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Trichostatin A improves insulin stimulated glucose utilization and insulin signaling transduction through the repression of HDAC2

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ABSTRACT

Previous study showed that Trichostatin A (TSA) could improve insulin receptor substrate 1 (IRS-1) phosphorylation at tyrosine in response to insulin evocation. However, the effects of TSA on insulin stimulated glucose utilization and insulin signaling transduction are still poorly understood. Here we showed that TSA significantly enhanced insulin stimulated glucose uptake, glycogen synthesis and glycogen synthase activities in C2C12 myotubes. In addition, the insulin stimulated phosphorylations in insulin receptor, Akt and GSK3β were remarkably increased in the TSA-treated cells. These improving effects of TSA were probably due to HDAC2 inhibition, since the enhanced expression of HDAC2 could abolish the TSA-induced improvement in the insulin signaling transduction. Moreover, HDAC2 knockdown as well as TSA treatment also improved insulin stimulated glycogen synthesis. Most importantly, no additional effect of TSA on insulin stimulated glycogen synthesis was observed in the HDAC2 downregulated cells. These data suggest that HDAC2 should be an important potential target for regulating insulin sensitivity.

1. Introduction

Diabetes mellitus, the most common endocrine disorder, is currently affecting over 170 million people world-wide and prospectively over 353 million in the year 2030 [1,2]. Two forms of diabetes, named type 1 and type 2, have been identified. Type 1 diabetes is primarily due to the autoimmune-mediated destruction of pancreatic β cells of the islets, resulting in absolute insulin deficiency. Type 2 diabetes is characterized by the resistance of target tissues to insulin stimulation, and more than 90% of the diabetic patients suffered from it [1]. The peptide hormone insulin lowers blood glucose levels by facilitating glucose uptake mainly into skeletal muscle and fat tissue and by inhibiting endogenous glucose production in the liver [3]. Insulin resistance occurs when a normal dose of the hormone is incapable of eliciting these metabolic responses [4]. Up to now, to alleviate insulin resistance by increasing insulin sensitivity is still one of the key avenues to cure type 2 diabetes [5].

Trichostatin A (TSA) is a natural product isolated from the metabolites of strains of *Streptomyces hygroscopicus* with antifungal antibiotic activities [6]. Subsequent investigations showed that TSA is a potent and specific inhibitor of histone deacetylase (HDAC) [7,8]. Histone acetylation/deacetylation has long been proposed to play a critical role in the regulation of transcription [9,10]. Hence, TSA may possess multiple biological functions by affecting HDAC activities. One of these important roles of TSA is to inhibit tumor cell growth based

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upon a growing number evidences [11]. Additionally, it has been demonstrated that HDAC inhibition by TSA prevents cytokine-induced toxicity in beta cells [12]. Another investigation showed that bone marrow stem cells could differentiate into islet-like clusters under appropriated culture conditions in the presence of TSA [13]. These results indicate that TSA produces some specific biological functions in the insulin-related cells and tissues. Recently, it has been demonstrated that TSA enhances insulin receptor substrate-1 (IRS-1) phosphorylation at tyrosine in response to insulin evocation in MCF-7 cells [14]. However, whether and how TSA affects glucose metabolism in some important insulin responsive cell lines such as C2C12 myotubes and HepG2 cells is still unclear.

To address such issue, we investigated the effects of TSA on insulin stimulated glucose utilization and insulin signaling transduction in C2C12 skeletal muscle cells and human hepatoma HepG2 cells. Our results showed that the insulin stimulated glucose uptake, glycogen synthesis and glycogen synthase activities, as well as the insulin signaling transduction, were improved by TSA in a dose-dependent manner. Further investigations showed that these improving effects are probably due to the repression of HDAC2 induced by TSA.

2. Materials and methods

2.1. Preparation of recombinant herpes simplex virus (HSV)

The HDAC2 cDNA was amplified by RT-PCR from mouse muscle tissue using GGATACGCGT CTTGAGCCCGTCGGCAGGGCA and GGAAGTCGACGGCAGTGTTACCCAGTCTGTCAG as primers, and then subcloned into Mlu I and Sal I sites in pHSVPrPUC-myc vector. The recombinant HSV-HDAC2 virus particles were prepared, amplified and titered as described previously [15].

