, blood glucose levels were measured at 0, 20, 40, 60, 80 and 120 min after subcutaneous injections of insulin (1.0 U kg^{-1}, Sigma).

Rectal temperatures

The rectal temperature was measured weekly before and during treatments with T3 using a *Reli On* Digital Thermometer (MABIS Healthcare, Waukegan, IL).

Blood pressure responses to cold exposure

Resting arterial systolic BP and heart rate were measured from the tail of each unanesthetized mouse using a CODA-6 Blood Pressure Monitoring system. Blood pressure responses to acute exposure to cold were tested before and during the treatments with T3. Briefly, arterial systolic blood pressure was measured after animals were exposed to a cold chamber (5°C) for 30 min.

Tissue collections

At the end of week 4 of treatments with T3, animals were killed with an overdose of sodium pentobarbital (100 mg·kg^{-1}, i.p.) and blood was collected in EDTA. Following blood collections, animals were perfused transcardially using heparinized saline. Skeletal muscle and adipose tissue were collected and stored in –80°C.

Plasma T3

Plasma T3 was measured using a T3 ELISA kit according to the manufacturer's instruction (Alpha Diagnostic International, San Antonio, TX, USA).

Plasma insulin

Plasma insulin levels were measured using an insulin (mouse) EIA kit according to the manufacturer's instruction (ALPCO Diagnostics, Salem, NH, USA).

Immunohistochemistry

Following perfusion, the pancreas was isolated and placed in 4% buffered paraformaldehyde (PFA) for 24 h and then embedded in paraffin.

For immunohistochemical analysis of insulin in pancreatic islets, the paraffin-embedded pancreas of each animal was cut at 100-μm intervals. Fifteen consecutive cross-sections (5 μm) of the whole pancreas were obtained. The cross-sections were incubated with anti-insulin or anti-NeuroD polyclonal antibody followed by anti-rabbit biotinylated secondary antibody. Stable diaminobenzidine (DAB) was used as substrate for peroxidase. Haematoxylin was used as counterstaining. Insulin staining was quantified using Image J (NIH freeware) as mean grey value/pixel. The number of cells with positive insulin-staining in the islet was counted. Image J was used to measure the mass size of islet of Langerhans by drawing a line along the islet of Langerhans. The selected area (μm^2) was measured after calibration of the scale.

NeuroD expression

Lysates of mouse pancreas were directly subjected to SDS-PAGE followed by Western blotting with antibody against NeuroD (Santa Cruz, Santa Cruz, CA, USA) and then with an antibody against β-actin (Santa Cruz) after stripping the blot. β-actin was used as an internal control.

Cell culture

The 3T3-L1 preadipocytes (ATCC) were cultured in DMEM (Cell Signaling) supplemented with 10% bovine calf serum, 100 $\mu g \cdot mL^{-1}$ of streptomycin and 100 $U \cdot mL^{-1}$ of penicillin at 37°C, 5% CO_2.

siRNA transfection

siRNA against mouse TRα1 (sense: CGCUCUUCCUGGAGGUCUUtt; antisense: AAGACCUCCAGGAAGAGCGtt) was selected, based on previous work (Hassani *et al.*, 2007). siRNA against mouse TRα1 was synthesized by Ambion (Austin, TX, USA). SiPORT Neo™ was used as a transfection reagent according to the instruction (Ambion). Preadipocytes were incubated with 90 nM control siRNA (Ambion) or TRα1 siRNA for 54 h and then incubated in DMEM containing 0.03% BSA, no phenol red, and no FCS for 18 h.

Western blotting and Immunoprecipitation

TRα1, IRS1 or IRβ were measured using Western blots as described earlier (Sun *et al.*, 2008; Wang and Sun, 2009). Briefly, confluent preadipocytes were incubated in DMEM containing 0.03% BSA, no phenol red, and no FCS for 18 h. Cells were incubated with vehicle or T3 at various concentrations for 15 min and then with vehicle or 10 nM insulin for 5 min. The cells were lysed. Some lysates were directly subjected to SDS-PAGE followed by Western blotting with antibody against TRα1. Some lysates were immunoprecipitated with antibodies against IRS-1 (Santa Cruz) or IRβ (Santa Cruz). Immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with antibody against tyrosine phosphorylation (Millipore) and then with antibodies against IRS-1 or IRβ (Santa Cruz) after stripping the blot.

PI3-kinase activity

Lysates of preadipocytes, mouse adipose tissue, or mouse skeletal muscles were immunoprecipitated with antibody against PI3-kinase (Millipore). PI3-kinase activity in the immunocomplexes was determined using a PI3-kinase ELISA kit (Echelon Biosciences).

Data analysis

Plasma T3, plasma insulin levels and PI3-kinase activities were analyzed by two-way ANOVA (strain × treatments). The remaining data were analyzed by a repeated measure one-way ANOVA. The Newman-Keuls procedure was used to reveal differences between groups. Data were expressed as mean ± SEM. A probability value with $P < 0.05$ was considered statistically significant.

Results

T3 decreased blood glucose levels in db/db *mice*

These 16-week old *db/db* mice were severely hyperglycemic, with blood glucose up to 5 $mg \cdot L^{-1}$. Surprisingly, a single intraperitoneal injection of T3 (7 $ng \cdot g^{-1}$, i.p.) significantly decreased the fasting blood glucose levels in *db/db* and lean mice within 2 h (Figure 1A). Pretreatment with the PI3-kinase inhibitor (LY294002, 3 $\mu g \cdot g^{-1}$) abolished this effect (Figure 1A). Therefore, T3 had a rapid blood glucose-lowering effect and this action is PI3-kinase dependent.

Daily injection of T3 at 14 $ng \cdot g^{-1} \cdot day^{-1}$ decreased the blood glucose levels dramatically in *db/db* mice (by ≈30.4% on Day 15) (Figure 1B). Interestingly, doubling of the doses of T3 further reduced the glucose levels in *db/db* mice (by ≈51.9% on Day 25) (Figure 1B). Pre-injection of LY294002 at the dose of 6 $\mu g \cdot g^{-1} \cdot day^{-1}$ and 9 $\mu g \cdot g^{-1} \cdot day^{-1}$ almost abolished the glucose-lowering effects of T3 at 14 and 28 $ng \cdot g^{-1} \cdot day^{-1}$, respectively, in *db/db* mice (Figure 1B). The *db/db* mice treated with LY294002 alone died within 2–3 days due to severe hyperglycemia (data not shown). Chronic treatments with T3 only slightly decreased blood glucose levels in lean mice, which was also abolished by LY294002 (Figure 1B). These results suggest that T3 can be used for long-term control of hyperglycemia in T2DM and these effects are PI3-kinase dependent.

T3 increased insulin sensitivity in db/db *mice*

Insulin sensitivity was decreased in *db/db* mice (Figure 2A–C). T3 enhanced insulin sensitivity in *db/db* mice during weeks 1, 2 and 3 of treatment (Figure 2A–C). It is noted that T3 at 28 $ng \cdot g^{-1} \cdot day^{-1}$ restored insulin sensitivity (Figure 2C). LY294002 completely blocked this effect in *db/db* mice (Figure 2A–C). These results suggested that T3 can enhance

