Hyperglycemia induced C2C12 myoblast cell cycle arrest and skeletal muscle atrophy by modulating sirtuins gene expression in rats

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Abstract

Diabetes is characterized by high blood glucose level termed hyperglycemia affecting skeletal muscle structure and function by an unclear molecular mechanism. This study aimed to investigate the effect and underlying mechanism(s) of hyperglycemia on skeletal muscle both in vitro and in vivo. Treatment with hyperglycemic condition (25 mM) for 48 h significantly inhibited C2C12 myoblast proliferation detected by MTT assay whilst flow cytometry revealed an interruption of the cell cycle at subG1 and G2/M phases. An exposure to hyperglycemic condition significantly decreased the myosin heavy chain (MHC) protein expression in the differentiated myotube and tibialis anterior (TA) muscle of Wistar rats. In addition, the muscle cross-section area (MCA) of TA muscle in diabetic rats were significantly decreased compared to the non-diabetic control. Western blotting analysis of C2C12 myoblasts and differentiated myotubes revealed the increased expressions of cleaved-caspase-9 and cleaved-caspase-3, but not cleaved-caspase-8. Of note, these caspases in the TA muscles were not changed under hyperglycemic condition. Quantitative real-time polymerase chain reaction (qRT-PCR) of the hyperglycemic myoblasts and TA muscles revealed modulation of the gene expression of sirtuins (SIRTs). In C2C12 myoblasts, the expressions of SIRT1, SIRT2 and SIRT4 were upregulated whilst SIRT7 was downregulated. Meanwhile, the expressions of SIRT1, SIRT2 in TA muscles were upregulated whilst SIRT4 was downregulated. Taken together, this study showed that hyperglycemia induced cell cycle arrest and apoptosis in myoblasts, and protein degradation and atrophy in skeletal muscle most likely via modulation of SIRTs gene expression.

Key words: diabetic mellitus, hyperglycemia, muscle atrophy, sirtuins, skeletal muscle
Introduction

Diabetes, which is increasing globally, is characterized by an excess level of glucose in bloodstream. The condition is known as hyperglycemia. Persistent hyperglycemia negatively affects a large number of tissues including skeletal muscle (Hirata et al. 2019). A previous study reported that hyperglycemic condition induced apoptosis in myoblasts by increasing the expression of Bax to Bcl-2 ratio (Ahangarpour et al. 2018). Moreover, in vivo study revealed that hyperglycemia stimulated skeletal muscle atrophy by decreasing muscle mass and muscle fiber cross-sectional area in extensor digitorum longus (EDL) muscle (Hirata et al. 2019). This condition also suppresses myogenesis by down-regulating Akt signaling (Luo et al. 2019). However, the mechanisms responsible for the effect of hyperglycemia on skeletal muscle and myoblasts are presently poorly understood.

Sirtuins are a conserved protein nicotinamide adenine dinucleotide (NAD)-dependent deacetylase consisted of 7 isoforms (SIRT1-7), which modulate distinct metabolic and stress response pathways. SIRT1 has been shown to promote myoblast cell proliferation and cell cycle progression (Rathbone et al. 2009), and plays critical role in metabolic function in liver, muscle, and adipose tissue (Chang and Guarente 2014). SIRT2 is a cytoplasmic protein that negatively regulates insulin resistance in C2C12 myoblasts (Arora and Dey 2014). SIRT3-5 are localized in mitochondria and function in oxidative stress modulation and metabolic enzyme activity (Verdin et al. 2010). Among these, SIRT6 exerts an important role in maintaining muscle mass (Samant et al. 2017) whilst SIRT7 induces gene expression involving age-related changes (Wronska et al. 2016). In response to cellular stress by external stimuli, up- and/or down-regulation of SIRTs mRNA have been reported in many cell types including endothelial cells (Wang et al. 2017) and skeletal muscle cells (Zhang et al. 2015) leading to different end points.

Therefore, we hypothesize that hyperglycemia would induce apoptosis in myoblasts and muscle fibers leading to an impairment of myogenesis and muscle atrophy. In this study, we provide insights into a possible mechanism of action of hyperglycemia on skeletal muscle via the modulation of sirtuins’ gene expression. This knowledge may contribute to the toxicological study of hyperglycemia on skeletal muscle.

