Biochimica et Biophysica Acta 1842 (2014) 1372–1378

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

SIRT2 negatively regulates insulin resistance in C2C12 skeletal muscle cells

Amita Arora, Chinmoy Sankar Dey *

Kusuma School of Biological Sciences, Indian Institute of Technology-Delhi, Hauz Khas, New Delhi 110016, India

ARTICLE INFO

Article history:
Received 28 February 2014
Received in revised form 25 April 2014
Accepted 28 April 2014
Available online 2 May 2014

Keywords:
Sirtuins
SIRT2
Insulin signaling
Insulin resistance
Skeletal muscle

ABSTRACT

SIRT2 is primarily a cytoplasmic protein deacetylase and is abundantly expressed in metabolically active tissues like adipocytes and brain. However, its role, if any, in regulating insulin signaling in skeletal muscle cells, is not known. We have examined the role of SIRT2 in insulin-mediated glucose disposal in normal and insulin resistant C2C12 skeletal muscle cells *in vitro*. SIRT2 was over expressed in insulin resistant skeletal muscle cells. Pharmacological inhibition of SIRT2 increased insulin-stimulated glucose uptake and improved phosphorylation of Akt and GSK3β in insulin resistant cells. Knockdown of endogenous SIRT2 and over expression of catalytically-inactive SIRT2 mutant under insulin-resistant condition showed similar amelioration of insulin sensitivity. Our results suggest that down-regulation of SIRT2 improved insulin sensitivity in skeletal muscle cells under insulin-resistant condition. Previously it has been reported that down-regulation of SIRT1 and SIRT3 in C2C12 cells results in impairment of insulin signaling and induces insulin resistance. However, we have observed an altogether different role of SIRT2 in skeletal muscle. This implicates a differential regulation of insulin resistance by sirtuins which otherwise share a conserved catalytic domain. The study significantly directs towards future approaches in targeting inhibition of SIRT2 for therapeutic treatment of insulin resistance which is the major risk factor in Type 2 diabetes.

1. Introduction

Mammalian sirtuins (SIRT 1–7) are NAD^+ dependent protein deacetylases, with a well-conserved catalytic domain. The variation in their N- and C-terminus regions results in differential sub-cellular localization and targets. Being dependent on NAD^+, the activity of these proteins is modulated with the changes in the level of NAD^+/NADH. They are expressed in all metabolically active tissues and play a crucial role in regulating various cellular processes. SIRT1 is primarily located in nucleus. It has been most elaborately studied for its crucial role in regulation of insulin resistance [1–3]. Overexpression of SIRT1 in insulin-resistant C2C12 myotubes rescues the cells from impaired insulin signaling [1]. SIRT1, therefore, is a possible therapeutic target for treatment of Type-2 diabetes [4]. SIRT3, a major mitochondrial deacetylase also plays a crucial role in regulation of insulin signaling. Expression of SIRT3 decreases in diabetic mouse model. Knockdown of SIRT3 in C2C12 cell line results in increased ROS levels, activation of stress kinase JNK resulting in impaired insulin signaling [5]. In contrast to this, SIRT6, another member of the sirtuin family, negatively regulates insulin-mediated glucose uptake in skeletal muscle *in vitro* as well as *in vivo* [6]. No information is available about the role of SIRT2 in regulating insulin sensitivity.

SIRT2 is primarily a cytoplasmic protein deacetylase and is abundantly expressed in metabolically active tissues like adipocytes [7] and brain [8]. SIRT2 regulates adipocyte differentiation through deacetylation of FOXO1 [7]. Knockdown of SIRT2 results in increased insulin-mediated phosphorylation of FOXO1, thus restricting it to cytoplasm. Expression of SIRT2 increases during caloric restriction and oxidative stress. SIRT2 then interacts with FOXO3a to promote cell death and overcome oxidative stress [9]. SIRT2 acts as a glucose sensor in liver cells [10]. Pharmacological inhibition of SIRT2 by AGK2, a potent and specific inhibitor of SIRT2, protects neurons from Huntingtin cytotoxity in animal models of Huntington's disease through modulation in sterol metabolism [11].

Given the fact that SIRT2 plays critical role in metabolic activities in liver, neurons and adipocytes and lack of report on the role of SIRT2 in skeletal muscle, which is the major site for glucose disposal, we sought to study the role of SIRT2 in insulin resistance and glucose disposal in skeletal muscle *in vitro*. For this, we used chronic hyperinsulinemia induced insulin-resistant model of skeletal muscle, previously generated and validated in our laboratory [12–15]. This model has been well-studied for understanding the role of kinases and phosphatases in insulin resistance [16,17]. In the present study, we observed a novel role of SIRT2 in regulation of insulin signaling pathway under insulin-resistant condition.

* Corresponding author. Tel.: +91 11 26597523.
E-mail address: csdey@bioschool.iitd.ac.in (C.S. Dey).

http://dx.doi.org/10.1016/j.bbadis.2014.04.027
0925-4439/

2. Material and methods

2.1. Cell culture

C2C12 skeletal muscle cell lines were cultured as described previously [12]. For differentiation, when the cells were 70% confluent, proliferation media was replaced with serum-free media containing equal mixture of MCDB 201 and Ham's F-12 medium with chronic presence of 100 nM insulin (MFI: insulin resistant) or without insulin (MF: insulin sensitive) as reported previously [12]. Cells were maintained in these media for 3 days with media replacement every 12 h. For inhibition of SIRT2, cells were treated with AGK2 during the last 12 h of differentiation.