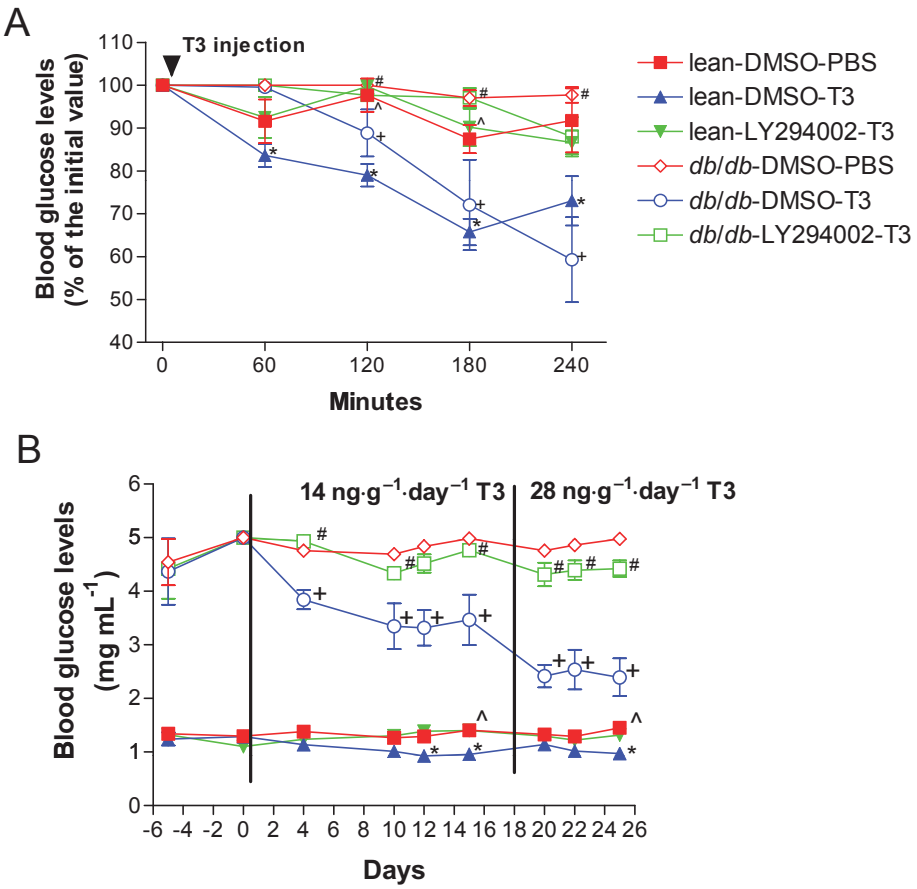

Figure 1

Antidiabetic effect of T3 in *db/db* mice. (A) Acute effect of T3 on blood glucose levels in *db/db* and lean mice. Three groups of each strain received i.p. injections of vehicle (35% DMSO and PBS), T3 in PBS (7 ng·g^{-1}), and LY294002 in 35% DMSO (3 μg·g^{-1}) followed by T3 in PBS (7 ng·g^{-1}), respectively. DMSO is a solvent for LY294002. (B) Chronic effect of T3 on blood glucose levels in *db/db* and lean mice. Three groups of each strain received daily i.p. injections of vehicle (35% DMSO and PBS), T3 (14 ng·g^{-1}) and LY294002 (3 μg·g^{-1}) followed by T3 (14 ng·g^{-1}), respectively, for 18 days. Animals were further treated with double doses of vehicle, T3 (28 ng·g^{-1}), and LY294002 (9 μg·g^{-1}) plus T3 (28 ng·g^{-1}) for 10 days. Data = mean ± SEM. N = 4–5 animals per group. *P < 0.05 versus the lean-DMSO-PBS group; ^P < 0.05 versus the lean-DMSO-T3 group; ^{+}P < 0.001 versus the *db/db*-DMSO-PBS group; #P < 0.001 versus *db/db*-DMSO-T3 group.

insulin sensitivity in *db/db* mice and this effect is PI3-kinase dependent. T3 did not affect insulin sensitivity in lean mice (Figure 2A–C), suggesting that the acute blood glucose-lowering effect of T3 (Figure 1A) may be due to rapid insulin release.

T3 normalized body temperature and blood pressure responses to cold exposure in db/db *mice*

Body weight was significantly higher in *db/db* mice compared with lean mice at the beginning of the experiment (Figure 3A). Body weights of all three groups of *db/db* mice decreased gradually to those of lean mice. The decline in body weight was not due to the treatment with T3 because it was also found in the *db/db* -DMSO-PBS group. Indeed, *db/db* mice show progressive body weight loss after 5 months of age (Sharma *et al.*, 2003). No difference in body weight was found among the three *db/db* groups with different treatments or among the three lean groups with different treatments (Figure 3A).

Rectal temperature (core body temperature) was significantly lower in *db/db* mice than that of lean mice before treatment with T3 (Figure 3B). Treatments with T3 at the dose of 14 ng·g^{-1}·day^{-1} increased rectal temperature, and doubling of the dose of T3 (28 ng·g^{-1}·day^{-1}) normalized rectal temperature in *db/db* mice (vs. lean mice) (Figure 3B). Thus, *db/db* mice had defects in thermogenesis which could be restored by treatments with T3. LY294002 did not modulate the effects of T3 on body temperature in *db/db* mice, suggesting that the thermogenic effect of T3 was not mediated by PI3-kinase (Figure 3B). Treatments with T3 did not affect rectal temperature in lean mice (Figure 3B).

Heart rate in *db/db* mice was slightly lower than that in lean mice (Figure 3C). T3 did not affect heart rate in either *db/db* or lean mice (Figure 3C). There was no significant difference in basal arterial systolic blood pressure (BP) between *db/db* and lean mice (Figure 3D). T3 did not change BP in *db/db* or lean mice (Figure 3D). Thus, T3 at the doses used did not produce obvious cardiovascular effects.

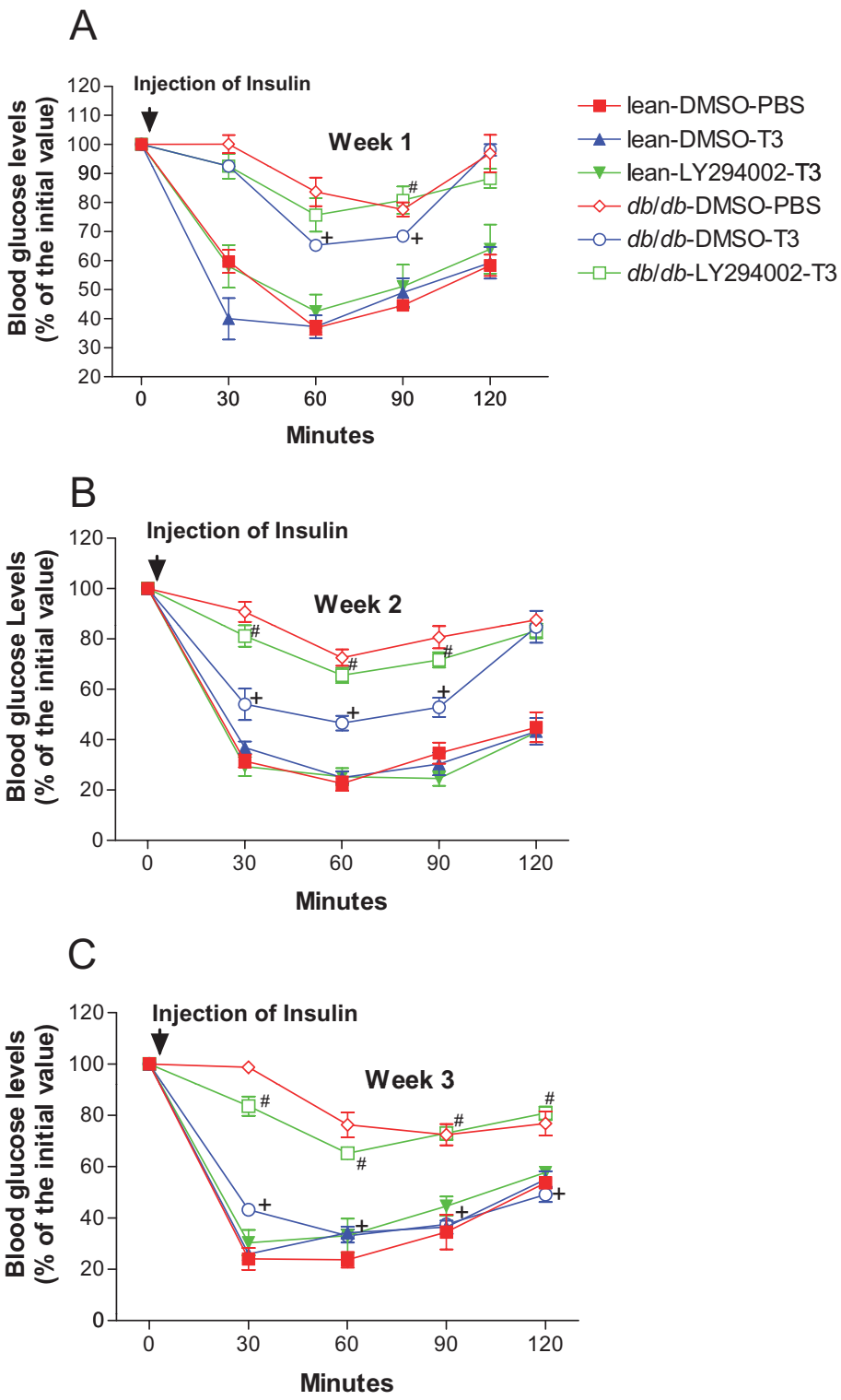

Figure 2

Effects of T3 on insulin sensitivity. Insulin sensitivity was tested during week 1 (A), week 2 (B), and week 3 (C) of treatment with T3. Insulin sensitivity was evaluated by the blood glucose-lowering effect of insulin following s.c. injection of insulin (1 U·kg^{-1}) in *db/db* and lean mice. Data = mean ± SEM. N = 4–5 animals per group. $^{+}P < 0.001$ versus the *db/db*-DMSO-PBS group; $\#P < 0.001$ versus *db/db*-DMSO-T3 group.

