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Thyroid Hormone Promotes Insulin-induced Glucose Uptake by Enhancing Akt Phosphorylation and VAMP2 Translocation in 3T3-L1 Adipocytes

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Abstract

The purpose of this study was to test a hypothesis that T3 promotes glucose uptake *via* enhancing insulin-induced Akt phosphorylation and VAMP2 translocation in 3T3-L1 adipocytes. T3 significantly enhanced insulin-induced phosphorylation of Akt, cytoplasm to cell membrane translocations of vesicle-associated membrane protein 2 (VAMP2) and glucose transporter 4 (GLUT4), and glucose uptake in adipocytes. Akt inhibitor X abolished the promoting effects of T3, suggesting that Akt activation is essential for T3 to enhance these insulin-induced events in adipocytes. Knockdown of VAMP2 using siRNA abrogated the effects of T3 on insulin-induced GLUT4 translocation and glucose uptake, suggesting that VAMP2 is an important mediator of these processes. Conclusions - These data suggest that T3 may promote glucose uptake *via* enhancing insulin-induced phosphorylation of Akt and subsequent translocations of VAMP2 and GLUT4 in 3T3-L1 adipocytes. Akt phosphorylation is necessary for the promoting effects of T3 on insulin-stimulated VAMP2 translocation. Further, VAMP2 is essential for T3 to increase insulin-stimulated translocation of GLUT4 and subsequent uptake of glucose in adipocytes.

Keywords

GLUT4; RNAi; T3; Akt; VAMP2

Introduction

The central cellular mechanism for removal of glucose from circulation is insulin-stimulated glucose transport majorly into skeletal muscles and adipose tissues (Zaid et al., 2008). The insulin-stimulated glucose uptake in mammalian cells is essential in whole-body glucose homeostasis. The chronically elevated level of blood glucose or hyperglycemia due to the impairment of insulin actions in peripheral tissues is the hallmark of type 2 diabetes mellitus (T2DM)(Lin and Sun, 2010a), which is predicted to affect about 150 million people worldwide (Das and Elbein, 2006; Zimmet et al., 2001).

Glucose uptake involves the transfer of glucose across cell membranes through integral transport proteins. The principal glucose transporter protein that mediates this uptake is

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glucose transporter 4 (GLUT4) in skeletal muscles and adipose tissues (Zaid et al., 2008). A key function of insulin is to promote translocation of GLUT4 to plasma membrane from GLUT4-containing vesicles, leading to uptake of glucose by these cells (Zaid et al., 2008). GLUT4 catalyzes glucose transporters across plasma membranes through an ATP-independent, facilitative diffusion mechanism (Hruz and Mueckler, 2001). The insulin signaling such as the insulin-insulin receptor (IR)-IR substrate-PI3 kinase-Akt axis is essential in regulating GLUT4 translocation and glucose uptake (Zaid et al., 2008). Several lines of evidences support an important role for Akt in insulin-stimulated GLUT4 translocation and glucose uptake (Leney and Tavaré, 2009; Zaid et al., 2008). Assembly of the soluble N-ethylmaleimide-sensitive factor-attachment protein (SNAP) receptor (SNARE) complex is a key step for GLUT4 translocation. A SNARE complex consists of vesicle SNARE (v-SNARE) proteins including syntaxin4, SNAP23 and cognate target SNARE (t-SNARE) proteins such as VAMP2. VAMP2 may be involved in insulin-stimulated GLUT4 translocation and glucose uptake (Kawaguchi et al., ; Olson et al., 1997; Williams and Pessin, 2008).