2.2. siRNA transfection

siRNA oligonucleotides against SIRT2 targeted sequence 5′-TGGCTA AATCAAATTAACCTA-3′ were designed and synthesized by Qiagen GmbH (Germany). C2C12 cells were transiently transfected with SIRT2 specific and non-specific siRNA (400 nmol/l) using RNAifect transfection reagent (Qiagen) in reduced-serum Opti-MEM media (Gibco BRL, USA) according to the manufacturer's instructions, with minor modifications.

2.3. Plasmid DNA transfection

The Flag-tagged SIRT2 Wild type and the Q130A deacetylase-inactive mutant plasmids were a kind gift from Dr. Toren Finkel. C2C12 skeletal muscle cells were transiently transfected with SIRT2-wild type (SIRT2wt) and the mutant plasmid (SIRT2Q130A) using Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to the manufacturer's instructions, with minor modifications.

2.4. Cell lysis for immunoblotting and immunoprecipitaion

Differentiated C2C12 cells were stimulated with or without insulin (100 nM) for 30 min. Cells were then lysed in lysis buffer containing 1% Triton X-100 as described previously [16] and equal amount of lysate proteins were resolved by SDS-PAGE followed by immunoblotting. For immunoprecipitation, 500 μg of protein was immunoprecipitated with the appropriate antibody using Protein A/G-agarose beads (Santa Cruz Biotechnology, USA). Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting as reported previously [12]. Quantification of Western immunoblots were performed using Quantity One 1-D analysis software (Bio-Rad, USA) as described previously [16]. The relative values of the samples were determined by giving an arbitrary value of 1.0 to the respective control samples of each experiment, keeping the background value as 0.

2.5. Glucose uptake

Glucose uptake was assayed using 2-NBDG as described earlier [18] with minor modifications. Briefly, media were removed and cells were washed with a buffer containing NaCl 140 mM, KCl 5 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1 mM, KH_2PO_4 1 mM, and HEPES 10 mM [pH 7.4] followed by stimulation with or without 100 nM insulin for 30 min. Cells were further incubated at 37 °C with 50 μM of 2-NBDG for 1 h, followed by lysis in lysis buffer (sodium deoxycholate 1%, Nonidet P-40 1%, KCl 40 mM, and Tris 20 mM [pH 7.4]) for 10 min. Lysed cells were homogenized, followed by centrifugation at 16,000 × *g* for 15 min at 4 °C. Fluorescence of aliquots from supernatants, containing equal amount of protein was measured using Fluorescence Spectrometer LS55 (Perkin Elmer, USA) at excitation and emission wavelengths of 485 nm and 535 nm, respectively.

2.6. Statistical analysis

The data are expressed as mean ± SE. For comparison of two groups, *p*-values were calculated by two-tailed unpaired Student's *t*-test. In all cases $p < 0.05$ was considered to be statistically significant.

2.7. Reagents

Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS) and trypsin-EDTA,2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose (2-NBDG) was purchased from Life Technologies (USA). AGK2, Nutrient Mixture F-12 Ham, MCDB201 medium, bovine albumin (cell culture grade), anti-SIRT2 antibody were from Sigma Chemical Company (USA). Anti-phospho-Akt (Ser473), Anti-Akt and Anti-phospho-GSK3β (Ser9), Anti-GSK-3β, were from Cell Signaling Technology (USA). Antibodies against IR, IRS-1, α-tubulin, phosphotyrosine, and anti-rabbit and anti-mouse IgG conjugated to alkaline phosphatase were from Santa Cruz Biotechnology (USA). Nitrocellulose membranes and TEMED were procured from Bio-Rad Laboratories (USA). Recombinant insulin was purchased from Calbiochem (USA). All the other reagents unless mentioned specifically were either from Sigma Chemical Company or Amresco (USA).

3. Results

3.1. Effect of insulin resistance on expression of SIRT2 in differentiated C2C12 skeletal muscle cells

To investigate whether SIRT2 has any association with insulin resistance, we first tested the expression of SIRT2 in C2C12 skeletal muscle cells differentiated in insulin sensitive (MF) and insulin resistant (MFI) conditions. The cellular lysates obtained from insulin sensitive (MF) and resistant (MFI) were immunoblotted and probed with anti-SIRT2 antibody. As shown in Fig. 1, SIRT2 expression was ~2.7 folds higher in insulin resistant cells as compared to the sensitive ($p < 0.01$), suggesting increase in SIRT2 protein expression under hyperinsulinemia-induced insulin resistant condition. This finding

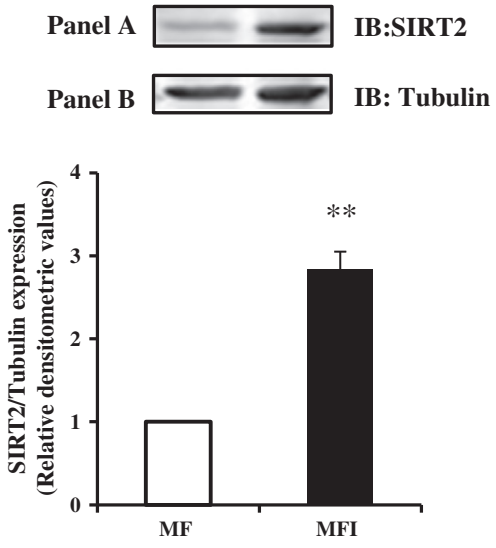


Fig. 1. Effect of skeletal muscle insulin resistance on expression of SIRT2. C2C12 cells were differentiated in serum free medium in absence (insulin sensitive; MF) or chronic presence of 100 nM insulin (insulin resistant; MFI) for 3 days. Cell lysates were subjected to Western immunoblotting and probed with anti-SIRT2 or anti-tubulin antibodies to determine the expression of SIRT2 in MF and MFI condition. Bar represents relative densitometric values of SIRT2 expression after normalizing with tubulin expression (SIRT2/tubulin). Experiments were done thrice and a representative result is shown. Values are mean ± SE. $^{**}p < 0.01$ compared with lane 1. Open bar, MF; filled bar, MFI; IB: immunoblot.