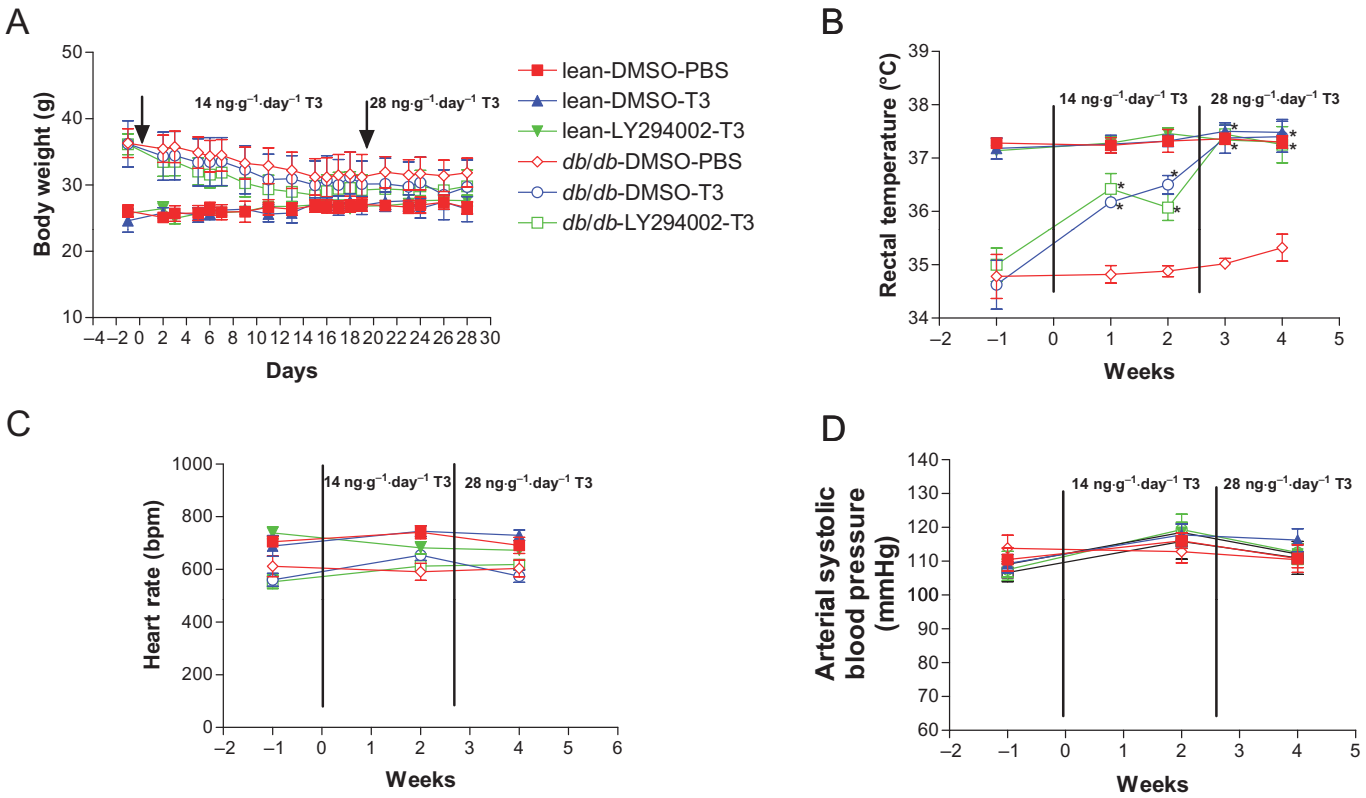

Figure 3

Effects of T3 on body weight, rectal temperature, arterial systolic blood pressure and heart rate. (A) Body weight was monitored daily for 4 weeks. (B) Rectal temperature was measured weekly. (C,D) Heart rate and arterial systolic blood pressure were measured using the tail-cuff system method one week before the treatment with T3 and during week 2 and 4 after the treatment. Data = mean ± SEM. $N = 4–5$ animals per group. *$P < 0.001$ versus the *db/db*-DMSO-PBS group.

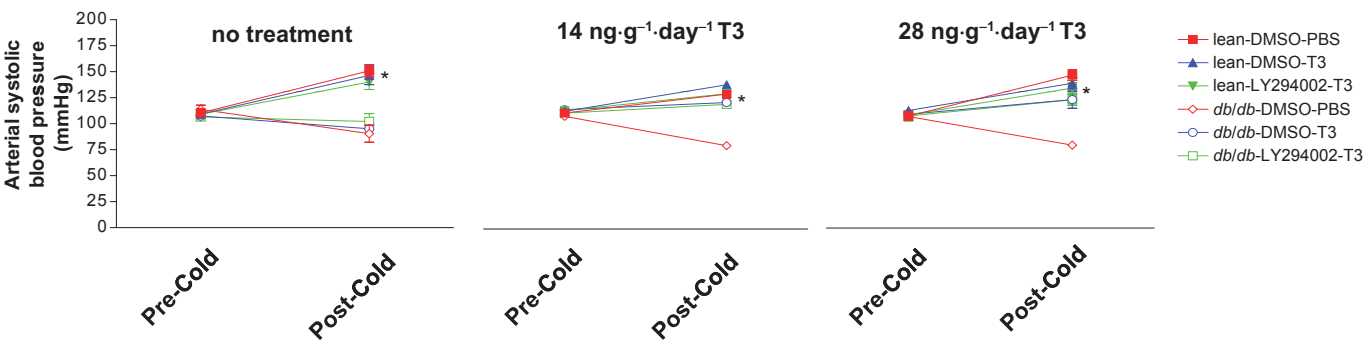

Figure 4

Effects of T3 on blood pressure responses to cold exposure. Blood pressure responses to 30 min exposure to cold temperatures (5°C) were measured before (Pre cold) and after the exposure (Post cold). Data = mean ± SEM. $N = 4–5$ animals per group *$P < 0.001$ versus the *db/db*-DMSO-PBS group.

A 30-min exposure to moderate cold (5°C) increased arterial systolic BP significantly in lean mice (Figure 4). In contrast, BP was decreased in *db/db* mice in response to cold exposure. T3 at doses of 14 and 28 ng·g^{-1}·day^{-1} increased and normalized BP responses to cold exposure, respectively, in *db/db* mice (Figure 4). LY294002 did not affect these effects in *db/db* mice (Figure 4), suggesting that the cardiovascular effects of T3 are independent of PI3-kinase.

T3 increased plasma insulin levels in db/db *mice*

Basal levels of T3 in *db/db* mice were significantly lower than those of lean mice (Figure 5A). Daily injections of T3 (28 ng·g^{-1}·day^{-1}) for 7 days increased plasma levels of T3 in *db/db* mice by 2.7 fold and in lean mice by 5 fold (Figure 5A).

There was no significant difference in plasma insulin between lean-DMSO-PBS and *db/db* -DMSO-PBS groups