Our recent study indicated that acute injections of T3 rapidly decreased blood glucose levels and that chronic injections of T3 increased insulin sensitivity in diabetic leptin receptor-deficient (db/db) mice (Lin and Sun, 2010b). Long-term treatment with thyroxine improves glucose dynamics in healthy adult horses (Frank et al., 2008). KB14, an analog of thyroid hormone, reduces plasma glucose in response to a glucose challenge or insulin tolerance test in diabetic leptin-deficient ob/ob mice (Bryzgalova et al., 2008). Thyroid hormone increased glucose uptake in heart and cultured cells (Romero et al., 2000; Segal, 1989; Shimizu and Shimazu, 2002; Weinstein et al., 1994). Thyroid hormones rapidly increased glucose uptake into cultured chick embryo heart cells and rat thymocytes (Segal and Gordon, 1977; Segal and Ingbar, 1980). Short-term treatments with T3 stimulated glucose uptake into L6 muscle cells (Gordon et al., 2006). Therefore, thyroid hormone may be involved in the regulation of glucose uptake in peripheral tissues. However, the underlying mechanism is not fully understood. The purpose of this study was to test a hypothesis that T3 promotes glucose uptake *via* enhancing insulin-induced Akt phosphorylation and VAMP2 translocation in 3T3-L1 adipocytes.

Materials and Methods

Cell culture

Murine 3T3L1 preadipocytes were purchased from the American Type Culture Collection (Manassas, VA). 3T3-L1 preadipocytes were cultured DMEM (Cell Signaling) supplemented with 10% bovine calf serum (BCS, ATCC), 100 µg/ml of streptomycin (Sigma) and 100 U/ml of penicillin (Sigma) at 37°C, 5% CO₂. Differentiation was induced in 3T3-L1 preadipocytes as described previously (Tordjman et al., 1989). Briefly, after confluence, cells were incubated with DMEM containing 5 µg/ml insulin (Sigma), 0.25 µM dexamethasone (Sigma), 0.5 mM isobutyl-1-methylxanthine (Sigma), and 10% BCS for 48 hours. Cells were then incubated with DMEM containing 5 µg/ml insulin and 10% BCS for another 48 hours, followed by DMEM containing with 10% BCS. Greater than 95% of the induced cells had displayed the adipocytes morphology (rounded with intracellular fat droplets under phase-contrast microscopy) by day 9. Mature adipocytes were used in experiments from 9 to 13 days after the initiation of differentiation.

siRNA transfection

Two duplex of siRNA sequences against mouse VAMP2 gene (Sequence A: GGTTCGAC TGAAAACCTTTC and Sequence B: GGAAGGATCTAATCTTTT) were used (Williams and Pessin, 2008). siRNA sequences were synthesized by Ambion. SiPORT NeoTM was

used as transfection reagent according to the instruction (Cat#: AM4510, Ambion). Mature adipocytes were incubated with transfection reagent alone, 90 nM control siRNA (Cat#: AM4611, Ambion), or 45 nM VAMP2 siRNA sequence A plus 45 nM VAMP2 siRNA sequence B, respectively, for 56 hours.

Measurement of glucose uptake

3T3-L1 cells were grown and differentiated in 12-well culture plates. Glucose uptake in these cells was measured using a modified procedure as described previously (Nakata et al., 2006). Briefly, confluent mature adipocytes were incubated in phenol-red-free DMEM containing 0.5% charcoal-stripped FBS for 16 hours and then in phenol-red-free DMEM with 0.05% BSA for 2 hours. After washing with Krebs–Ringer phosphate buffer (KRB) containing glucose at the concentration of 2.8 mmol/L, cells were incubated in 500 μ l of KRB for 30 min at 37°C. Subsequently, cells were incubated with T3 for 30 minutes and then stimulated with insulin (1 nmol/L) (Sigma) for 10 min in KRB at 37°C. In some experiments, Akt inhibitor X at the concentration of 20 μ M (Santa Cruz Biotechnology) was added 15 minutes before the incubation of cells with T3. Uptake was initiated by addition of a fluorescent derivative of 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) at 600 μ mol/l (Invitrogen) in KRB containing 2.8 mmol/l glucose. After 15 min, the reaction was terminated by quickly washing the cells with ice-cold KRB. Cells were lysed by freeze–thaw. Fluorescence of 2-NBDG was measured using fluorescence spectrophotometer SynergyTM2 (BioTek Instruments, Vermont) at a wavelength of 528 nm (excitation wavelength at 485 nm).