indicates the possible involvement of SIRT2 in insulin-resistant skeletal muscle cells.

3.2. Effect of SIRT2 inhibition on insulin stimulated deoxyglucose uptake

To further investigate the functional contribution of this altered SIRT2 protein expression in skeletal muscle insulin resistance, glucose uptake was measured in SIRT2 inhibited cells under insulin-sensitive and insulin-resistant condition. C2C12 cells differentiated under MF or MFI condition and treated with 50 μM of selective inhibitor of SIRT2, AGK2, a vinyl nitrile [19] that binds the conserved pocket that participates in NAD^+ binding and catalysis [20,21]. Cells were then stimulated with or without insulin and uptake of glucose by cells was measured. Insulin-mediated glucose uptake was significantly impaired in cells differentiated under chronic hyperinsulinemic conditions (MFI) as compared to control MF cells (Fig. 2, lane 6 *vs* lane 2). Inhibition of SIRT2 under MFI condition improved insulin-stimulated glucose uptake by 97.8 ± 0.47% (Fig. 2, lane 6 *vs* lane 8, $p < 0.01$), resulting in comparable glucose uptake as observed under MF condition (Fig. 2, lane 2 *vs* lane 8). A small but significant increase of 47.3 ± 0.32% in glucose uptake was also observed in AGK2 treated MFI samples without insulin stimulation (Fig. 2, lane 5 *vs* lane 7, $p < 0.05$). Surprisingly, there was an increase of 38.1 ± 0.45% in glucose uptake in MF condition without insulin stimulation on AGK2 treatment (Fig. 2, lane 1 *vs* lane 3, $p < 0.05$), however insulin stimulation did not increase glucose uptake further (Fig. 2, lane 2 *vs* lane 4). The result suggests that SIRT2 plays a key role in regulating glucose disposal in skeletal muscle cells and inhibition of SIRT2 sensitizes the cells to insulin under insulin-resistant condition.

3.3. Effect of SIRT2 inhibition on insulin signaling

We next investigated the plausible molecular mechanism for protection of cells from insulin resistance under SIRT2 inhibited condition. For this, we tested the effect of SIRT2 downregulation on insulin signal transduction cascade under normal (MF) and insulin resistant (MFI) condition with or without insulin stimulation. A significant reduction of 76% was observed in insulin-stimulated Akt phosphorylation under MFI condition (Fig. 3A, Panel A, lane 6 *vs* lane 2, $p < 0.001$). In accord with the glucose uptake data, inhibition of SIRT2 alone improved

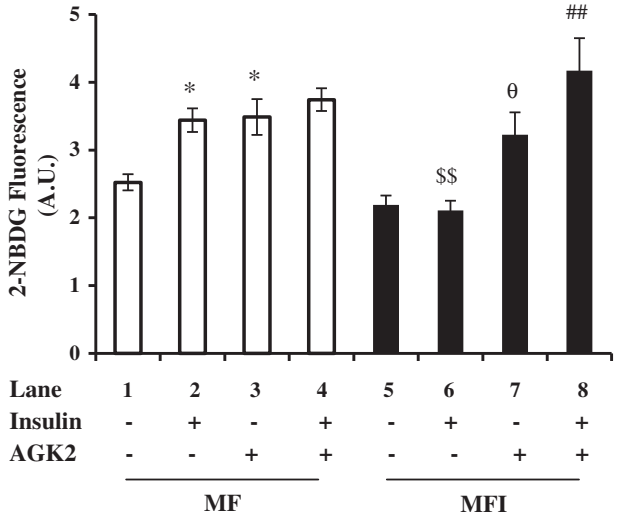


Fig. 2. Effect of inhibition of SIRT2 on glucose uptake. C2C12 cells were treated with or without AGK2 (50 μM) were stimulated with (100 nM) or without insulin for 30 min, followed by addition of 2-NBDG. Uptake of 2-NBDG was measured in 20 μg cell lysates. Bar represents 2-NBDG fluorescence. Experiments were done thrice and a representative result is shown. Values are mean ± SE. $^{*}p < 0.05$ compared with lane 1; $^{\$\$}p < 0.01$ compared with lane 2; $^{\theta}p < 0.05$ compared with lane 5; $^{\#\#}p < 0.01$ compared with lane 6. Open bars, MF; filled bars, MFI; A.U.: arbitrary units.