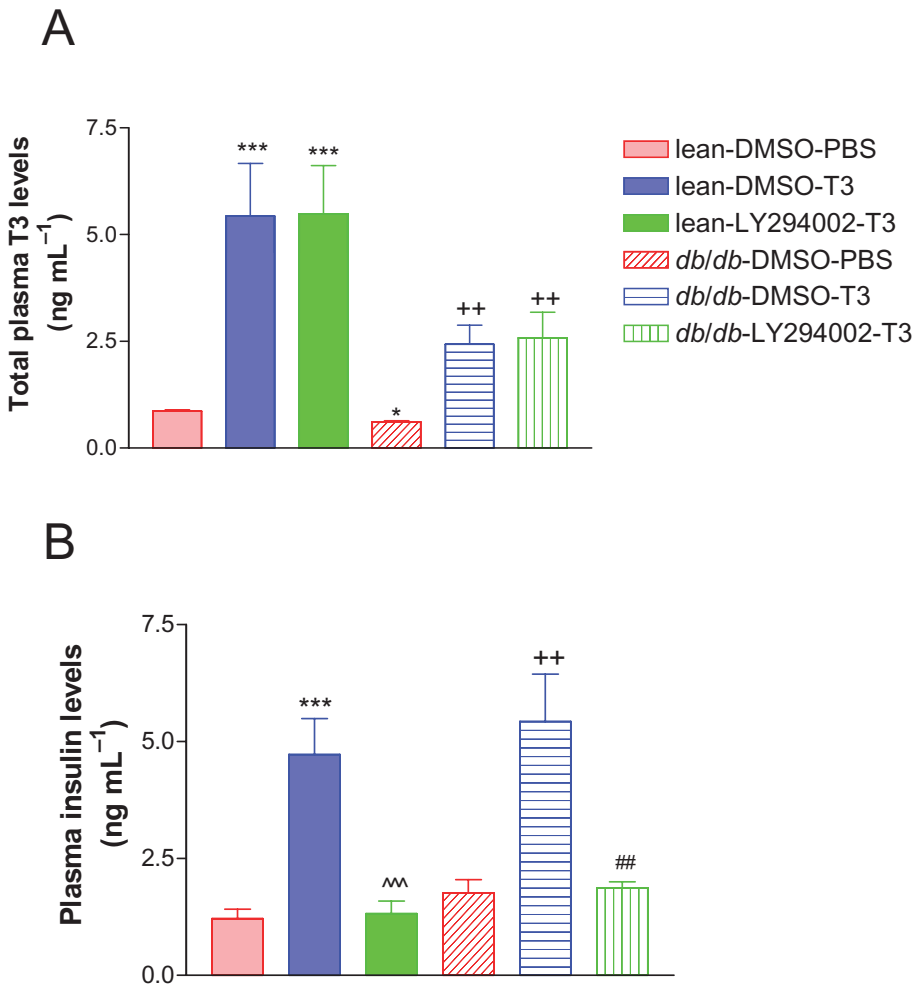

Figure 5

Plasma levels of T3 and insulin and the activities of PI3-kinase in skeletal muscle and adipose tissue. (A) Plasma T3 levels were measured after treatments with T3 (28 ng·g^{-1}·day^{-1}) for 10 days using a total T3 ELISA kit. (B) Plasma insulin levels were measured using an Insulin EIA kit. Data = mean ± SEM. N = animals per group. *P < 0.05, ***P < 0.001 versus the lean-DMSO-PBS group; ^P < 0.05, ^^^P < 0.001 versus the lean-DMSO-T3 group; ^{+}P < 0.05, ^{++}P < 0.01 versus *db/db*-DMSO-PBS group; #P < 0.05, ##P < 0.01 versus the *db/db*-DMSO-T3 group.

(Figure 5B), which was in sharp contrast with severe hyperglycemia in *db/db* -DMSO-PBS group (Figure 1B). Treatments with T3 increased plasma insulin levels by 2.07 fold in *db/db* mice and by 2.88 fold in lean mice (Figure 5B). LY294002 abolished these effects in both *db/db* and lean mice (Figure 5B). Therefore, T3 increased insulin release in *db/db* and lean mice and these effects were PI3-kinase dependent.

T3 increased PI3-kinase activities in skeletal muscle and adipose tissue of db/db *mice*

The basal PI3-kinase activity, measured as production of phosphatidylinositol (3,4,5)-trisphosphate: (PIP_3) was significantly lower in skeletal muscles (Figure 6A) and adipose tissue (Figure 6B) in *db/db* mice than that in lean mice. Treatment with T3 increased PI3-kinase activity significantly in both tissues in *db/db* mice and lean mice (Figure 6A,B), suggesting that T3 activated PI3-kinase. LY294002 abolished these effects of T3.

Immunohistochemical analysis of mouse pancreatic islets of Langerhans

Insulin staining in pancreatic islets of Langerhans in *db/db* mice was significantly lower compared with that of lean mice (Figure 7A,B). T3 increased insulin staining in pancreatic islets of *db/db* mice by 54.5% (Figure 7A,B). Inhibition of PI3-kinase by LY294002 abolished this effect, suggesting that T3 increased insulin synthesis *via* activation of PI3-kinase. Injections of T3 did not increase insulin staining in pancreatic islets in lean mice (Figure 7A,B).

The number of cells with positive insulin-staining in pancreatic islets in *db/db* mice was significantly lower than that in lean mice (Figure 7C). Daily treatments with T3 increased the number of cells with positive insulin-staining in pancreatic islets by 2.62 fold in *db/db* mice, suggesting that T3 increased the number of functional β cells (Figure 7C). This effect was mediated by PI3-kinase as it was abolished by LY294002. T3

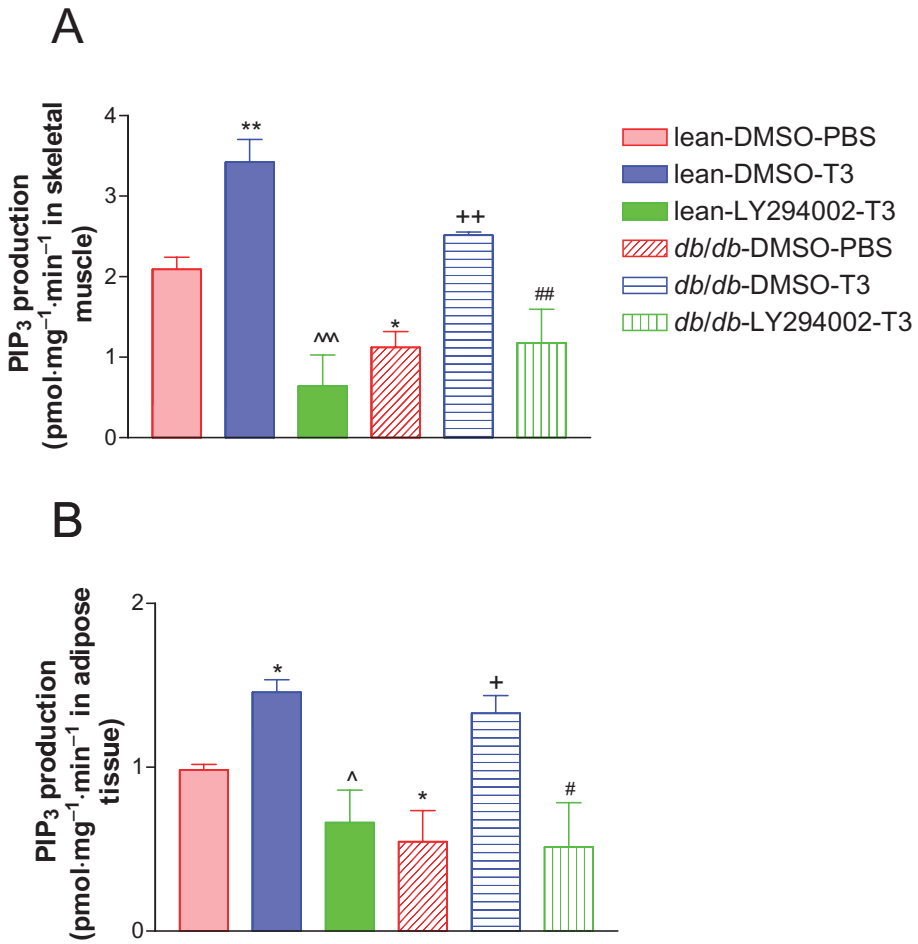

Figure 6

Effects of T3 on PI3-kinase activity. Activities of PI3-kinase in skeletal muscle (A) and adipose tissue (B) were measured using an immunoassay kit after tissue lysates were immunoprecipitated with antibody against PI3-kinase. Data = mean ± SEM. N = 4–5 animals per group. *P < 0.05, ***P < 0.001 versus the lean-DMSO-PBS group; ^P < 0.05, ^^^P < 0.001 versus the lean-DMSO-T3 group; ^{+}P < 0.05, ^{++}P < 0.01 versus *db/db*-DMSO-PBS group; #P < 0.05, ##P < 0.01 versus the *db/db*-DMSO-T3 group.

did not alter the number of β cells with positive insulin-staining in pancreatic islets in lean mice (Figure 7C).

There was no significant difference in the size of pancreatic islets in *db/db* mice and lean mice (Figure 7D). T3 increased the size of pancreatic islets in *db/db* mice by 1.42 fold but did not affect the size of pancreatic islets in lean mice (Figure 7D). This effect of T3 in *db/db* mice may also be mediated by PI3-kinase as it was abolished by LY294002 (Figure 7D).

T3 upregulated NeuroD expression in pancreas from db/db *mice*

In order to understand the molecular mechanism for the T3-induced increase in insulin storage/synthesis in islets of pancreas in diabetic mice treated with T3, we assessed the protein expression of NeuroD, a key transcription factor for the regulation of insulin synthesis (Naya *et al.*, 1997; Kaneto *et al.*, 2009). NeuroD staining was significantly lower in pancreatic islets of Langerhans in *db/db* mice compared with that of the lean mice (Figure 8A,B). T3 restored the NeuroD staining in *db/db* mice (Figure 8A,B). Western blot analysis confirmed that T3 recovered the suppressed NeuroD protein expression in the pancreas of *db/db* mice (Figure 8C,D). These effects of T3 could be abolished by LY294002 (Figure 8A–D), suggesting that T3 increased NeuroD expression *via* activation of PI3-kinase.

T3 enhanced insulin signaling in 3T3-L1 preadipocytes

To determine whether T3 affects insulin signaling, we serum-starved 3T3-L1 preadipocytes for 18 h and then pretreated cells with various concentrations of T3 for 15 min, followed by incubation of cells with 10 nM insulin for 5 min. Pretreatments with T3 enhanced insulin-induced tyrosine phosphorylation of IRS-1, while T3 alone did not affect tyrosine phosphorylation of IRS-1 (Figure 9A).