2.2. Cell culture and treatments

The mouse myoblastoma C2C12 and human hepatoma (HepG2) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). C2C12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, and differentiated in DMEM with 2% horse serum after reaching confluence. After 4 days, the C2C12 cells were differentiated into myotubes, and treated with TSA (Sigma) for 24 h at indicated concentrations. For glucose deprivation, C2C12 myotubes were incubated for 3 h in glucose-free DMEM supplemented with 10% dialyzed fetal bovine serum and nonessential amino acids. Subsequently, the treated cells were stimulated with 20 nM insulin for 20 min, and then cells were applied for glucose uptake and glycogen synthesis assays and blotting. HepG2 cells were maintained in DMEM with high glucose (4 g/l) supplemented with 10% (v/v) fetal calf serum (Gibco) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The cells were grown in 5% CO_2:95% air at 37 °C and passaged using a split ratio at 1:4 after trypsinization. After transfection with siRNA against HDAC2, HepG2 cells were treated with TSA at 100 μM for 24 h. The treated cells were maintained in DMEM with low glucose (1 g/l) for 12 h before glycogen synthesis assay.

2.3. Glucose uptake assay

Glucose uptake was determined by applying the method described previously [16] with some modifications. In brief, cells were incubated for 20 min at 37 °C in the absence or presence of 20 nM insulin in Krebs–Ringer phosphate buffer (128 mM NaCl, 4.7 mM KCl, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, pH 7.4). Then ^{3}H-2-deoxy-glucose (Amersham) and 2-deoxyglucose were added to a final concentration of 1 μCi/ml and 0.1 mM, respectively, and incubated for 5 min. After washing with ice-cold PBS for four times, the cells were lysed with 0.5 M NaOH for 30 min. The radiolabeled glucose in lysates was measured in a high flash-point scintillation cocktail (PerkinElmer) using a liquid scintillation counter (PerkinElmer).

2.4. Glycogen synthesis assay

Glycogen synthesis was determined using a modified method described previously [17]. The cells cultured in 24-well plates were treated with 20 nM insulin and 1 μCi/ml ^{3}H-glucose in DMEM with 5 mM glucose for 3 h. Then the cells harvested for glycogen synthesis assay. Each well was lysed in 200 μl 30% KOH with 5 mg/ml glycogen at 60 °C for 30 min. The cell lysates were collected, and glycogen was precipitated overnight at −20 °C by adding 1 ml of ethanol. Glycogen was separated by centrifugation at 5000 × *g* for 10 min, and the pellets were washed twice with 75% ice-cold ethanol. Finally, the pellets was solubilized in 200 μl of 0.1 M HCl, and measured by a liquid scintillation counter (PerkinElmer) by adding 0.7 ml of high flash-point scintillation cocktail.

2.5. Measurement of glycogen synthase activity

Glycogen synthase activity was determined as described previously [18]. Briefly, cells in 100 mm dishes were treated with or without TSA at different concentrations as indicated for 24 h. Then, the cells were serum-deprived in Krebs–Ringer buffer with 30 mM Hepes (KRPH; pH 7.4) with 0.5% bovine serum albumin in the absence of glucose for 3 h, and then incubated with insulin (20 nM) for 20 min at 37 °C. After three washes with ice-cold PBS, cells were scraped into 500 μl of glycogen synthase assay buffer (50 mM Tris–HCl, pH 7.8, 10 mM EDTA and 100 mM KF) and homogenized with a glass–glass dounce homogenizer prior to centrifugation (10,000 × *g*, 20 min). To measure glycogen synthase activity, 50 μl of the supernatant (100–200 μg of protein) was added to an equal volume of original buffer containing 10 mM UDP-[^{14}C]glucose (PerkinElmer) and 15 mg/ml rat glycogen (Sigma), in the presence of absence of 10 mM glucose 6-phosphate (Sigma). After 15-min incubation at 37 °C, assay tubes were chilled for 15 min in an ice bath. Tube contents were then spotted on prelabeled Whatman filter papers (GF/A; 2.4 cm) which were immediately immersed in 400 ml of 70% ethanol (4 °C), mixed 40 min, then washed three more times in 200 ml of 70% ethanol (30 min each) to remove unincorporated substrate from precipitated glycogen. Filters were air-dried, and radioactivity was counted with 5 ml of scintillation cocktail using a liquid scintillation counter (PerkinElmer).