Fractionation of plasma membrane proteins

A differential centrifugation procedure was used to isolate crude plasma membrane proteins from mature adipocytes as described previously (Elmendorf, 2003). Briefly, cells in one 100-mm dish were rinsed twice in cold HES buffer (20 mM HEPES, 1 mM EDTA, 250 mM sucrose, pH 7.4) and homogenized in HES buffer containing protease inhibitors (Sigma) (2 ml/dish) by 10 strokes with a pestle (Pyrex). The homogenate was centrifuged at 19,000g in a fixed-angle rotor (JA-20.1 rotor, Beckman) for 20 minutes at 4°C. The pellet was resuspended in HES buffer and centrifuged again at 19,000g for 20 minutes. The pellet was resuspended again in HES buffer, and layered onto a sucrose cushion (38.5%) and centrifuged for 60 minutes at 100,000g in a swing-out rotor (SW41; Beckman, Fullerton, CA). The plasma membrane fraction was collected from the top of the sucrose cushion (white fluffy band at sucrose cushion interface), resuspended in HES, and repelleted by centrifugation at 40,000g for 20 minutes. The pellet of plasma membrane fractions were resuspended in HES buffer containing protease inhibitor and store at –20°C. The protein concentration of these membrane fractions was determined using the BCA assay (Pierce).

Western blotting analysis

Confluent mature adipocytes were incubated in phenol-red-free DMEM containing 0.5% charcoal-stripped FBS for 16 hours and then in phenol-red-free DMEM with 0.05% BSA for 2 hours. Cells were incubated with the control vehicle or T3 at various concentrations for 30 minutes and then incubated with the control vehicle or 1 nM insulin for 15 minutes. In some experiments, cells were incubated with Akt inhibitor X (20 μ M) for 15 minutes before adding T3 or control vehicle. The cells were then lysed with Ripa buffer containing protease inhibitor cocktail (Cat: P8340; 1:100 dilution, Sigma), 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 2 mM sodium vanadate, 1 mM EDTA, and 1 mM EGTA. The protein concentration was measured with Pierce BCA assay (Thermo Scientific). Lysates (40 μ g protein/well) under the reducing condition were directly subjected to SDS-PAGE (4–20% Tris-HCL precast gel, Bio-Rad) followed by Western blotting with antibody against phosphor-Akt (Ser 473) (Cat: 4051, Cell Signaling Technology). The same blot was re-

probed with antibody against Akt (Cat: 4685, Cell Signaling Technology) after stripping the blot.

For the cells transfected with siRNA or control reagents for 74 hours (including the initial transfection and the preparation time), the cells were lysed with above Ripa buffer. Lysates (40 µg protein/well) were subjected with SDS-PAGE followed by western blotting with rabbit polyclonal antibody against VAMP2 (Abcam). The same blot was then re-probed with mouse monoclonal antibody against β -actin after stripping as an internal control (Santa Cruz biotechnology).

For plasma membrane proteins after fractionation, 5 µg of protein under the reducing condition was loaded on each well of SDS-PAGE, followed by western blotting with antibody against VAMP2 or GLUT4 (mouse monoclonal, R&D Systems), respectively. The same blot was re-probed with rabbit polyclonal antibody against insulin receptor β (Santa Cruz Biotechnology) after stripping as an internal control.

In some experiments, proteins from whole cell lysates with various treatments under the reducing condition was loaded on each well (40 µg protein/well) of SDA-PAGE, followed by western blotting with antibody against VAMP2 or GLUT4 (mouse monoclonal, R&D Systems), respectively.

Plasma membrane lawn assay and immunofluorescence staining analysis

Plasma membrane sheets were prepared from mature adipocytes as previously described (Robinson et al., 1992). In brief, after the appropriate treatment, coverslips were rinsed with PBS and incubated with 0.5 mg/ml poly-L-lysine (Sigma) in PBS for 30 seconds. Cells were then swollen in a hypotonic buffer (1/3 x Buffer A) by three successive rinses with Buffer A (70 mM KCl, 30 mM Hepes, 5 mM MgCl₂, 3 mM EGTA, pH 7.4). The swollen cells were sonicated in Buffer A. The bound plasma membrane sheets were washed twice with Buffer A and fixed with 4% PFA for 20 minutes at 4°C. The plasma membrane sheets were then blocked using 10% goat serum and 1% BSA. The coverslips were then incubated in antibody against GLUT4 (Cat: MAB1262, R&D Systems) for overnight at 4°C. After three washing with PBS solution, the sheets were incubated with goat anti-mouse IgG FITC conjugate (Cat: F4143, Sigma) for 1 hour at room temperature. After washing, the coverslips were mounted on the glass slide with PBS containing 90% glycerol and 0.1% p-phenylenediamine. The sample was sealed with clear nail polish.