TRα1 is the dominant isoform in 3T3-L1 preadipocytes (Jiang *et al.*, 2004). To determine whether TRα1 mediates the potentiating effect of T3 on insulin signaling, we transfected 3T3-L1 preadipocytes with TRα1 siRNA or control siRNA for

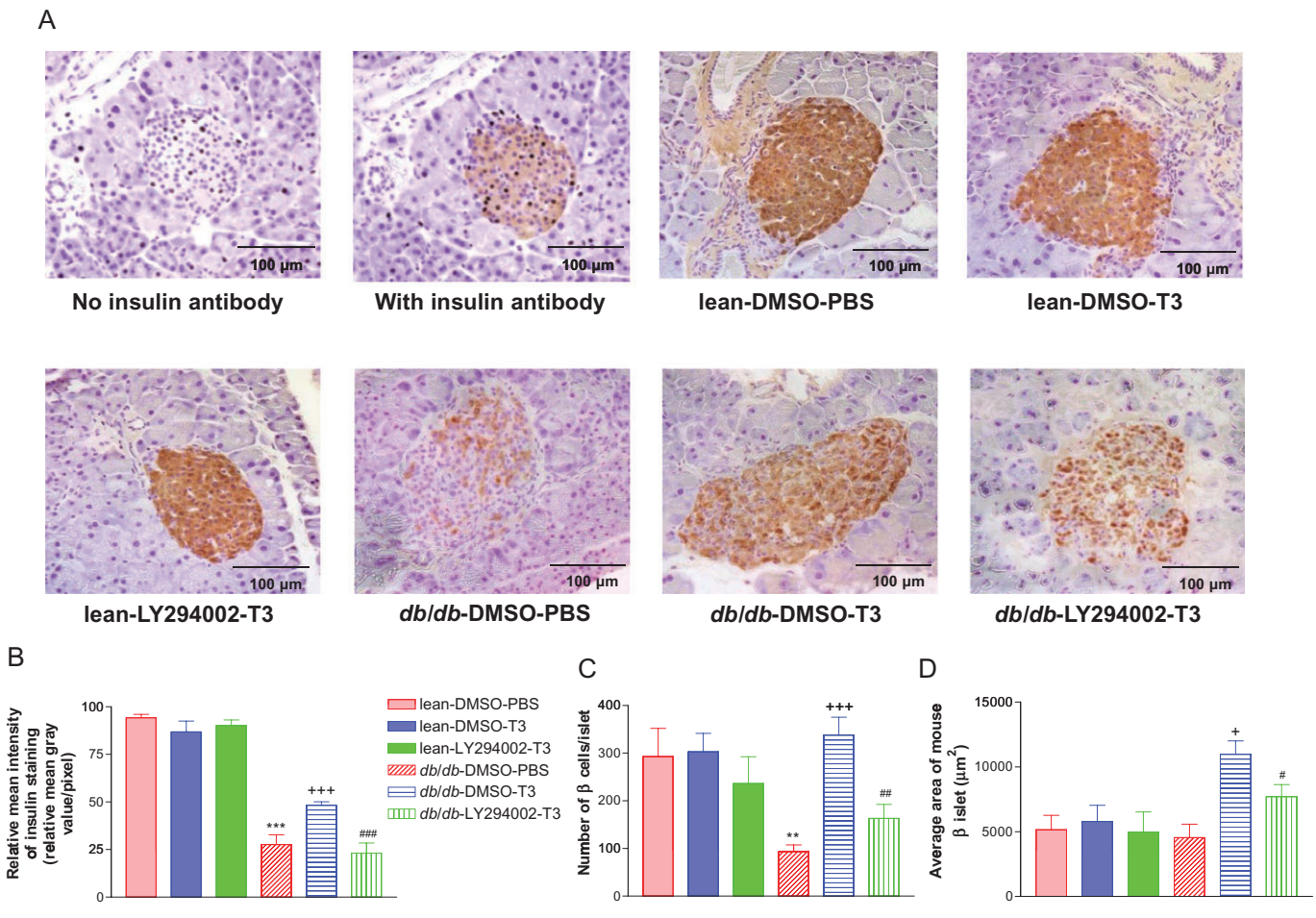

Figure 7

Effects of T3 on pancreatic islet characteristics. Staining for insulin was carried out after treatment with T3 for 4 weeks. A total of 15 consecutive cross sections (5 μm) from the pancreas of each animal were stained with antibody against insulin. Images of the 15 cross sections from each animal were collected at the same exposure conditions and at the same magnification (20×). (A) Representative images of insulin staining of cross-sections of islets of Langerhans. (B) Insulin staining in pancreatic islets was quantified using the NIH Image J software (mean grey value/pixel). (C) The number of cells with positive insulin-staining in pancreatic islets was counted using the NIS-Elements BR 3.0 software. (D) The size of islet of Langerhans was measured using Image J. Data = mean ± SEM. $N = 4–5$ animals per group. $^{**}P < 0.01$, $^{***}P < 0.001$ versus the lean-DMSO-PBS group; $^{+}P < 0.05$, $^{+++}P < 0.001$ versus the *db/db*-DMSO-PBS group; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ versus the *db/db*-DMSO-T3.

72 h. Transfection with TRα1 siRNA significantly decreased TRα1 expression, indicating effective knockdown of TRα1 (Figure 9B). Knock down of TRα1 abolished the potentiating effect of T3 on insulin-induced tyrosine phosphorylation of IRS-1 in 3T3-L1 preadipocytes (Figure 9C).

T3 did not affect tyrosine autophosphorylation of insulin receptor (IRβ) or insulin-induced tyrosine phosphorylation of IRβ in 3T3-L1 preadipocytes (Figure 9D).

To determine whether T3 also affects the downstream insulin signaling, we measured PI3-kinase activity. Pretreatment with T3 enhanced insulin-induced PI3-kinase activity by 48% although T3 alone did not affect PI-3 kinase activity (Figure 9E). Knockdown of TRα1 abolished the potentiating effect of T3 on insulin-induced activation of PI3-kinase (Figure 9E).

These data indicate that T3 enhanced insulin signaling in mouse 3T3-L1 preadipocytes *via* potentiating insulin-induced tyrosine phosphorylation of IRS-1 and activation of PI3-kinase and these effects are mediated by TRα1.

Discussion

The *db/db* mice were severely hyperglycemic at the age of 16 weeks. It was surprising that injection of T3 resulted in a rapid and profound attenuation in hyperglycemia in *db/db* mice. This finding was unexpected because such effects of T3 have never been reported in *db/db* diabetic animals. A single injection of T3 rapidly decreased blood glucose level within 2 h, suggesting that the acute effect of T3 may occur at the functional level of the signaling pathway that mediated glucose homeostasis. PI3-kinase, a key enzyme in the insulin signaling pathway, may mediate the blood glucose-lowering effect of T3 in *db/db* mice because this effect can be abolished by a