2.6. HDAC2 knockdown by small interference RNA (siRNA)

Double stranded RNA duplex was designed against the *HDAC2* gene to recognize the sequence 5′-AATCCGCATGACCCA-TAACTT-3′. For complementary siRNA transfection, we used the Cell Line Nucleofector Kit V (Amaxa) according to the manufacturer's instructions. Briefly, the dissociated HepG2 cells were resuspended in transfection medium, mixed with siRNAs (200 nM), and electroporated using the fixed programme (H-22) for optimal transfection. Cells were then quickly centrifuged, resuspended and plated. The knockdown efficiency was determined by Western blotting of extracts from transfected cultures. Scrambled siRNA with non-silencing effect on *HDAC2* expression was also synthesized and used as a control.

2.7. Western blot

Protein samples were analyzed with antibodies against insulin receptor, Tyr1146-phosphorylated insulin receptor, Ser473-phosphorylated Akt, Akt, Ser9-phosphorylated GSK-3β, (Cell Signaling); HDAC2 (Santa Cruz); β-tubulin (Sigma). The immune complexes were detected using a horseradish peroxidase-conjugated secondary antibody and visualized with a chemiluminescence reagent (Pierce). Each blot shown in the figures is representative of at least three experiments. Protein quantification was performed by Quantity One software (Bio-Rad), and the intensity values were normalized to insulin receptor, or Akt, or tubulin.

2.8. Statistics

Data are expressed as means ± S.D. of at least three independent experiments. Statistical significance was assessed by Student's *t*-test. Values were considered statistically significant when $p < 0.05$.

3. Results

3.1. Time course of the effects of TSA on insulin stimulated glucose uptake and insulin receptor phosphorylation

To choose an appropriate treating time, we treated C2C12 myotubes with TSA by different time durations and investigated time course of the effects of TSA on insulin stimulated glucose uptake and insulin receptor phosphorylation. The insulin stimulated glucose uptake was increase gradually upon the treating time till to 24 h, however, when the time prolonged further to 36 h, the glucose uptake was decreased slightly (Fig. 1A). Statistical analysis showed TSA significantly enhanced insulin stimulated glucose uptake with 24 h duration. Additionally, the insulin receptor phosphorylation at tyrosine in response to insulin stimulation was remarkably increased in the TSA-treated cells for 12 and 24 h (Fig. 1B). No significant alteration was occurred in the TSA-treated cells with 6 or 36 h duration. Based upon the above data, we choose 24 h as an appropriate treating time to investigate the effects of TSA on insulin stimulated glucose utilization and insulin signaling transduction in the following experiments.

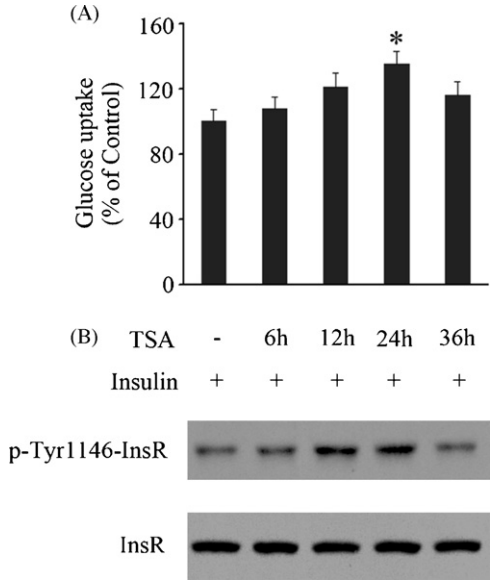

Fig. 1 – The time-course effects of TSA on insulin stimulated glucose uptake and insulin receptor phosphorylation. (A) Glucose uptake was determined as described in Section 2 by using ^{3}H-2-deoxy-glucose. $^*p < 0.05$ vs. cells only treated insulin. Values represent means ± S.D. of two independent determinations, each performed in triplicate. (B) The protein level was analyzed by Western blotting with the antibodies directly against p-Try1146-InsR and InsR. Representative experiments are shown. The applied TSA and insulin concentrations were 100 and 20 nM, respectively.