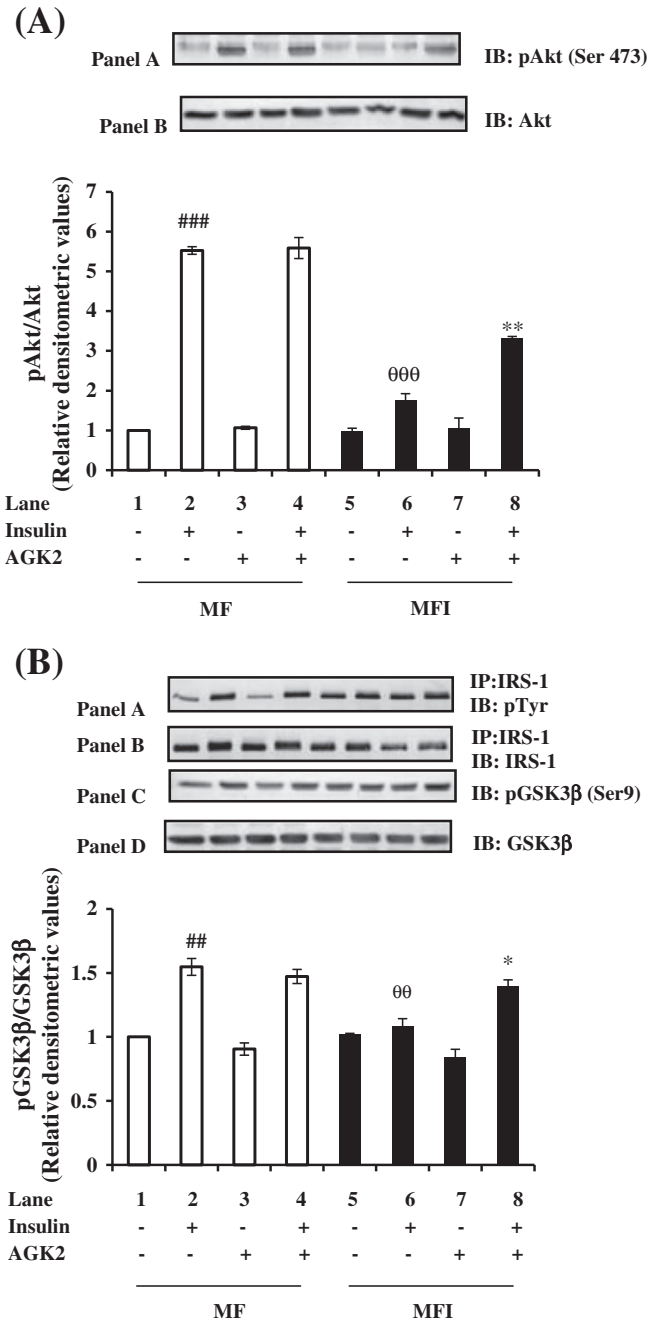


Fig. 3. Effect of inhibition of SIRT2 on insulin signaling. (A) C2C12 were treated with or without AGK2 (50 μM) during the last 12 h of differentiation, followed by stimulation with or without insulin (100 nM) for 30 min. Cell lysates were subjected to Western immunoblotting and probed with anti-phospho-Akt (ser473) or anti-Akt antibodies. Bar represents relative densitometric analysis of phospho-Akt after normalizing with Akt expression (pAkt/Akt). (B) The effect of AGK2 on IRS-1 and GSK3β phosphorylation was examined as in (A). Total cell lysates (500 μg) were immunoprecipitated with anti-IRS-1 (for Panels A and B) antibody, subjected to Western immunoblotting and probed with anti-phosphotyrosine (Panel A), or anti-IRS1 (Panel B) antibodies. Cell lysates were subjected to Western immunoblotting and probed with anti-phospho-GSK3β (Ser9) (Panel C) or anti-GSK3β (Panel D) antibodies. Bar represents relative densitometric analysis of phospho-GSK3β after normalizing with GSK3β expression (pGSK3β/GSK3β). Experiments were done thrice and a representative result is shown. Values are mean ± SE. $^{\#\#\#}p < 0.001$ compared with lane 1; $^{\theta\theta\theta}p < 0.001$ compared with lane 2; $^{**}p < 0.01$ compared with lane 6; $^{\#\#}p < 0.01$ compared with lane 1; $^{\theta\theta}p < 0.01$ compared with lane 2; $^{*}p < 0.05$ compared with lane 6. Open bars, MF; filled bars, MFI. IB: immunoblot; IP: immunoprecipitation.

insulin-stimulated Akt phosphorylation by ~1.4 folds under insulin-resistant condition as compared to the respective untreated control (Fig. 3A, Panel A, lane 6 *vs* lane 8 and bar diagram, $p < 0.01$). Under normal (MF) condition, insulin stimulation increased the phosphorylation of Akt by ~5.5 folds (Fig. 3A, Panel A, lane 1 *vs* lane 2, $p < 0.001$) however, there was no further change in phosphorylation on AGK2 treatment (Fig. 3A, Panel A, lane 2 *vs* lane 4). Expression of Akt remained the same under all conditions tested (Fig. 3A, Panel B). The data suggests that SIRT2 regulates insulin signaling pathway under insulin-resistant condition.

To ascertain the role of SIRT2 in insulin signaling, phosphorylation of IRS-1, the signaling molecule upstream to Akt, and GSK3β, the immediate downstream target of Akt was tested. Insulin-stimulated tyrosine phosphorylation of IRS-1 was impaired under MFI condition as compare to respective MF sample (Fig. 3B, Panel A, lane 6 *vs* lane 2). However, there was no further change in tyrosine phosphorylation of IRS-1 in samples treated with AGK2 under MF or MFI condition (Fig. 3B, Panel A, lane 4 *vs* lane 2 and lane 8 *vs* lane 6).