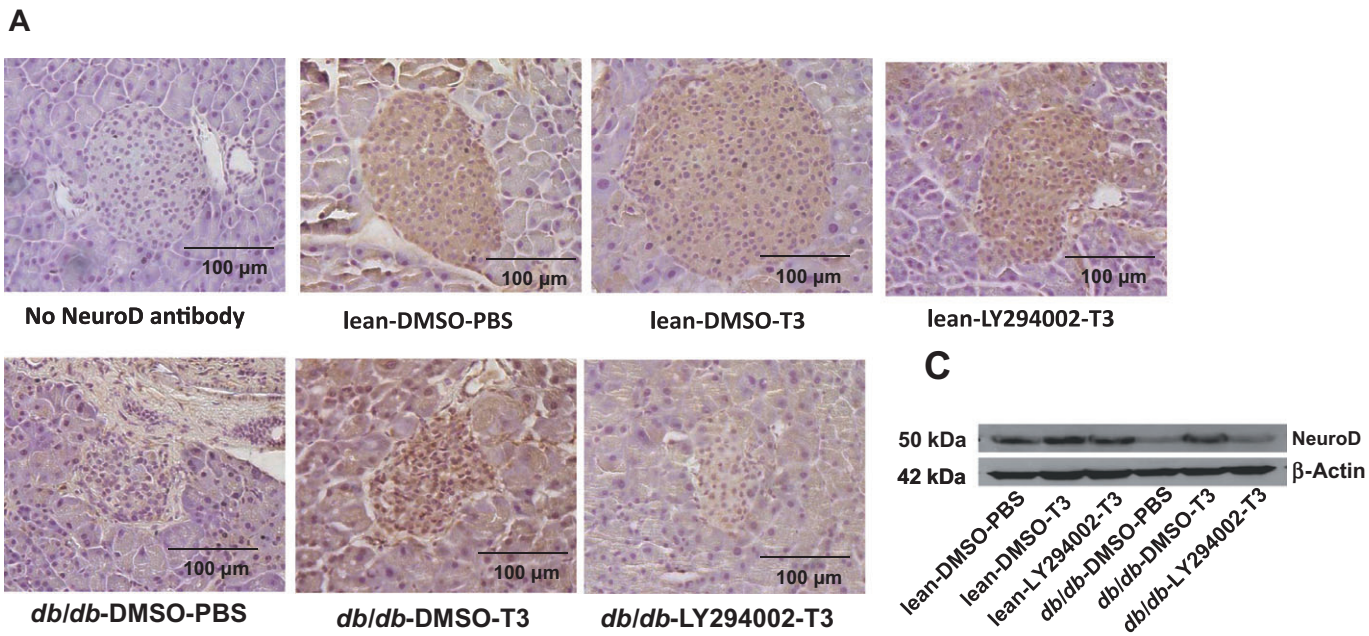

Figure 8

Effects of T3 on NeuroD expression in pancreatic islets. A total of 15 consecutive cross-sections (5-μm) across the pancreas of each animal were stained with antibody against NeuroD. Images of the islets from each animal were collected at 20× magnification. (A) Representative images of NeuroD staining of cross-sections of islets of Langerhans. (B) Semi-quantitative analysis of NeuroD staining in pancreatic islets (mean gray value/pixel). (C) Western blot analysis of NeuroD expression in pancreatic lysates. Data = mean ± SEM. N = 4–5 animals per group. *P < 0.01, ***P < 0.001 versus the lean-DMSO-PBS group; ^{+++}P < 0.001 versus the *db/db*-DMSO-PBS group; ###P < 0.001 versus the *db/db*-DMSO-T3 group.

PI3-kinase inhibitor. Daily treatments with T3 dose-dependently attenuated hyperglycemia and enhanced insulin sensitivity in *db/db* mice. Exogenous insulin fails to control hyperglycemia in *db/db* mice (Robertson and Sima, 1980), suggesting impaired insulin sensitivity. Notably, T3 at the dose of 28 ng·g^{-1}·day^{-1} reversed the impaired insulin sensitivity in *db/db* mice. The chronic beneficial effect of T3 may also be PI3-kinase-dependent because pre-treatment with the PI3-kinase inhibitor eliminated the effects of T3 on hyperglycemia and insulin resistance in *db/db* mice. Indeed, PI3-kinase activity was markedly suppressed in skeletal muscle and adipose tissue of *db/db* mice, which could be reversed by T3. On the other hand, the PI3-kinase inhibitor failed to worsen hyperglycemia in *db/db* mice treated with T3, suggesting that T3 may offer some protection to these mice against this inhibitor.

This study showed, for the first time, that T3 rapidly increased insulin-induced tyrosine phosphorylation of IRS-1 and activation of PI3-kinase in 3T3-L1 preadipocytes, whereas T3 alone did not affect the activities of IRS-1 and PI3-kinase. Therefore, T3 enhanced the insulin signaling by potentiating insulin-induced activation of IRS-1 and PI3-kinase rather than directly activating the insulin signaling pathway. The potentiating effect of T3 on the insulin signaling may not occur at the insulin receptor level because T3 did not affect tyrosine autophosphorylation or insulin-induced phosphorylation of IRβ. TRα1 is the dominant thyroid hormone receptor in adipocytes. Knockdown of TRα1 abolished effects of T3 on insulin-induced tyrosine phosphorylation of IRS-1 and activation of PI3-kinase, indicating that these effects of T3 may be mediated by TRα1. Incubation of 3T3-L1 adipocytes with T3 has been shown to increase glucose uptake, expres-

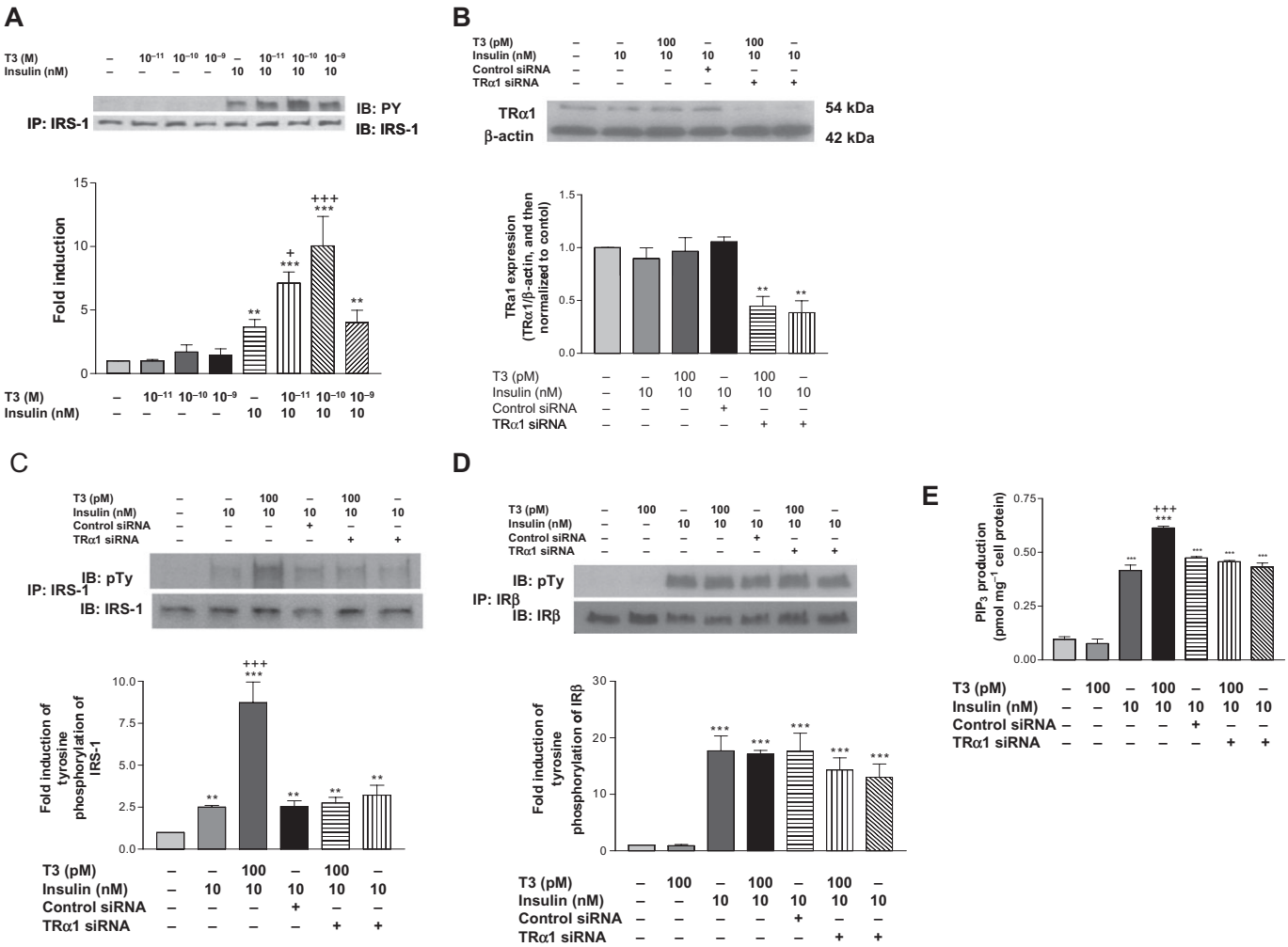

Figure 9

Effects of T3 on insulin signaling in 3T3-L1 preadipocytes. (A) Cells were starved overnight before experiment. Following treatment with T3 (30 min) and/or Insulin (5 min), cells were lysed and the cell lysates were immunoprecipitated with antibody against IRS-1. The immunoprecipitates were detected with antibody against tyrosine phosphorylation before incubation with antibody against IRS-1 after stripping the blot. The Western blot bands showed tyrosine phosphorylation (pTY) (top) and protein (bottom) of IRS-1. The bar graph showed quantification of tyrosine phosphorylation in IRS-1. Tyrosine phosphorylation of IRS-1 was normalized with IRS-1 protein, which was then calculated as fold changes of the control (vehicle). IP, immunoprecipitation. (B) After transfection and treatments, cells were lysed. Cell lysates were detected with antibody against TRα1 and antibody against β-actin. The Western blot bands showed TRα1 and β-actin. TRα1 expression was normalized with β-actin, which was then calculated as fold changes of the control (vehicle). (C) Cell lysates were immunoprecipitated with antibody against IRS-1. The immunoprecipitates were detected with antibody against tyrosine phosphorylation and then with antibody against IRS-1 after stripping the blot. Western blot bands showed tyrosine phosphorylation (pTy) (top) and protein (bottom) of IRS-1. The tyrosine phosphorylation of IRS-1 was normalized with IRS-1, which was then calculated as fold changes of the control (vehicle). (D) Cell lysates were immunoprecipitated with antibody against insulin receptor β (IRβ). The immunoprecipitates were detected with antibody against tyrosine phosphorylation and then with antibody against IRβ after stripping the blot. Western blot bands showed tyrosine phosphorylation (pTy) (top) and protein (bottom) of IRβ. Tyrosine phosphorylation of IRβ was normalized with IRβ, which was then calculated as fold changes of the control (vehicle). (E) Cell lysates were immunoprecipitated with antibody against PI3-kinase. PI3-kinase activity in the immunoprecipitates was determined using a PI3-kinase ELISA kit and expressed as the relative production of PIP3. Data = mean ± SEM. $N = 3–4$ independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control (vehicle); $^{+}P < 0.05$, $^{+++}P < 0.001$ versus insulin alone.