3.2. TSA improved insulin stimulated glucose uptake, glycogen synthesis and glycogen synthase activity

To evaluate the effects of TSA on glucose utilization, we first measured glucose uptake and glycogen synthesis in C2C12 myotubes. As shown in Fig. 2A, TSA significantly increased glucose uptake in C2C12 myotubes with insulin stimulation in a dose-dependent manner. However, the basal uptake was not affected by TSA. Similarly, the insulin stimulated glycogen synthesis also was significantly increased by TSA in a dose-dependent manner (Fig. 2B). TSA failed to enhance glycogen synthesis in HepG2 cells without insulin stimulation. The glycogen synthesis was improve by TSA, which prompt us to investigate whether TSA affect glycogen synthase activity. To address this speculation, we treated C2C12 myotubes with TSA at different concentrations. As we expected, the glycogen synthase activities were significantly activated by TSA with insulin stimulation in C2C12 myotubes (Fig. 3).

3.3. TSA increased insulin signaling transduction

The increases in glucose uptake, glycogen synthesis and glycogen synthase activities induced by TSA raised a possibility that the insulin signaling transduction also may be strengthened by TSA. Thus, we next investigated whether TSA affected the levels of some phosphorylated proteins involved

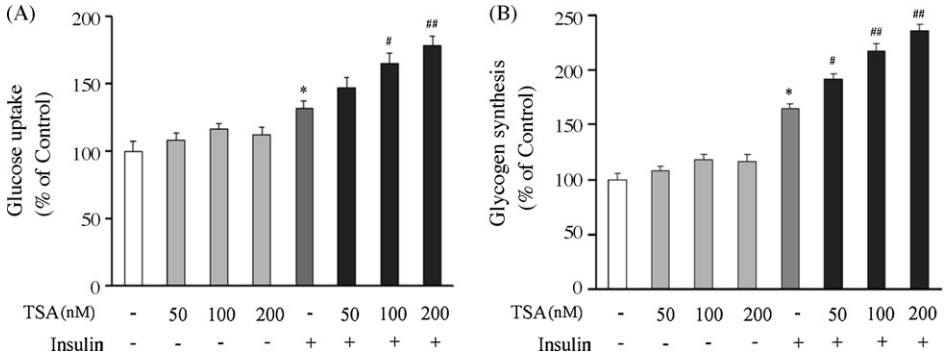

Fig. 2 – TSA increased insulin stimulated glucose uptake (A) and glycogen synthesis (B) in C2C12 myotubes in a dose-dependent manner. Glucose uptake and glycogen synthesis assays were performed as described in Section 2. The applied insulin concentration was 20 nM. $^{*}p < 0.05$ vs. cells without any treatment. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. cells only treated with insulin. Values represent means ± S.D. of two independent determinations, each performed in triplicate.

in insulin signaling pathway by Western blotting. The basal level of phosphorylated insulin receptor (p-Tyr1146-InsR), as well as phosphorylated Akt (p-Ser473-Akt) and GSK3β (p-Ser9-GSK3β), was very low and their levels were not altered by TSA. Upon insulin challenge, however, these phosphorylated protein levels were significantly increased in the TSA-treated cells especially at higher concentrations (Fig. 4A–C).