Fluorescence images of plasma membrane sheets from 2 to 4 random fields for each sample were collected at equal exposure conditions under Nikon Eclipse Ti microscopy (FITC, magnification x400) with the software NIS-Elements BR 3.0 (Nikon). Mean fluorescence density from at least 8 representative cells per sample was analyzed using NIS-Elements BR 3.0.

Statistical Analysis—Data for phosphorylation of Akt, glucose uptake, GLUT4 translocation, and VAMP2 translocation were analyzed by one-way ANOVA, followed by Newman-Keuls test. Data were expressed as mean $\pm$ SEM. A probability value with $p < 0.05$ was considered as statistically significant.

Results

T3 potentiated insulin-induced glucose uptake and GLUT4 translocation in adipocytes

We first examined glucose uptake using 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)] amino]-2-deoxy-D-glucose (2-NBDG) fluorescence assay. Treatments of cells with T3 alone at the concentrations of 1 to 100 nM did not affect glucose uptake significantly in adipocytes.

Treatments of cells with 1 nM insulin for 10 minutes significantly increased glucose uptake compared with the control group. Pretreatments of cells with T3 at the concentrations of 1 to 100 nM for 30 minutes increased insulin-stimulated glucose uptake significantly (Fig. 1). Next we investigated GLUT4 translocation using plasma membrane lawn assay. Treatments of cells with T3 alone at the concentrations of 1 to 100 nM did not affect GLUT4 translocation in adipocytes. Treatments of cells with insulin at 1 nM for 5 to 30 minutes significantly increased GLUT4 translocation to plasma membrane (Fig. 2). Pretreatments of cells with 10 nM T3 for 30 minutes enhanced insulin-stimulated GLUT4 translocation significantly, starting from 5-minute after insulin treatment (Fig. 2).

T3 enhanced insulin-induced Ser 473 phosphorylation of Akt in adipocytes

Ser/Thr-protein kinase Akt is essential in insulin signaling in adipocytes (Zaid et al., 2008). To determine whether T3 affects insulin signaling, we measured Ser 473 phosphorylation of Akt, which is required for full activation of Akt (Song et al., 2005). Treatments of cells with T3 alone at the concentrations of 1 to 100 nM did not significantly affect Ser 473 phosphorylation of Akt in adipocytes. Treatments of cells with 1 nM insulin for 15 minutes significantly increased Ser 473 phosphorylation of Akt compared with the control group (Figs. 3A–B). Pretreatments of adipocytes with T3 at the concentrations of 1 to 100 nM for 30 minutes enhanced insulin-induced Ser 473 phosphorylation of Akt (Figs. 3A–B).

Akt activation was required by T3 to enhance insulin-induced GLUT4 translocation and glucose uptake in adipocytes

To investigate the roles of Akt activation in the regulation of insulin-induced glucose uptake and GLUT4 translocation by T3, we used Akt inhibitor X to block Akt activation (Thimmaiah et al., 2005). Akt inhibitor X at the concentration of 20 μ M abrogated the promoting effects of T3 on insulin-stimulated Ser 473 phosphorylation of Akt (Figs. 3C–D). Treatments with Akt inhibitor X at the concentration of 20 μ M abolished the promoting effect of T3 on insulin-induced glucose uptake, suggesting that Akt activation is required by T3 to regulate insulin-induced glucose uptake in adipocytes (Fig. 3E). Then, we investigated the GLUT4 translocation using both plasma membrane sheet assay and western-blotting after fractionation of plasma membrane proteins. Treatments with Akt inhibitor X abolished the promoting effect of T3 on insulin-induced GLUT4 translocation as confirmed by both assays (Fig. 4A–D). Since insulin receptor β subunit (IR β) on plasma membrane in adipocytes does not display significant change after insulin stimulation (Fagerholm et al., 2009), we used IR β as a loading control in western-blotting. GLUT4 content in whole cell lysates remains constant in the same conditions (figure not shown). These data indicated that Akt activation was required by T3 to enhance insulin-induced GLUT4 translocation.