Materials and Methods

Reagents

Otherwise indicated, cell culture reagents were from Gibco (Life Technologies) and other basic chemicals were purchased from Sigma-Aldrich. The anti-caspase 3, anti-caspase 8, and anti-caspase 9 antibodies were received from cell signaling technology. The anti-MHC and anti-tubulin antibodies (Millipore), ECL prime western blotting detection reagent (GE Healthcare) and Luna universal RT-qPCR (New England BioLabs) were used in this study.

Animals and treatments

The animal ethical committee specifically reviewed and approved this study (MOE 0521.11/490). The 8-week-old male Wistar rats purchased from the Southern Laboratory Animal Facility and were housed in a 12-h light/dark cycle at 25±2°C, with food (rat chow) and water ad libitum. The hyperglycemic rats were induced by a single dose intraperitoneal injection of streptozotocin (60 mg/kg) dissolved in citrate buffer. Control rats were similarly injected with the vehicle. Rats with blood sugar level higher than 250 mg/dL were classified as hyperglycemic group. These rats were kept in the housing for another 8 weeks, then were sacrificed and dissected for the TA muscles.

Cell culture

C2C12 myoblast cell line (ATCC) were maintained in growth medium (GM) composed of DMEM supplemented with 10% fetal bovine serum (FBS) in a humidified CO₂ incubator at 37°C. To generate differentiated myotubes, the 80% confluence myoblasts were shifted to differentiation medium (DM) composed of DMEM supplemented with 2% horse serum for 5 days.

To find the optimum concentration for hyperglycemic condition (HG), the C2C12 myoblasts were cultured in GM containing different concentrations of 2-deoxy ribose (0, 10, 15, 20, 25, 30 mM) for 48 h before subjected to MTT assay.

For hyperglycemic treatment, the sub-confluence myoblasts and 5 days differentiated myotubes were shifted to GM containing 2-deoxy ribose (25 mM) for 48 h before subjected to other assays.

MTT assay

The treated cells were incubated with 0.5 mg/mL MTT solution for 3 h in the CO₂ incubator. After incubation, the MTT solution were replaced with 100 µL solubilizer with constant agitation. The absorbance was measured at 570-630 nm using a microplate reader.

Flow cytometry

The treated myoblast cells were trypsinized and fixed in ice-cold 70% alcohol overnight. The fixed cells were washed with phosphate buffer saline (PBS)
and incubated in propidium iodide (PI) staining solution (10 µg/mL PI, 100 µg/mL RNase in PBS) for 30 min in the dark. The cell cycle stages were determined using BD FACSCanto™ flow cytometer and analyzed with BD FACSDiva version 6.1.1 software.

**Immunofluorescence staining**

The treated cells were fixed in ice-cold methanol for 10 min and washed and then rehydrated in PBS. The cells were subsequently blocked and incubated with anti-MHC overnight at 4°C. After several washed with PBS, the cells were incubated with Alexa488-conjugated secondary antibody for 45 min at RT. The signal was visualized under a fluorescence microscope (Olympus IX73).

**Histological study**

The tibialis anterior (TA) muscles from the left leg were dissected and immediately fixed in 10% formalin overnight. Tissues were prepared by standard paraffin technique and were sectioned at 5 µm. The sections were stained with hematoxylin and eosin. The muscle fiber cross-sectional area (MCA) was measured with ImageJ software.

**Western blotting**

The treated cells and skeletal muscle were subjected to protein extraction and concentration determination. An equal amount of protein was separated on SDS-PAGE and subsequently transferred onto PVDF membrane. The membrane was incubated with the desired antibodies; anti-MHC, anti-caspase 3, anti-caspase 8, anti-caspase 9, and anti-tubulin overnight at 4°C. After several washes, the membrane was probed with an appropriate HRP-conjugated secondary antibody. The signal was visualized using ECL prime western blotting detection reagents under the gel documentation system.

**Real-time polymerase chain reaction (real-time PCR)**

The RNA was extracted in TRizol reagent and was converted to cDNA using cDNA synthesis kit. Real-time PCR was performed using Luna universal RT-qPCR with BioRad CFX96 Touch Real Time PCR machine. Relative gene expressions were normalized to GAPDH and expressed by 2^{\text{-ΔΔCT}} method. The primer sequences for each gene were as follows: SIRT1: Fw5'-TACAGGCCCAATGTCACTCA-3' and Rw5'-CTTCGACAGCCGTCATGTA-3', SIRT4: Fw5'-GTCGTTCCTGTTTGCAGGAC-3' and Rw5'-AGAATGCCTATGGGAGGTT-3', SIRT5: Fw5'-AGCAAGATCTGCTACCATC-3' and Rw5'-GCCTGCCATTTTCACTTGTA-3', SIRT6: Fw5'-AGGCCTCTGCTGATGTCAC-3' and Rw5'-GCACACCTACCTCAACAGGATGTC-3', SIRT7: Fw5'-AGCCATCCACTCCACCACATG-3' and Rw5'-GGTGAGGCCCATCACAGGTTC-3', and GAPDH: Fw5'-TGCGACTTCAACAGCAACTC-3′ and Rw5′-GCCTCTTGGTCAGTGGTC-3′ (Ban et al. 2013, Quinn et al. 2017).