3.4. *Increased expression of HDAC2 blocked insulin signaling transduction*

TSA is a potent inhibitor of histone deacetylases (HDACs) [7,8], and HDAC2 was previously characterized as an important regulator of insulin signaling transduction by affecting IRS1 acetylation [14]. Thus, we proposed that the improving effects of TSA on glucose utilization and insulin signaling transduction might be dependent upon HDAC2 repression. To ascertain this speculation, we upregulated *HDAC2* expression in C2C12 cells by infection of herpes simple virus bearing *HDAC2* (HSV-HDAC2) and investigated the effects of TSA on insulin signaling pathway in HDAC2-upregulated cells. HDAC2 protein level was significantly increased in HSV-HDAC2 infected cells compared to the control virus (helper) infected cells (Fig. 5A and B). The level of phosphorylated insulin receptor (p-Tyr1146-InsR) in response to insulin stimulation in the TSA-treated cells, as well as the levels of phosphorylated Akt (p-Ser473-Akt) and GSK3β (p-Ser9-GSK3β), was blocked largely in the HSV-HDAC2 infected cells (Fig. 5C–E). These results suggest the improving effects of TSA on insulin signaling transduction was probably dependent upon its inhibitory effects on HDAC2.

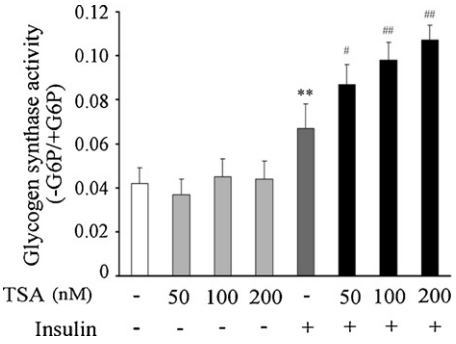

Fig. 3 – TSA activated glycogen synthase activities in C2C12 myotubes under insulin stimulation in a dose-dependent manner. Glycogen synthase activity assays were performed as described in Section 2. $^{**}p < 0.01$ vs. cells without any treatment. The applied insulin concentration was 20 nM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. cells only treated with insulin. G6P, glucose 6-phosphate. Shown are the means ± S.D. of three separate experiments, each performed in duplicate.

3.5. *Effects of HDAC2 knockdown by siRNA on insulin stimulated glycogen synthesis*

To further confirm the effects of TSA on insulin stimulated glucose utilization via HDAC2 repression, we down regulated HDAC2 expression by siRNA in HepG2 cells and investigate the effects of TSA on glycogen synthesis in HDAC2 knockdown cells. As shown in Fig. 6A, HDAC2 protein level was decreased significantly in the HDAC2 siRNA transfected cells. We next assayed insulin stimulated glycogen synthesis in these cells. Similar to TSA treatments mentioned above, HDAC2 knockdown also significantly enhanced insulin stimulated glycogen synthesis. Most importantly, TSA could not further promote insulin stimulated glycogen synthesis in the HDAC2 down-regulated cells (Fig. 6B). These data strongly indicate that the effects of TSA on insulin stimulated glucose utilization were dependent upon HDAC2 repression.

4. Discussion

TSA treatments significantly improved insulin stimulated glucose uptake and glycogen synthesis in C2C12 skeletal muscle cells (Fig. 2A and B). Skeletal muscle accounts for more than 80% of insulin-dependent glucose disposal [19]. The skeletal muscle cells are therefore considered as important experimental tissues for investigating insulin stimulated