T3 promoted insulin-induced VAMP2 translocation to plasma membrane in adipocytes

VAMP2 is key to the regulation of insulin-induced GLUT4 translocation and glucose uptake *via* forming the SNARE complex, which is one of the important steps in regulation of GLUT4 translocation (Kawaguchi et al., ; Olson et al., 1997; Williams and Pessin, 2008). We measured VAMP2 in plasma membrane using subcellular fractionation assay to obtain plasma membrane proteins, followed by western-blotting. Treatments of cells with T3 alone at the concentration of 10 nM did not significantly affect VAMP2 translocation in adipocytes. Treatments of cells with 1 nM insulin for 30 minutes significantly increased VAMP2 translocation compared with the control group (Fig.5B). Pretreatments with T3 at the concentration of 10 nM for 30 minutes enhanced insulin-induced VAMP2 translocation in adipocytes (Fig. 5B). Treatments with Akt inhibitor X abolished the promoting effect of T3 on insulin-induced VAMP2 translocation (Fig. 5B). Again we did not notice any significant change in total VAMP2 in adipocytes in response to all these short-term

treatments (data not shown). These data indicated that Akt activation is necessary for T3 to upregulate insulin-induced VAMP2 translocation to plasma membrane in adipocytes.

VAMP2 was indispensable for T3 to promote insulin-induced GLUT4 translocation and glucose uptake in adipocytes

To further study the role of VAMP2 in mediating the potentiating effect of T3 on insulin-induced GLUT4 translocation and glucose uptake, we transfected adipocytes with siRNA against VAMP2 gene. Transfection with VAMP2-siRNA for 74 hours decreased the protein levels of VAMP2 by 70% (Fig. 6B). Transfection with VAMP2-siRNA abolished the promoting effect of T3 on insulin-induced GLUT4 translocation and glucose uptake (Fig. 6C and Fig. 6E), suggesting that VAMP2 is indispensable for T3 to enhance insulin-induced GLUT4 translocation and glucose uptake. We also noticed that transfection with VAMP2-siRNA did not completely abolish insulin-induced glucose uptake and GLUT4 translocation although it eliminated the promoting effect of T3. These results suggest that either the residual level of VAMP2 or a VAMP2-independent pathway may partially mediate insulin-stimulated glucose uptake and GLUT4 translocation in these cells.

Discussion

The present study demonstrated that T3 increased insulin-induced glucose uptake in 3T3-L1 adipocytes. This result supports our recent findings that injections of T3 decreased hyperglycemia and increased insulin sensitivity in diabetic db/db mice (Lin and Sun, 2010b). It was reported that treatments with a T3 analog reduced plasma glucose levels and increased insulin sensitivity in diabetic leptin-deficient ob/ob mice (Bryzgalova et al., 2008). The impaired insulin action in peripheral tissues is a hallmark of T2DM (Das and Elbein, 2006; Lin and Sun, 2010a). Clinical studies indicated that the serum free T3 level was about 47% lower in patients with T2DM compared to non-diabetic patients (Islam et al., 2008). Exercise-induced improvements in insulin resistance are significantly blunted in subjects with subclinical hypothyroidism (Amati et al., 2009). The insulin-induced glucose disposal was decreased in patients with hypothyroidism (Dimitriadis et al., 2006). In humans, a decrease in the production of T3 due to genetic deficiency of iodothyronine deiodinase 2 (DIO2) in skeletal muscle is associated with greater insulin resistance in patients with T2DM (Canani et al., 2005). Therefore, it is interesting to test the therapeutic effect of T3 on hyperglycemia and insulin resistance in patients with T2DM.