Further, insulin-mediated GSK3β phosphorylation was significantly impaired under MFI condition (Fig. 3B, Panel C and bar diagram, lane 2 *vs* lane 6, $p < 0.01$). SIRT2 inhibition under such condition significantly restored its phosphorylation (increased by 28.8 ± 0.05%) on insulin-stimulation (Fig. 3B, Panel C and bar diagram, lane 6 *vs* lane 8, $p < 0.05$). There was no change in insulin-mediated GSK3β phosphorylation in insulin sensitive (MF) cells with AGK2 treatment. Expression of GSK3β remained the same under all conditions tested (Fig. 3B, Panel D).

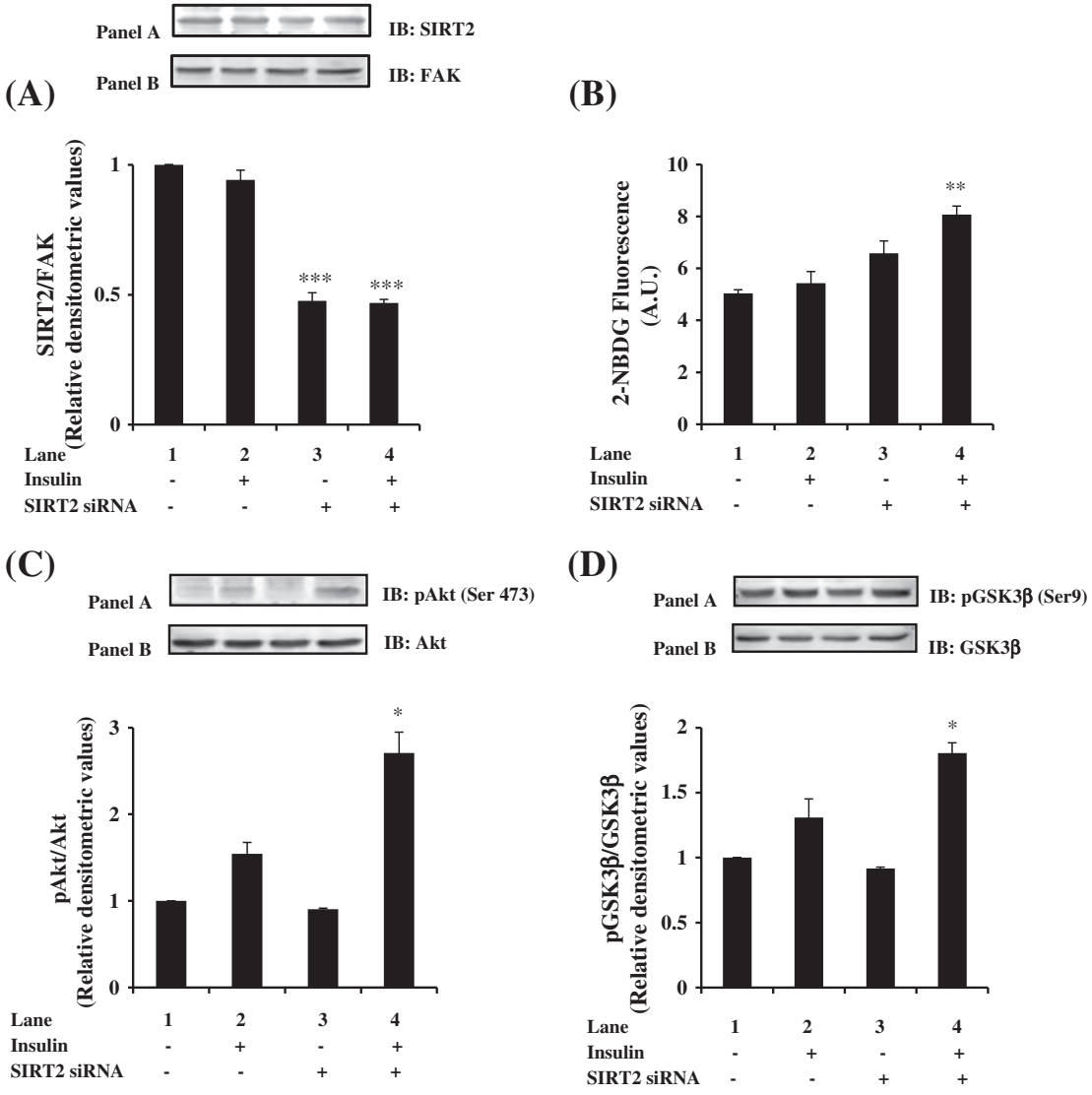
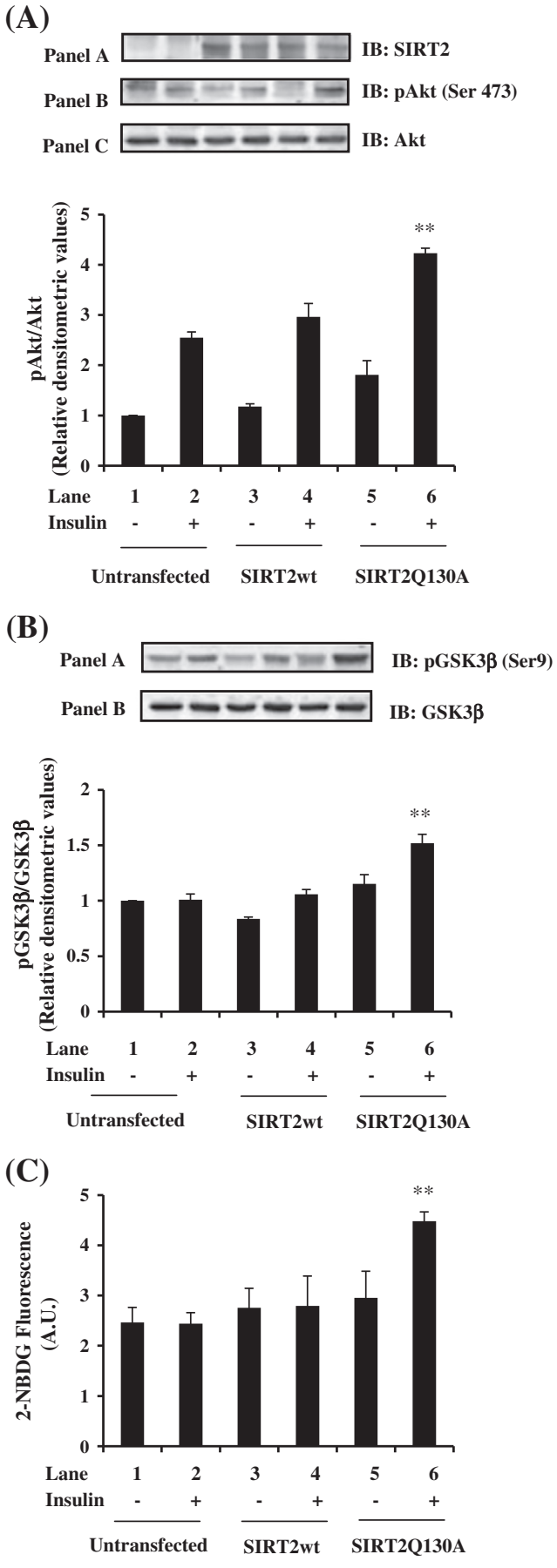