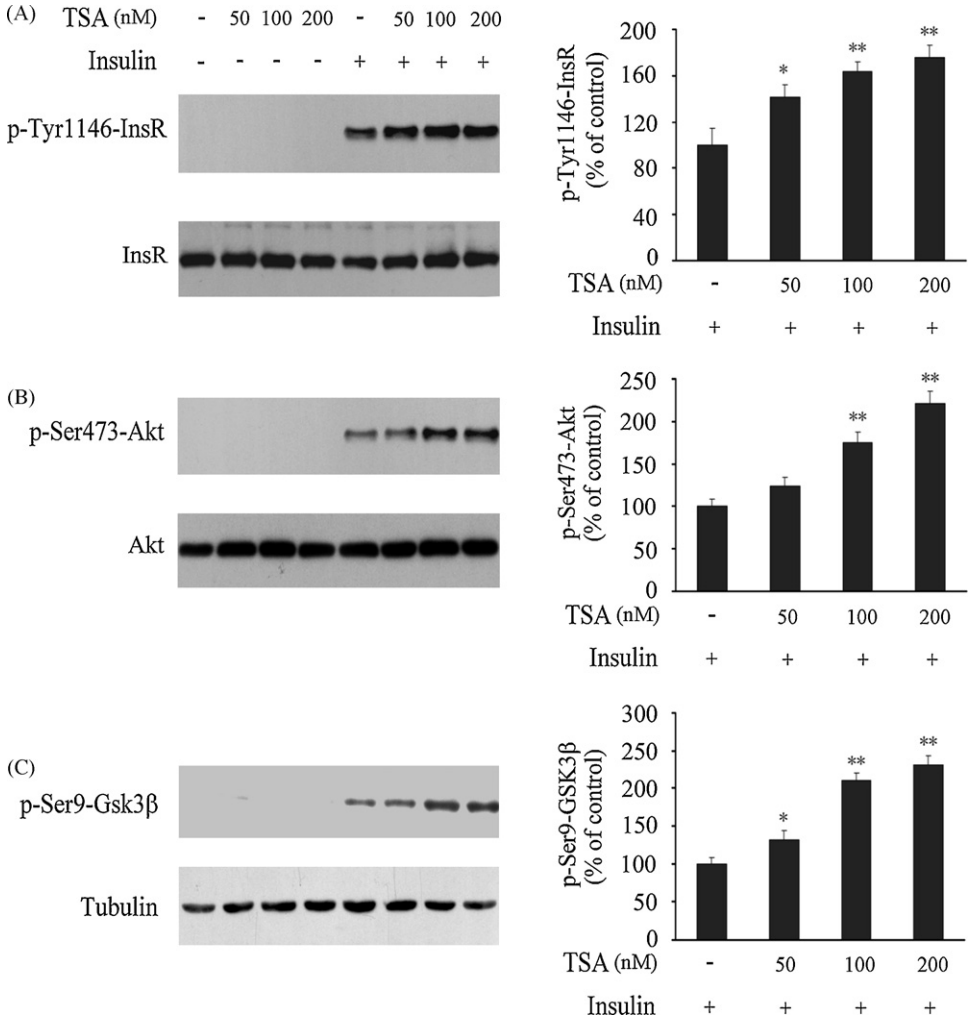

Fig. 4 – Insulin signaling transduction was improved by TSA in C2C12 myotubes. (A) TSA enhanced insulin receptor phosphorylation at tyrosine (p-Tyr1146-InsR) in response to insulin evocation. (B) TSA promoted Akt phosphorylation at serine (p-Ser473-Akt) with insulin stimulation. (C) TSA increased Gsk3β phosphorylation at serine (p-Ser9-Gsk3β) with insulin stimulation. Protein levels were analyzed by Western blotting. Tubulin was used as a loading control. Representative experiments are shown. Data are expressed as means ± S.D. of three different experiments after normalization. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the respective control cells only treated with insulin.

glucose metabolism [20–22]. C2C12 is a widely recognized mouse skeletal muscle cell line for studying glucose metabolism and insulin signaling pathway in response to insulin stimulation [23–26]. With insulin stimulation, glucose was transported into skeletal muscle cells for glycogen synthesis and thus to maintain postprandial glucose homeostasis [3]. In type 2 diabetic patients, the improving functions of insulin on glucose uptake and glycogen synthesis are blunted and eventually result in hyperglycemia [27]. With the improving effects on insulin stimulated glucose utilization, TSA is therefore considered as a drug or lead-compound for enhancing insulin sensitivity to maintain normal blood glucose level. Corresponding to the effects of TSA on glucose uptake and glycogen synthesis, we also found that TSA could significantly activate glycogen synthase activities in insulin-treated C2C12 myotubes (Fig. 3). Glycogen synthase is a rate-limiting enzyme involved in glycogen synthesis processes [28]. In agreement with our findings, it has been demonstrated that insulin sensitizers commonly could activate glycogen synthase activities [29,30].