**Statistical analysis**

Data are presented as means ± SEM from at least three independent experiments. The data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. Also, the Student’s T-test was used where appropriate in this experiment. Statistical analyses were carried out with GraphPad Prism version 5.00. Statistical significance is displayed as follows: * p<0.05, ** p<0.01, *** p<0.001.

**Results**

**Hyperglycemia induced C2C12 myoblast cell cycle arrest at subG1 and G2/M phases**

After treatment with different concentrations of 2-deoxy ribose (0-30 mM) for 48 h, the morphology of C2C12 myoblasts gradually changed in a concentration-dependent manner. Treatment with 10 mM, however, showed no effect on cell morphology. The cells were round or star shape as in the non-treatment control. The cells elongated at the concentrations of 15 and 20 mM. After treatment with 25 and 30 mM solutions, further elongation and the appearance of cytoplasmic extension (Fig. 1A, arrow heads) were observed. Moreover, the cell density also gradually decreased when the level of hyperglycemia increased (Fig. 1A). MTT results revealed significant decreases in the absorbance after treatment with concentrations higher than 15 mM, compared to the control (Fig. 1B). The results suggest that hyperglycemia caused cytotoxicity and/or inhibition of cell proliferation. Thus, significant accumulation of cells in the subG1 and G2/M phases was noted, whereas the cell population in G0/G1 and S phases significantly decreased compared to control (Fig. 1C and 1D).
Hyperglycemia induced skeletal muscle atrophy

In *in vivo* study, the MCA of TA muscle of diabetic rats compared to control rats was examined. As shown in Fig. 2A and 2B, the MCA of TA muscle of diabetic rats was significantly reduced compared to that in the control rats. The expression of MHC, the major contractile protein of skeletal muscle, in the diabetic group was also significantly reduced compared to the control group (Fig. 2C). In parallel with the *in vivo* experiment, treatment of the differentiated myotubes in *in vitro* with hyperglycemic condition (25 mM) significantly decreased the size and the expression of MHC protein of differentiated myotubes (Fig. 2D). Immunofluorescence staining for MHC protein showed clumping and scattering (arrowhead) arrangement in the myotubes of the

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**Fig. 1.** Hyperglycemia inhibited C2C12 myoblast proliferation by inducing cell cycle arrest. C2C12 myoblasts were treated with different concentrations of 2-deoxy ribose (0, 10, 15, 20, 25, 30 mM) in growth medium for 48 h. C2C12 myoblast cell morphology and density after treatment were observed (A). Cell viability was measured with MTT assay (B) and cell cycle stages were elucidated with flow cytometry (C, D). * p<0.05, *** p<0.001 compared with the control. Scale bars = 200 μm.
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hyperglycemic group compared to the continuous band throughout the myotubes in the control group. Also, the fluorescence intensity of MHC protein in hyperglycemic myotubes was significantly reduced compared to control (Fig. 2E). Consistent with the fluorescence data, the Western blot data of MHC protein in the myotube cultured under hyperglycemic condition were significantly reduced compared to that in the normal growth medium (Fig. 2F).

Hyperglycemia modulated the expression of apoptotic proteins

Since both in vivo and in vitro experiments showed that hyperglycemia caused atrophy of TA muscle, along with disruption and suppression of MHC of myotubes, the cellular and molecular bases for these effects were further studied. In order to determine whether muscle atrophy is associated with apoptosis, the expressions of cleaved-caspase proteins were assayed by Western blot (Fig. 3A). As illustrated in Fig. 3B, under the hyperglycemic condition (25 mM) for 48 h, the levels of cleaved-caspase 3 were significantly increased in both C2C12 myoblasts and differentiated myotubes, but not in TA muscle. Also, the expression of cleaved-caspase 9 was significantly enhanced only in C2C12 myoblasts and differentiated myotubes. However, the levels of cleaved-caspase 8 expression were not altered in any of the experimental groups.