Fig. 4. Effect of modulation of SIRT2 expression on insulin signaling and glucose uptake under insulin-resistant condition. Scramble and SIRT2 siRNA transfected cells were differentiated in serum free medium in chronic presence of 100 nM insulin (MFI) for 3 days and then stimulated with (100 nM) or without insulin for 30 min. (A) Lysates were subjected to western immunoblotting and probed with anti-SIRT2 or anti-FAK antibody (FAK was used as an unrelated protein to determine loading control) to examine the expression of SIRT2 in scramble and SIRT2-siRNA-transfected cells. Bar represents relative densitometric values of SIRT2 expression after normalizing with FAK expression (SIRT2/FAK). (B) Insulin-mediated 2-NBDG uptake was measured in C2C12 cells were transfected with scramble or SIRT2 siRNA, followed by differentiation in serum free medium containing chronic presence of 100 nM insulin (MFI) for 3 days. Uptake of 2-NBDG was measured in 20 μg cell lysates. Bar represents 2-NBDG fluorescence. (C) Cell lysates were subjected to Western immunoblotting and probed with anti-phospho-Akt (ser473) or anti-Akt antibodies. Bar represents relative densitometric analysis of phospho-Akt after normalizing with with Akt expression (pAkt/Akt). (D) Cell lysates were subjected to Western immunoblotting and probed with anti-phospho-GSK3β (Ser9) or anti-GSK3β antibody. Bar represents relative densitometric analysis of phospho-GSK3β after normalizing with GSK3β expression (pGSK3β/GSK3β). All experiments were done thrice and a representative result is shown. Values are mean ± SE. $^{***}p < 0.001$ as compared to lane 1; $^{**}p < 0.01$ as compared to lane 2; $^{*}p < 0.05$ as compared to lane 2. Filled bars, MFI. IB: immunoblot, A.U.: arbitrary units.



The data establishes that the increased glucose uptake due to SIRT2 inhibition is associated with concomitant increase in glycogen synthesis.

3.4. Knockdown of SIRT2 under insulin-resistant condition improves insulin sensitivity

To validate the hypothesis that inhibition of SIRT2 improves insulin sensitivity in skeletal muscle cells, C2C12 cells differentiated under insulin-resistant (MFI) conditions, with or without insulin stimulation were transfected with SIRT2 specific siRNA and silenced SIRT2 expression by 50% (Fig. 4A). We determined the effect of downregulation of endogenous SIRT2 on insulin-stimulated deoxyglucose uptake under the insulin resistant condition. Consistent with the AGK2 mediated inhibition results, silencing of SIRT2 resulted in significant increase (48.4 ± 0.33%) in insulin-stimulated glucose uptake under insulin resistant condition (Fig. 4B, lane 4 *vs.* lane 2, $p < 0.01$).

We further examined whether the depletion of this sirtuin could also ameliorate the decreased insulin-mediated phosphorylation of Akt and GSK3β. Knockdown of SIRT2 significantly improved insulin-stimulated Akt phosphorylation by 75.4 ± 0.24% (Fig. 4C, lane 4 *vs* lane 2 and bar diagram, $p < 0.05$) and GSK3β phosphorylation by 37.9 ± 0.07 (Fig. 4D, lane 4 *vs* lane 2 and bar diagram, $p < 0.05$) under insulin-resistant condition as compared to scramble-transfected controls. Expression of Akt (Fig. 4C, Panel B) and GSK3β (Fig. 4D, Panel B) remained the same under all conditions tested. The data strongly supports that SIRT2 negatively regulates insulin signaling in C2C12 skeletal muscle cells.