sions of glucose transporters GLUT1 and GLUT4, and their partitioning to plasma membrane (Romero *et al.*, 2000). The present findings also suggest that the potentiating effects of T3 on the insulin signaling may contribute to its beneficial effect on hyperglycemia and impaired insulin sensitivity in *db/db* mice. T3 at the concentrations of 100 pM to 10 nM has been shown to activate and phosphorylate Akt *via* binding to PI3-kinase in endothelial cells in coimmunoprecipitation assay and in GST pull-down assay (Hiroi *et al.*, 2006). The present study revealed, for the first time, that T3 can activate IRS-1, an upstream factor of the insulin signaling pathway. The mechanism by which T3 potentiated insulin-induced tyrosine phosphorylation of IRS-1 *via* TRα1 in 3T3-L1 preadipocytes remains to be determined.

β-cell dysfunction, including decreased β-cell mass and insulin secretion, is central to the development of T2DM (Marchetti *et al.*, 2008). It has been shown that there is no hyperglycermia without β-cell dysfunction (Ferrannini and Mari, 2004). In fact, most patients who have insulin resistance can increase their insulin secretion appropriately and preserve glucose homeostasis for years without the occurrence of diabetes (Marchetti *et al.*, 2008). Insulin secretion deteriorates in advanced stages of T2DM in humans. Here, we found that *db/db* mice aged 20 weeks showed decreases in plasma T3, insulin storage in pancreatic islets, and the number of β cells in pancreatic islets. In addition, plasma insulin levels were not elevated in the *db/db* mice at this age even though they were severely hyperglycemic, suggesting impaired insulin secretion. *db/db* mice show an uncontrolled rise in blood glucose, severe depletion of insulin-producing β cells (Hummel *et al.*, 1966), and a marked decrease in islet mass (Hellerstrom *et al.*, 1976). Therefore, the pancreatic β cells in *db/db* mice at this age are losing the capacity to compensate for insulin resistance and hyperglycaemia. This is, to our knowledge, the first study showing that T3 increased the insulin storage in pancreatic β cells, the number of β cells in pancreatic islet, and plasma insulin levels in *db/db* mice. The present data suggested that a decrease in plasma T3 may contribute to the deterioration of β cell function and further that T3 may restore the capacity of β cells to compensate for insulin resistance and hyperglycemia in *db/db* mice. Plasma levels of free T3 have been reported to be positively associated with insulin secretion in euthyroid individuals (Ortega *et al.*, 2008). It will be interesting to further determine the mechanism that mediates the beneficial effects of T3 on insulin secretion in β cells.

NeuroD is a key transcription factor for pancreatic β cell differentiation and mature β cell function *via* binding to the promoter region of the insulin gene (Kaneto *et al.*, 2009). NeuroD has also been shown to have beneficial effects in animal models of type 1 diabetes due to its insulin-inducing function (Kojima *et al.*, 2003; Huang *et al.*, 2007). The present study demonstrated that NeuroD expression was suppressed in pancreatic islets of diabetic mice and therefore this transcription factor could be involved in β cell dysfunction and insulin depletion in *db/db* mice. We found that exogenous T3 enhanced NeuroD expression in *db/db* mice, and effect which was PI3-kinase-dependent. It has been reported that T3 is a survival factor for human and rodent pancreatic β cells undergoing apoptosis *in vitro* (Verga Falzacappa *et al.*, 2006). Treatment of human pancreatic duct cells with T3 promotes cell differentiation into insulin-producing β-cells, upregulates insulin and glucose transporter-2 transcripts, and increases the insulin release into the medium (Misiti *et al.*, 2005). T3 enhanced insulin release in rat isolated pancreatic islets exposed to glucose concentrations of 2–8 mM (Cortizo *et al.*, 1987). T3 activates Akt in the islet β cell lines such as rRINm5F and hCM through the interaction between TRβ1 and PI3-Kinase p85α (Verga Falzacappa *et al.*, 2007). Protein kinase Akt has been shown to be important in the regulation of β cell growth and survival in a mouse model of streptozotocin-induced diabetes (Tuttle *et al.*, 2001). Therefore, T3 may promote NeuroD expression *via* the up-regulation of the PI3-kinase/Akt pathway, leading to increases in β cell survivability and insulin biosynthesis and storage in diabetic mice. This, however, needs to be tested.

On the basis of our present understanding of T3 signaling, the effects of T3 on T2DM could not be predicted (Baxter and Webb, 2009). Hyperglycemia has been found in patients with hyperthyroidism due to an increase in hepatic glucose production (Crunkhorn and Patti, 2008). Treatments with a large dose of T3 have been shown to increase plasma glucose levels in humans (Dimitriadis *et al.*, 1985). However, moderate doses of T3 have beneficial effects on plasma glucose levels in animal models (Levin *et al.*, 1982; Bryzgalova *et al.*, 2008). Dietary supplement with 0.03% powdered thyroid (equivalent to T3 25 $ng \cdot g^{-1} \cdot day^{-1}$) decreased plasma glucose levels in Zucker *fa/fa* rats (Levin *et al.*, 1982). KB-141, an analogue of T3 (54–328 $ng \cdot g^{-1} \cdot day^{-1}$), decreased plasma glucose levels and increase insulin sensitivity in obese mice (Bryzgalova *et al.*, 2008). The present data revealed that moderate doses of the thyroid hormone T3 ameliorated β-cell dysfunction and reversed insulin resistance, which may offer a new therapeutic strategy for delaying or preventing the development of T2DM. It has been reported that serum levels of free T3 were decreased significantly in patients with T2DM, compared to non-diabetic patients (Islam *et al.*, 2008). It should be mentioned that *db/db* mice are generated by genetic mutation of leptin receptors which results in overeating and subsequently obesity and diabetes. Although it has been reported that copy number variation at the leptin receptor gene locus is associated with metabolic traits and the risk of T2DM in human subjects (Jeon *et al.*, 2010), the *db/db* model may not fully replicate T2DM in humans. The aetiology of human T2DM may involve genetic and environmental factors. Therefore, to increase the therapeutic potential of T3, further studies are required to validate its anti-hyperglycemic effect in other models of insulin resistance or obesity (e.g. the diet-induced obese model).

The major side effect of T3 treatments is tachycardia (Grover *et al.*, 2003). However, T3 at the doses used here did not affect heart rate significantly in *db/db* or lean mice, although it did increase plasma levels of T3. Similar findings have been reported, that is, T3 at doses lower than $65\ ng \cdot g^{-1} \cdot day^{-1}$ did not alter heart rate in Sprague-Dawley rats (Erion *et al.*, 2007). Female *db/db* mice aged 7 weeks have lower serum T4 levels and lower T3 levels in liver and brain, probably due to impaired de-iodination in these tissues of *db/db* mice (Kaplan and Young, 1987). Nevertheless, the mechanism responsible for the low level of T3 in *db/db* mice is not fully understood. The present study suggest that there may be an increase in T3 metabolism associated with the *db/db* mice because the same dose of exogenous T3 increased plasma T3 levels to a lesser degree in *db/db* mice, compared to the lean mice. As reported earlier (Masaki *et al.*, 2000), the present study showed that *db/db* mice had lower body temperatures. Interestingly, T3 increased rectal temperature in *db/db* mice at a dose of $14\ ng \cdot g^{-1} \cdot day^{-1}$ and restored it at a dose of $28\ ng \cdot g^{-1} \cdot day^{-1}$. The latter result suggested that T3 normalized thermogenesis in *db/db* mice. Although T3 did not change basal arterial blood pressure in *db/db* or lean mice, it restored the impaired blood pressure responses to cold exposure in *db/db* mice. In addition, T3 did not alter body weights in lean or *db/db* mice. Thus, T3 at the doses used was safe and restored physiological function in *db/db* mice.

In summary, T3 rapidly decreased blood glucose levels in *db/db* mice. Chronic treatments with T3 attenuated hyperg-

lycemia and improved insulin sensitivity in *db/db* mice. T3 enhanced insulin signaling by potentiating insulin-induced activation of IRS-1 and PI3-kinase *via* TRα1 in preadipocytes. T3 also increased insulin levels in plasma and insulin synthesis and storage in pancreatic islets in *db/db* mice. The anti-diabetic effects of T3 in *db/db* mice were PI3-kinase dependent.