In order to gain more insights into the mechanisms of the promoting effects of T3 on insulin-induced glucose uptake, we assessed Akt phosphorylation and GLUT4 translocation in adipocytes, the major cascade events of insulin stimulation (Martin et al., 1998; Martin et al., 1996; Zaid et al., 2008). Interestingly, the enhancing effect of T3 on insulin-induced glucose uptake and GLUT4 translocation was abolished by inhibition of Akt. This result suggests, for the first time, that Akt is required by T3 to modulate these insulin-induced events in adipocytes. Since Akt inhibitor X used in the experiments may block more than one isoform of Akt, e.g., Akt1, Akt2, and Akt3 (Thimmaiah et al., 2005), an additional experiment is required to further assess the specific isoform(s) of Akt that mediate the promoting effect of T3 on insulin signaling in adipocytes.

Previous attention was given to the role of VAMP2 in the regulation of GLUT4 translocation (e.g. vesicle docking and fusion) whereas few studies have focused on VAMP2 translocation in response to insulin signaling (Martin et al., 1996; Zaid et al., 2008). The present findings revealed that insulin induced VAMP2 translocation and that T3 greatly enhanced this process. Inhibition of Akt eliminated not only insulin-induced VAMP2 translocation but the enhancing effect of T3 on insulin-induced VAMP2 translocation as well. These results revealed a previously unidentified role of Akt phosphorylation/activation

in insulin-induced VAMP2 translocation. More importantly, VAMP2 may be necessary for T3 to promote insulin-induced GLUT4 translocation and glucose uptake because RNAi knockdown of VAMP2 abrogated the promoting effects of T3 on these processes. These findings suggest a new pathway (e.g., Akt-VAMP2-GLUT4) in mediating the promoting effects of T3 on insulin-stimulated glucose uptake in adipocytes.

The molecular process that mediates the promoting effect of T3 on insulin-induced phosphorylation of Akt is not fully understood. It has been well documented that insulin binds to the α -subunits of the heterotetrameric insulin receptor (IR; $\alpha, \alpha, \beta, \beta$), 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 (Czech and Corvera, 1999; Saltiel and Kahn, 2001; Virkamaki et al., 1999). Tyrosine phosphorylation of IRS-1 as a docking protein binds to the p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase), leading to the activation of the p110 catalytic subunit of PI3-kinase (Czech and Corvera, 1999; Saltiel and Kahn, 2001; Virkamaki et al., 1999). PI3-kinase generates PI triphosphate from PI-bisphosphate, leading to the phosphorylation and activation of Akt (Czech and Corvera, 1999; Saltiel and Kahn, 2001; Virkamaki et al., 1999). T3 was shown to rapidly increase the association of thyroid hormone receptor (TR) $\alpha 1$ with the p85 subunit of PI3-kinase, resulting in activation and phosphorylation of Akt in endothelial cells and cardiomyocytes (Hiroi et al., 2006; Iordanidou et al.). We noticed that T3 stimulated Akt phosphorylation and glucose uptake in cardiac myocytes (Iordanidou et al.) whereas T3 alone did not affect these processes in adipocytes in the current experiments. T3 increased Akt phosphorylation and glucose uptake only in the presence of insulin. This finding may reflect the differential responses of adipocytes and cardiac myocytes to T3 although the detailed molecular mechanism needs to be determined. Therefore, the current study suggests that T3 may promote insulin-induced Akt phosphorylation rather than directly stimulate Akt phosphorylation in adipocytes. Our recent study (Lin and Sun, 2010b) showed that T3 enhanced insulin-induced activation of PI3 kinase via TR $\alpha 1$. It is known that PI3 kinase could activate Akt. A further study is needed to assess if PI3 kinase may be involved in the promoting effect of T3 on insulin-induced activation of the Akt-VAMP2 pathway.

The promoting effect of T3 on the insulin signaling may not be due to changes in transcription mechanism of TR because the effects of T3 occurred within 30 min. Instead, we believe that T3 may enhance the insulin signaling at the functional level. This hypothesis is supported by the current data that T3 increased insulin-induced phosphorylation of Akt but did not alter the total Akt level. In another word, the effect of T3 may be mediated by its cytosolic action rather than nuclear action ("non-genomic").