3.5. Overexpression of catalytically-inactive SIRT2 mimics the effect of pharmacological inhibition of SIRT2

To further confirm that SIRT2 indeed regulates insulin resistance, C2C12 cells were transfected with plasmid containing wild type (SIRT2wt) and mutated (SIRT2Q130A) SIRT2 gene, followed by differentiation in MFI condition that resulted in overexpression of both SIRT2 (Fig. 5A, Panel A). We examined the effect of overexpression on activity of insulin signaling molecules. Overexpression of catalytically-inactive mutant resulted in amelioration of Akt phosphorylation on insulin stimulation by 65.9 ± 0.9% (Fig. 5A, Panel B and bar diagram, lane 2 *vs* lane 6, $p < 0.01$). Also, GSK3β phosphorylation improved by 50.3 ± 0.08% on insulin stimulation (Fig. 5B, upper panel and bar diagram, lane 2 *vs* lane 6, $p < 0.01$). However, there was no further change in phosphorylation of GSK3β on overexpression of wild type form. There was no change in expression of AKT (Fig. 5A, Panel C) or GSK3β (Fig. 5B, Panel B) under any condition. The result further proves that SIRT2 negatively regulates insulin resistant C2C12 cells.

Further, we examined whether the over expression of catalytically-deficient mutant could also ameliorate the decreased insulin-stimulated glucose uptake under the insulin resistant condition. Consistent with the above results, overexpression of catalytic mutant SIRT2 led to a significant enhancement 83.6 ± 0.18% of insulin-stimulated glucose uptake under MFI condition (Fig. 5C, lane 2 *vs.* lane 6, $p < 0.01$). However,

Fig. 5. C2C12 cells were transfected with SIRT2 wt and Q130A mutant plasmid. Transfected cells were differentiated in serum free medium containing chronic presence of 100 nM insulin (MFI) for 3 days, followed by stimulation with or without insulin for 30 min and cell lysis. (A) Lysate was subjected to Western immunoblotting and probed with anti-SIRT2 antibody (Panel A), anti phospho-Akt (Ser473, Panel B), anti Akt (Panel C). Bar represents relative densitometric values of pAkt after normalizing with Akt expression (pAkt/Akt). (B) Lysate was subjected to Western immunoblotting and probed with anti-pGSK3β antibody (upper panel), anti-GSK3β (lower panel) as in (A). Bar diagram representing relative densitometric values of pGSK3β (Ser-9) after normalizing with GSK3β expression (pGSK3β/GSK3β). (C) Insulin-stimulated 2-NBDG uptake was measured in plasmid DNA transfected cells differentiated in serum free medium containing chronic presence of 100 nM insulin (MFI) for 3 days. Uptake of 2-NBDG was measured in 20 μg cell lysates. Bar represents 2-NBDG fluorescence. All experiments were done thrice and a representative result is shown. Values are mean ± SE. $^{**}p < 0.01$ compared with lane 2. Filled bars, MFI. IB: immunoblot, A.U.: arbitrary units.

there was no further change in glucose disposal in insulin-resistant C2C12 cells on overexpression of wild type SIRT2 (Fig. 5C, lane 2 *vs.* lane 4). Results conclusively prove that SIRT2 negatively regulates insulin resistance in C2C12 skeletal muscle cells.

Overall, these results suggest that SIRT2 plays a key role in regulating glucose disposal and glycogen synthesis under insulin resistant condition in skeletal muscle cells by suppressing phosphorylation of Akt, GSK3β and subsequently glucose uptake. Downregulation of SIRT2 under such conditions sensitizes the cells to insulin.

4. Discussion

In the present study, we have identified the role of SIRT2 in insulin resistance in skeletal muscle cells. Expression of SIRT2 increases significantly when the cells are differentiated under chronic hyperinsulinemic condition. Under such conditions, inhibition of SIRT2 either by AGK2 treatment or siRNA mediated knockdown improved insulin sensitivity with significant amelioration of insulin-stimulated uptake of glucose by the cells. This observation was supported by data obtained from over-expression of catalytically inactive mutant of SIRT2. The molecular mechanism for insulin sensitization on SIRT2 inhibition involved insulin-mediated modulation of Akt and GSK3β activity. Interestingly, we found that modulation of SIRT2 activity under insulin-sensitive condition did not interfere with insulin action as there was no change in insulin-mediated glucose disposal and Akt and GSK3β activity on AGK2 treatment. This clearly indicates that SIRT2 acts as a key component of a signaling network required to maintain the state of insulin resistance and downregulation of SIRT2 improves insulin sensitivity under such condition. The increased level of endogenous SIRT2 under chronic hyperinsulinemic condition was sufficient to maintain insulin resistance and hence exogenous expression of wild-type SIRT2 did not further aggravate this condition.

A recent study by Choi et al. [22] suggests that ERK1/2 interacts with SIRT2 in HEK293 cells and regulates SIRT2 protein level. Also, ERK1/2 signaling is deregulated in insulin-resistant condition in skeletal muscle cells and targeting ERK1/2 is proposed to be of potential therapeutic application for treatment of insulin resistance [23]. Therefore, to understand the increased expression of SIRT2 under insulin resistant condition (Fig. 1A), we immunoprecipitated SIRT2 with anti-ERK1/2 antibody and *vice versa* in MF and MFI conditions. However, under our experimental conditions we did not observe any interaction between SIRT2 with ERK1/2 under normal or hyperinsulinemic condition (data not shown). Therefore the precise reason for an increased expression of SIRT2 under insulin-resistant condition remains to be clarified.