Acknowledgements

This work was supported by NIH R01 HL-077490 (to Z.S.).

Conflicts of interest

None to disclose.

References

Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC), 4th edn. Br J Pharmacol 158 (Suppl. 1): S1–S254.

Baxter JD, Webb P (2009). Thyroid hormone mimetics: potential applications in atherosclerosis, obesity and type 2 diabetes. Nat Rev 8: 308–320.

Bryzgalova G, Effendic S, Khan A, Rehnmark S, Barbounis P, Boulet J *et al*. (2008). Anti-obesity, anti-diabetic, and lipid lowering effects of the thyroid receptor beta subtype selective agonist KB-141. J Steroid Biochem Mol Biol 111: 262–267.

Cheng SY (2005). Thyroid hormone receptor mutations and disease: beyond thyroid hormone resistance. Trends Endocrinol Metab 16: 176–182.

Cortizo AM, Chazenbalk GD, de Gagliardino EE, Garcia ME, Pisarev MA, Gagliardino JJ (1987). Thyroid hormone binding and deiodination by pancreatic islets: relationship with the in vitro effect upon insulin secretion. Acta Endocrinol (Copenh) 116: 66–72.

Crunkhorn S, Patti ME (2008). Links between thyroid hormone action, oxidative metabolism, and diabetes risk? Thyroid 18: 227–237.

Das SK, Elbein SC (2006). The genetic basis of type 2 diabetes. Cellscience 2: 100–131.

Dimitriadis G, Baker B, Marsh H, Mandarino L, Rizza R, Bergman R *et al*. (1985). Effect of thyroid hormone excess on action, secretion, and metabolism of insulin in humans. Am J Physiol 248 (5 Pt 1): E593–E601.

Erion MD, Cable EE, Ito BR, Jiang H, Fujitaki JM, Finn PD *et al*. (2007). Targeting thyroid hormone receptor-beta agonists to the liver reduces cholesterol and triglycerides and improves the therapeutic index. Proc Natl Acad Sci U S A 104: 15490–15495.

Ferrannini E (1998). Insulin resistance versus insulin deficiency in non-insulin-dependent diabetes mellitus: problems and prospects. Endocr Rev 19: 477–490.

Ferrannini E, Mari A (2004). Beta cell function and its relation to insulin action in humans: a critical appraisal. Diabetologia 47: 943–956.

Grover GJ, Mellstrom K, Ye L, Malm J, Li YL, Bladh LG *et al*. (2003). Selective thyroid hormone receptor-beta activation: a strategy for reduction of weight, cholesterol, and lipoprotein (a) with reduced cardiovascular liability. Proc Natl Acad Sci U S A 100: 10067–10072.

Hassani Z, Francois JC, Alfama G, Dubois GM, Paris M, Giovannangeli C *et al*. (2007). A hybrid CMV-H1 construct improves efficiency of PEI-delivered shRNA in the mouse brain. Nucleic Acids Res 35: e65.

Hellerstrom C, Andersson A, Gunnarsson R (1976). Regeneration of islet cells. Acta Endocrinol Suppl (Copenh) 205: 145–160.

Hiroi Y, Kim HH, Ying H, Furuya F, Huang Z, Simoncini T *et al*. (2006). Rapid nongenomic actions of thyroid hormone. Proc Natl Acad Sci U S A 103: 14104–14109.

Huang Y, Chen J, Li G, Cheng TY, Jiang MH, Zhang SY *et al*. (2007). Reversal of hyperglycemia by protein transduction of NeuroD *in vivo*. Acta Pharmacol Sin 28: 1181–1188.

Hubbard SR, Wei L, Ellis L, Hendrickson WA (1994). Crystal structure of the tyrosine kinase domain of the human insulin receptor. Nature 372: 746–754.

Hummel KP, Dickie MM, Coleman DL (1966). Diabetes, a new mutation in the mouse. Science 153: 1127–1128.

Islam S, Yesmine S, Khan SA, Alam NH, Islam S (2008). A comparative study of thyroid hormone levels in diabetic and non-diabetic patients. Southeast Asian J Trop Med Public Health 39: 913–916.

Jeon JP, Shim SM, Nam HY, Ryu GM, Hong EJ, Kim HL *et al*. (2010). Copy number variation at leptin receptor gene locus associated with metabolic traits and the risk of type 2 diabetes mellitus. BMC Genomics 11: 426.

Jiang W, Miyamoto T, Kakizawa T, Sakuma T, Nishio S, Takeda T *et al*. (2004). Expression of thyroid hormone receptor alpha in 3T3-L1 adipocytes; triiodothyronine increases the expression of lipogenic enzyme and triglyceride accumulation. J Endocrinol 182: 295–302.

Kahn SE (2003). The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. Diabetologia 46: 3–19.

Kaneto H, Matsuoka TA, Katakami N, Matsuhisa M (2009). Combination of MafA, PDX-1 and NeuroD is a useful tool to efficiently induce insulin-producing surrogate beta-cells. Curr Med Chem 16: 3144–3151.

Kaplan MM, Young JB (1987). Abnormal thyroid hormone deiodination in tissues of ob/ob and db/db obese mice. Endocrinology 120: 886–893.

Klein I, Danzi S (2007). Thyroid disease and the heart. Circulation 116: 1725–1735.

Kojima H, Fujimiya M, Matsumura K, Younan P, Imaeda H, Maeda M *et al*. (2003). NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. Nat Med 9: 596–603.

Levin BE, Triscari J, Sullivan AC (1982). Sympathetic activity in thyroid-treated Zucker rats. Am J Physiol 243: R170–R178.

Marchetti P, Dotta F, Lauro D, Purrello F (2008). An overview of pancreatic beta-cell defects in human type 2 diabetes: implications for treatment. Regul Pept 146: 4–11.

Masaki T, Yoshimatsu H, Chiba S, Sakata T (2000). Impaired response of UCP family to cold exposure in diabetic (db/db) mice. Am J Physiol Regul Integr Comp Physiol 279: R1305–R1309.

Misiti S, Anastasi E, Sciacchitano S, Verga Falzacappa C, Panacchia L, Bucci B *et al*. (2005). 3,5,3′-Triiodo-L-thyronine enhances the differentiation of a human pancreatic duct cell line (hPANC-1) towards a beta-cell-Like phenotype. J Cell Physiol 204: 286–296.

Naya FJ, Huang HP, Qiu Y, Mutoh H, DeMayo FJ, Leiter AB *et al*. (1997). Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. Genes Dev 11: 2323–2334.

Ortega E, Koska J, Pannacciulli N, Bunt JC, Krakoff J (2008). Free triiodothyronine plasma concentrations are positively associated with insulin secretion in euthyroid individuals. Eur J Endocrinol 158: 217–221.

Rhodes CJ (2005). Type 2 diabetes-a matter of beta-cell life and death? Science 307: 380–384.

Robertson DM, Sima AA (1980). Diabetic neuropathy in the mutant mouse [C57BL/ks(db/db)]: a morphometric study. Diabetes 29: 60–67.

Romero R, Casanova B, Pulido N, Suarez AI, Rodriguez E, Rovira A (2000). Stimulation of glucose transport by thyroid hormone in 3T3-L1 adipocytes: increased abundance of GLUT1 and GLUT4 glucose transporter proteins. J Endocrinol 164: 187–195.

Sharma K, McCue P, Dunn SR (2003). Diabetic kidney disease in the *db/db* mouse. Am J Physiol Renal Physiol 284: F1138–F1144.

Sun Z, Bello-Roufai M, Wang X (2008). RNAi inhibition of mineralocorticoid receptors prevents the development of cold-induced hypertension. Am J Physiol Heart Circ Physiol 294: H1880–H1887.

Tuttle RL, Gill NS, Pugh W, Lee JP, Koeberlein B, Furth EE *et al*. (2001). Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. Nat Med 7: 1133–1137.

Verga Falzacappa C, Panacchia L, Bucci B, Stigliano A, Cavallo MG, Brunetti E *et al*. (2006). 3,5,3′-triiodothyronine (T3) is a survival factor for pancreatic beta-cells undergoing apoptosis. J Cell Physiol 206: 309–321.

Verga Falzacappa C, Petrucci E, Patriarca V, Michienzi S, Stigliano A, Brunetti E *et al*. (2007). Thyroid hormone receptor TRbeta1 mediates Akt activation by T3 in pancreatic beta cells. J Mol Endocrinol 38: 221–233.

Wang Y, Sun Z (2009). Klotho gene delivery prevents the progression of spontaneous hypertension and renal damage. Hypertension 54: 810–817.

Zimmet P, Alberti KG, Shaw J (2001). Global and societal implications of the diabetes epidemic. Nature 414: 782–787.