Hyperglycemia modulated the expression of sirtuin genes

In order to determine the involvement of sirtuins in the responses of C2C12 myoblasts and TA muscle in hyperglycemia, we investigated the sirtuin mRNA expression using real-time PCR, since mRNA expressions are the early response to a wide variety of cellular stimuli. The results showed that the mRNA expression levels of sirtuins genes (SIRTs) were altered under hyperglycemic conditions. In myoblasts treatment under hyperglycemic condition (25 mM) for 48 h, the expressions of SIRT1, SIRT2 and SIRT4 were significantly increased, while that of SIRT7 was significantly...
decreased (Fig. 4A). In the skeletal muscle, on the other hand, the expressions of SIRT1 and SIRT2 were significantly enhanced but SIRT4 was significantly suppressed (Fig. 4B). However, hyperglycemia showed no effect on the expressions of SIRT3, SIRT5 and SIRT6 in both myoblasts and skeletal muscle.
Discussion

In the present study, we reported the adverse effects of hyperglycemia on skeletal muscle in both in vitro and in vivo experiments. Our findings revealed morphological changes of myoblasts after culture in hyperglycemic condition. These changes are in concordance with toxicological studies reporting that exposure to toxic chemicals stimulated changes in cell shape and morphology, development of elongated cytoplasmic extensions/gripping spicules, and cell detachment (Buranasin et al. 2018, Surinlert et al. 2020). These phenomena could be explained by alterations or damage to the cell membrane structure leading to changes in the capacity of cells to adhere to the basement membrane. The decrease in density of myoblasts after treatment is consistent with previous reports that hyperglycemia significantly impaired cell proliferation in human gingi-

Fig. 4. Hyperglycemia modulated the expressions of sirtuins genes in myoblasts and TA muscle. The expressions of sirtuins (SIRT1-7) genes in the control and hyperglycemic C2C12 myoblasts (A) and tibialis anterior muscle (B) were analyzed by real-time PCR. * p<0.05 compared with the control.
val fibroblasts (Buranasin et al. 2018), renal proximal tubule cells (Park et al. 2001) and human osteoblast-like cells MG63 (Linxi et al. 2015). The underlying mechanism was proposed to be involved with the induction of cellular oxidative stress and secretion of transforming growth factor-β1 (TGF-β1) via the protein kinase C (PKC)-oxidative stress pathway (Buranasin et al. 2018, Park et al. 2001). Even though hyperglycemia has been reported to stimulate MG63 osteoblasts’ cell cycle arrest in the G1 phase (Linxi et al. 2015), but in our experiments, hyperglycemia stimulated C2C12 myoblasts’ accumulation in G2/M and sub G1 phases. It has been previously reported that hyperglycemia facilitates cell cycle arrest via TGF-β1 and ERK1/2 signaling cascade pathways (Luo et al. 2012). Whether this pathway is also involved in the myoblast cell arrest requires further studies.

The adverse effect of hyperglycemia was shown to be mainly due to induction of oxidative stress by reactive oxygen species (ROS), which impair cellular functions and finally lead to cellular apoptosis (Felice et al. 2010). The decreases in the size and MHC protein expression in both TA muscle and differentiated myotubes are consistent with the previous report that hyperglycemia accelerated the protein degradation in soleus, EDL and plantaris muscles (Wang et al. 2006). Likewise, hyperglycemia stimulated gastrocnemius muscle atrophy in rats characterized by decreases in MCA (Reddy et al. 2019). Possible mechanisms of hyperglycemia-induced muscle atrophy and apoptosis have been reported to occur through the stimulation of ER stress, an increase in ER-associated protein degradation (ERAD), an increase in apoptosis mediator proteins and the activation of the ubiquitin-proteasome pathway (Wang et al. 2006, Reddy et al. 2019). These findings are in agreement with our results that C2C12 myoblasts and differentiated myotubes cultured in hyperglycemic condition expressed elevated levels of cleaved-caspase 3 and 9, the key mediator proteins of apoptosis. On the other hand, hyperglycemia did not stimulate the expression of apoptotic markers in TA muscles in this study, even though the MCA was significantly decreased, suggesting that atrophy of skeletal muscle fiber in our experiment did not occur via apoptosis induction. It is possible that the accelerated activation of muscle protein degradation occurred via the ubiquitin-proteasome pathway (Wang et al. 2006). The ubiquitin-proteasome system has been reported to play an important role in controlling muscle protein degradation (Galban et al. 2001). Further studies are required to investigate the mechanisms of hyperglycemia-induced skeletal muscle atrophy and apoptosis.