Insulin initiates glucose uptake by binding to insulin receptor (InsR) and resulting in its autophosphorylation. Activated InsR leads a series of protein phosphorylations including Akt and GSK3β. Finally, glucose transporters translocate to the plasma membrane for accelerating glucose uptake [3]. TSA increased the responses of InsR and Akt to insulin stimulation at tyrosine 1146 and serine 473, respectively (Fig. 4A–C), which should be, at least in part, a mechanism responsible for the improving effects of TSA on insulin stimulated glucose uptake. The excessive glucose transported into skeletal muscle cells was mainly synthesized as glycogen. GSK3β is a key enzyme for regulating glycogen

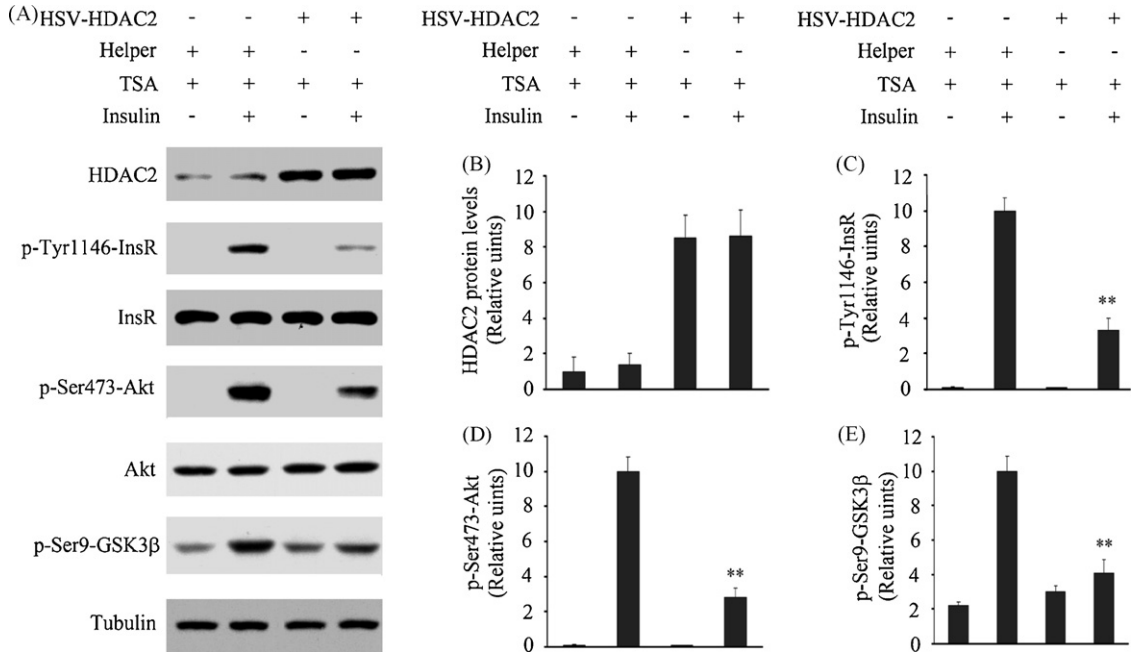

Fig. 5 – Increased expression of HDAC2 antagonized the improving effects of TSA on insulin signaling transduction in C2C12 myotubes. (A) The improved protein levels of p-Tyr1146-InsR, p-Ser473-Akt and p-Ser9-GSK3β by TSA were abolished by increased expression of HDAC2 in C2C12 myotubes. The increased expression of HDAC2 was mediated by HSV (herpes simplex virus) infection. Helper was used as control virus. Both HSV-HDAC2 and helper viruses were kept uniformly at m.o.i. = 1 (m.o.i. means multiplicity of infection). The applied TSA and insulin concentrations were 100 and 20 nM, respectively. Protein levels were analyzed by Western blotting. Tubulin was used as a loading control. Representative experiments are shown. The protein levels of HDAC2 (B), p-Tyr1146-InsR (C), p-Ser473-Akt (D) and p-Ser9-GSK3β (E) were quantified after normalization. Data are expressed as means ± S.D. of three different experiments. $^{**}p < 0.01$ vs. TSA-treated and helper virus infected cells with insulin stimulation.

synthesis by controlling glycogen synthase activity [31]. GSK3β can block glycogen synthesis process by phosphorylating glycogen synthase. However, phosphorylated GSK3β caused by insulin stimulation fails to phosphorylate glycogen synthase. TSA increased GSK3β phosphorylation in response to insulin evocation and thus increased insulin stimulated glycogen synthesis. These results suggest that the improving effects of TSA on glucose uptake and glycogen synthesis were probably due to TSA-induced enhancement in the insulin signaling transduction.