Serum concentration of free T3 normally is in picomolar range *in vivo* as most of T3 is bound to carrier proteins such as thyroxine-binding globulin (Williams, 2000; Yen, 2001). The concentrations of T3 used in the present study (1 nM to 100 nM) are higher and actually within the dissociation constant for thyroid hormone receptor (i.e., 0.1–1 nM). Given that the serum free T3 level was about 47% lower in patients with T2DM and that some patients suffered thyroid hormone resistance in peripheral tissues (Agrawal et al., 2008; Islam et al., 2008), super-physiological concentrations of thyroid hormone may be required to achieve the therapeutic effects. It remains to be determined why super-physiological concentrations of T3 are required for its promoting effects on insulin-induced events in mature adipocytes.

In summary, the present results indicated that T3 enhanced insulin-induced phosphorylation of Akt, translocations of VAMP2 and GLUT4 to plasma membrane, and glucose uptake in 3T3-L1 adipocytes. The novel finding of this study is that Akt phosphorylation is necessary

for the promoting effects of T3 on insulin-stimulated VAMP2 translocation in adipocytes. Further, VAMP2 is essential for T3 to increase insulin-stimulated translocation of GLUT4 and glucose uptake in adipocytes. These findings would deepen our understanding of the anti-diabetic effects of T3. These findings also provided experimental evidence for further testing if T3 would enhance the therapeutic effect of insulin in diabetic patients.

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Figure 1.

Effects of T3 on insulin-induced glucose uptake in 3T3-L1 adipocytes. Serum-starved cells were pretreated with or without T3 for 30 minutes and then incubated with insulin at 1 nM for 10 minutes. The uptake of 2-NBDG in cells was measured. Results were standardized to the control treatment after subtracting the reading from the sample in the absence of 2-NBDG. Each measurement was performed in duplicate and the results were obtained from 3 independent experiments. ** $p < 0.01$, *** $p < 0.001$ vs control vehicle; + $p < 0.05$, ++ $p < 0.01$ vs insulin alone.

Figure 2. Effects of T3 on insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. **A**, Representative plasma membrane sheets stained with mouse antibody against GLUT4, followed by FITC-conjugated goat anti-mouse IgG. Serum-starved cells were pretreated with or without T3 for 30 minutes and then incubated with or without insulin at 1 nM for different intervals. **B**, Relative fluorescence intensity of GLUT4 on the plasma membrane. Results were standardized to the control treatment and were obtained from 3 to 4 independent experiments. *** $p < 0.001$ vs control vehicle; +++ $p < 0.001$ vs insulin for 5 minutes; ^^^ $p < 0.001$ vs insulin for 15 minutes; ### $p < 0.001$ vs insulin for 30 minutes.

Figure 3.

Effects of T3 on insulin-induced Ser-473 phosphorylation of Akt and effects of Akt inhibitor X on the potentiation of insulin-stimulated glucose uptake by T3. **A**, Ser-473 phosphorylation of Akt in cell lysates was immunoblotted (upper panel). Akt protein was re-probed (lower panel). Serum-starved cells were incubated with the control vehicle or T3 for 30 minutes and then incubated with the control vehicle or 1 nM insulin for 15 minutes. **B**, Quantification of Ser-473 phosphorylation of Akt. Results were standardized to Akt protein level and then to the control vehicle ($n = 3$). * $p < 0.05$, *** $p < 0.001$ vs control vehicle; + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ vs insulin alone. **C**, Ser-473 phosphorylation of Akt in cells treated with and without Akt inhibitor X (upper panel). Akt protein was re-probed (lower panel). **D**, Quantification of Ser-473 phosphorylation of Akt in cells treated with or without Akt inhibitor X ($n = 3$). * $p < 0.05$, *** $p < 0.001$ vs control vehicle; + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ vs insulin alone; ### $p < 0.001$ vs T3 plus insulin. **E**, Akt inhibitor X abolished the effects of T3 on insulin-stimulated glucose uptake. Cells were first pretreated with or without Akt inhibitor X for 15 minutes, then treated with or without T3 for 30 minutes, and incubated with insulin at 1 nM for additional 10 minutes. The uptake of 2-NBDG in cells was measured ($n=4$). ** $p < 0.01$, *** $p < 0.001$ vs control vehicle; +++ $p < 0.001$ vs insulin alone; ### $p < 0.001$ vs T3 plus Insulin.