We observed that SIRT2 affects insulin signaling downstream to IRS-1 as there was no change in tyrosine phosphorylation of IRS-1 on inhibition of SIRT2 in C2C12 cells (Fig. 1C, Panel A). IRS-1-independent Akt phosphorylation and subsequently glucose uptake have been previously reported in L6 skeletal muscle cell line [24]. We also observed that inhibition of SIRT2 enhanced glucose uptake in cells even without insulin stimulation (Fig. 1D, lane 3 vs lane 1 and lane 7 vs lane 5; Fig. 2D, lane 3 vs lane 1). A plausible explanation could be an increase in the activity of insulin-independent glucose transporter GLUT1 on inhibition of SIRT2. Earlier, Xiao et al. [6] have shown that knockdown of SIRT6 in C2C12 skeletal muscle cells results in significant increase in expression and membrane translocation of GLUT1, which was perhaps responsible for increased uptake of glucose at the basal level.

SIRT2 directly interacts and deacetylates Akt which subsequently affects glycogen synthase kinase-3β-catenin signaling pathway to regulate motility of hepatocellular carcinoma cells [25]. Besides, SIRT1 directly interacts and deacetylates Akt on stimulation with growth factors [26]. Deacetylation of Akt by SIRT1 thus enhances its binding to PIP_3 and membrane localization, thereby activating Akt. Recently, SIRT2 has been identified as the major interacting partner of AKT in insulin-responsive HEK-293T cells [27]. To understand whether a direct interaction between SIRT2 and Akt is responsible for change in insulin sensitivity under our experimental conditions, we immunoprecipitated SIRT2 with anti-Akt antibody and *vice versa*, however, we did not observe any such interaction between Akt and SIRT2 (data not shown). Similar to Ramakrishnan et al. [27], we also did not observe any change in acetylation of Akt in MF or MFI with or without insulin stimulation (data not shown). One possible reason for this could be tissue-dependent interactions of sirtuins. For example, SIRT1 interacts and deacetylates histone H3 to regulate insulin sensitivity in C2C12 myotubes [1]. However, in adipocytes, SIRT1 targets NF-κB deacetylation to improve insulin sensitivity [2]. In hepatocytes, SIRT1 interacts with nuclear respiratory factor-1 (NRF-1) to regulate insulin sensitivity [28]. In hypothalamic cells, SIRT1 interacts with IRS-1 to regulate insulin sensitivity [29]. Therefore, tissue-specific functionality happens to be a common mode of regulation by sirtuins. Further studies are required to elucidate the precise target of SIRT2 by which it affects Akt phosphorylation and subsequently insulin sensitivity under insulin-resistant condition in skeletal muscle cells.

A recent study by Jiang et al. [10] proposes SIRT2 as a critical sensor of cellular glucose level. In HEK293T cells, SIRT2 deacetylates and stabilizes PEPCK1, an enzyme that catalyzes the rate-limiting step of gluconeogenesis. Since deregulation of gluconeogenesis is marker for initiation of Type 2 diabetes and the critical role of SIRT2 is regulation of this pathway, this study identifies inhibition of SIRT2 as a therapeutic approach against Type 2 diabetes. Another study shows that expression of SIRT2 decreases in adipose tissue of diet-induced obese mice therefore resulting in deregulation of PGC1α mediated mitochondrial energy expenditure in adipocytes, resulting in glucose intolerance and insulin resistance [30]. This identifies SIRT2 as a positive regulator of metabolic activities and augmentation of SIRT2 activity has been proposed as a therapeutic approach for obesity and Type 2 diabetes. Our study adds another perspective to the understanding of the role of SIRT2 in insulin resistance. We propose that under normal condition SIRT2 does not interfere with insulin action, however once the cells are resistant to insulin action, SIRT2 negatively regulates insulin action and inhibition of SIRT2 improves insulin sensitivity in skeletal muscle cells. Future studies might help in understanding the interplay between SIRT2 and other sirtuins like SIRT1 and SIRT3 that positively regulate insulin signaling in skeletal muscle.

In summary, we have unraveled a novel role of SIRT2 as a negative regulator of insulin resistance in skeletal muscle cells. This finding establishes the importance of SIRT2 in pathophysiology of insulin resistance which is of great significance as insulin resistance is a major cause for Type 2 diabetes. Approaches targeting inhibition of SIRT2 may be beneficial for therapeutic treatment of Type 2 diabetes in the near future.

Statement of conflicts of interest

None.

Acknowledgments

We gratefully acknowledge Dr. Toren Finkel of the National Heart, Lung and Blood Institute, NIH Bethesda, Maryland 20892, USA for providing Flag-tagged SIRT2 (wild type) and the Q130A deacetylase-inactive mutant plasmids as a kind gift. Amita Arora is the recipient of a Senior Research Fellowship from the Council of Scientific and Industrial Research, Government of India, New Delhi. This study was supported by a grant from the Department of Science and Technology, Government of India, New Delhi to Chinmoy Sankar Dey (SR/S2/JCB-24/2008(G), dated 30.12.2008).

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