The mechanism responsible for the improved insulin signaling transduction caused by TSA still remains elusive. Recently, it has been demonstrated that HDAC2 can deacetylate insulin receptor substrate 1 (IRS-1) and thus blocks tyrosine phosphorylation of IRS-1 in response to insulin stimulation [14]. Besides, TSA was a well-recognized inhibitor of HDAC2 [7,8,32]. Based upon these evidences, we proposed that the effects of TSA on insulin stimulated glucose utilization probably due to its inhibiting ability on HDAC2. In the HDAC2 upregulated cells, we found that TSA lost its improving effects on insulin signaling transduction (Fig. 5). Moreover, as well as TSA treatment, HDAC2 knockdown also promoted insulin stimulated glycogen synthesis (Fig. 6B). Most importantly, no additional effect of TSA on glycogen synthesis was observed in the HDAC2 downregulated cells. These findings demonstrated that the effects of TSA on insulin stimulated glucose utilization were dependent upon HDAC2 repression. However, the HDAC2 target(s) was still not clear. Our findings present here found that TSA could increase insulin receptor phosphorylation at tyrosine with insulin stimulation. Previous study showed that HDAC2 knockdown enhanced acetylation of IRS-1 and thus improved IRS-1 phosphorylation at tyrosine upon insulin stimulation [14]. Therefore, we speculate that insulin receptor, as well as IRS-1, is another target of HDAC2. The project regarding whether and how HDAC2 deacetylates insulin receptor are going to proceed.

In conclusion, we have shown that TSA treatments significantly increased insulin stimulated glucose uptake, glycogen synthesis and glycogen synthase activity in C2C12 skeletal muscle cells. Moreover, the insulin signaling transduction also was improved by TSA. Enhanced expression of HDAC2 impaired the improving effects of TSA on insulin signaling transduction. Most importantly, HDAC2 knockdown also could enhance insulin stimulated glycogen synthesis, and no additional effect of TSA on glycogen synthesis in the HDAC2 downregulated cells was observed. These data strongly indicated that the effects of TSA on insulin stimulated glucose utilization were dependent upon HDAC2 repression. Our results suggest that HDAC2 should be a novel target for

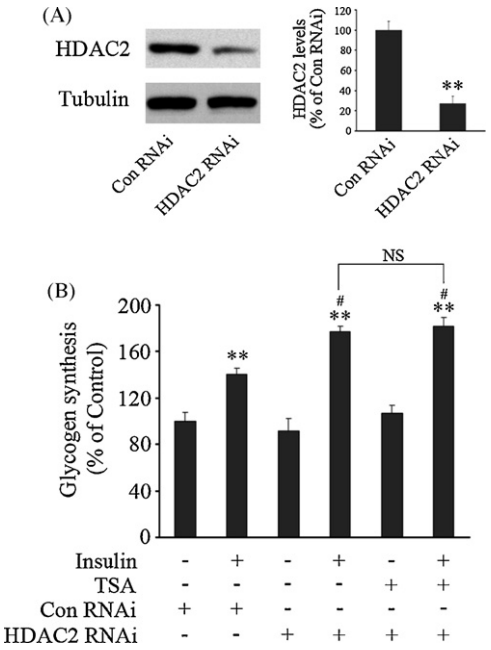

Fig. 6 – Effects of HDAC2 knockdown and TSA on insulin stimulated glycogen synthesis in HepG2 cells. (A) Representative Western blots and quantitative analysis showing downregulation of HDAC2 by siRNA. $^{**}p < 0.01$ vs. Con RNAi. (B) HDAC2 knockdown improved insulin stimulated glycogen synthesis and TSA treatment could not further improve glycogen synthesis in the HDAC2 downregulated cells. $^{**}p < 0.01$ vs. the respective control cells without insulin stimulation. $^{\#}p < 0.05$ vs. the cells with insulin and Con RNAi treatments. NS: Not significant.

regulating insulin sensitivity to combat insulin resistance associated diseases.

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