Figure 4.

Effects of Akt inhibitor X on the potentiation of insulin-stimulated GLUT4 translocation by T3 (fluorescence assay and western blot assay). Cells were first pretreated with or without Akt inhibitor X for 15 minutes, then treated with or without T3 for 30 minutes, and incubated with or without insulin for additional 30 minutes. **A**, Representative plasma membrane sheets stained with antibody against GLUT4, followed by FITC-conjugated goat anti-mouse IgG (images without insulin treatment not shown here). **B**, Relative fluorescence intensity of GLUT4 in the plasma membrane ($n = 4$). *** $p < 0.001$ vs control vehicle; +++ $p < 0.001$ vs insulin alone; ### $p < 0.001$ vs T3 plus insulin. **C**, GLUT4 in plasma membrane crude fractionation was blotted with antibody against GLUT4 (upper panel). The same blot was re-probed with antibody against insulin receptor β after stripping (Lower panel). **D**, Quantification of GLUT4 in crude plasma membrane fractionation. Results were standardized to the IR β level and then to the control vehicle ($n = 3$). * $p < 0.05$, *** $p < 0.001$ vs control vehicle; + $p < 0.05$, +++ $p < 0.001$ vs insulin alone; ### $p < 0.001$ vs T3 plus insulin.

Figure 5.

Effects of T3 on insulin-induced VAMP2 translocation: role of Akt. Western-blotting analysis VAMP2 after fractionation of plasma membrane proteins from cells. Cells were first pretreated with or without Akt inhibitor X for 15 minutes, then treated with or without T3 for 30 minutes, and further incubated with or without insulin for 30 minutes. Crude plasma membrane proteins were prepared. **A**, VAMP2 in plasma membrane crude fractionation was blotted with antibody against VAMP2 (upper panel). The same blot was re-probed with antibody against IR β after stripping (Lower panel). **B**, Quantification of VAMP2 in crude plasma membrane fractionation. Results were standardized to the IR β level and then to the control vehicle (n = 3). * p < 0.05, *** p < 0.001 vs control vehicle; + p < 0.05, +++ p < 0.001 vs insulin alone; ### p < 0.001 vs T3 plus insulin.

Figure 6.

Effects of RNAi knockdown of VAMP2 on the potentiation of insulin-stimulated glucose uptake and GLUT4 translocation by T3. Cells were transfected for 56 hours and then were prepared. **A**, Western-blotting for VAMP2 after transfection (upper panel). The same blot reprobed with antibody against β -actin (lower panel). **B**, Quantification of VAMP2 in cells transfected with VAMP2 siRNA. Results were standardized to β -actin protein level and then to the transfection reagent alone ($n = 3$). *** $p < 0.001$ vs transfection reagent alone; +++ $p < 0.001$ vs control siRNA. **C**, Serum-starved cells transfected with siRNAs and were pretreated with or without T3 for 30 minutes, and then incubated with insulin for 10 minutes. The uptake of 2-NBDG in 3T3-L1 cells was measured ($n=4$). *** $p < 0.001$ vs control vehicle in the control siRNA groups; + $p < 0.05$, ++ $p < 0.01$ vs insulin alone in the control siRNA groups; ^^^ $p < 0.001$ vs control vehicle in the VAMP2 siRNA groups; ### $p < 0.001$ vs T3 plus Insulin in the control siRNA groups. **D**, Representative plasma membrane sheets stained with antibody against GLUT4, followed by FITC-conjugated goat anti-mouse IgG. Transfected cells were pretreated with or without T3 for 30 minutes and then incubated with or without insulin for 30 minutes. **E**, Relative fluorescence intensity of GLUT4 in the plasma membrane. Results were standardized to the control vehicle ($n=4$). *** $p < 0.001$ vs control vehicle in the control siRNA groups; +++ $p < 0.001$ vs insulin alone in the control siRNA groups; ^^ $p < 0.01$, ^^^ $p < 0.001$ vs control vehicle in the VAMP2 siRNA groups; ### $p < 0.001$ vs T3 plus Insulin in the control siRNA groups.