In the present study, the mRNA expression of sirtuins in both C2C12 myoblasts and TA were changed under hyperglycemic conditions. In C2C12 myoblasts, the up-regulation of SIRT1 and SIRT2 mRNA and down-regulation of SIRT7 mRNA occurred corresponding with the up-regulation of apoptotic marker proteins, cleaved-caspase 9 and 3. The activation of SIRT1 has been shown to induce the expression of cleaved-caspase 3 protein by deacetylating p65 subunit of NF-κB complex in human chondrosarcoma cells, resulting in cellular apoptosis (Chao et al. 2017). SIRT2 has been reported to enhance apoptosis in the model of Parkinson’s disease by deacetylating Foxo3a and increase RNA and protein levels of Bim (Liu et al. 2014). However, the results are in contrast with other studies which showed that hyperglycemia down-regulates the expression of SIRT1 protein in human endothelial cells (Orimo et al. 2009, Wang et al. 2017) and SIRT2 protein in dorsal root ganglion neurons (Scharton et al. 2018) leading to impairment of mitochondrial function and cellular apoptosis. A possible reason for this discrepancy may be the different cell types being used. It has been reported that the function of sirtuins may be different, depending on cell types (Chen et al. 2014). Moreover, the down-regulation of SIRT7 mRNA in myoblasts in this study would favor the myoblast apoptosis under hyperglycemic condition. It has previously been reported that lack of SIRT7 suppressed the cellular capacity to resist the oxidative stress and increased the rate of cellular apoptosis up to 200% in primary cardiomyocytes via the activation of p53 protein (Vakhruševa et al. 2008). On the other hand, upregulation of SIRT4 in myoblasts may be associated with the defense mechanism against cellular stress and apoptosis caused by hyperglycemia, since overexpression of SIRT4 has been reported to inhibit hyperglycemia-induced podocyte cell death by inhibiting the activation of p38, increasing the expression of anti-apoptotic protein Bcl-2, decreasing the expression of pro-apoptotic protein Bax, and suppressing pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) (Shi et al. 2017).

In TA muscle, even though the expressions of SIRT1 and SIRT2 mRNA were upregulated as in myoblasts, the expression of apoptotic marker proteins was not significantly altered. This discrepancy may be due to differences in the response of cells between in vitro and in vivo experimental conditions. It should be pointed out that although the in vitro system is a powerful tool to mimic the animal model, it does not fully reproduce the biocomplexity of the living animal. In vivo experiment involved with adaptation processes which do not establish in the cell cultures (Doktorova et al. 2012, Porreca et al. 2016). Thus, the hyperglycemia-induced skeletal muscle atrophy in living organisms did not occur via apoptosis. Instead, it was involved with the enhancement of muscle protein.
degradation and inhibition of protein synthesis (Wang et al. 2006, Sharples et al. 2015). In support to this notion, SIRT1 has been reported to be implicated with muscle protein degradation via the modulation of transcription factor forkhead box protein O (FOXO) (Sharples et al. 2015), which causes skeletal muscle atrophy by inducing ubiquitin ligase atrogin-1 expression (Sandri et al. 2004). In addition, SIRT1 activation has been previously reported to negatively regulate the mammalian target of rapamycin (mTOR) activity which subsequently suppressed the phosphorylation of S6 kinase and 4E-binding protein 1 (4E-BP1) proteins finally leading to the inhibition of muscle protein synthesis (Liu et al. 2010, Sharples et al. 2015). Similarly, the activation of SIRT2 has been reported to deacetylate FOXO protein in response to oxidative stress leading to an increase in the DNA binding and elevations of the expression of FOXO target genes (Wang et al. 2007). Unlike myoblasts, the expression of SIRT4 in TA muscle was down-regulated whilst that of SIRT7 was not changed. This discrepancy cannot be explained at present. It may be due to the different state of development of muscle or other factors. On the other hand, the expressions of SIRT3, SIRT5 and SIRT6 mRNA in both myoblasts and TA muscle were not changed in our study which suggest that these genes play no role in muscle atrophy in hyperglycemia condition.

Conclusion
In conclusion, this study provides the novel information for the possible mechanism of hyperglycemia on skeletal muscle. Our study shows that hyperglycemia modulates the expression of sirtuin genes that are associated with cell cycle arrest and apoptosis in myoblasts or protein degradation and muscle atrophy. This information may be useful for further development of food supplements and/or drugs to prevent muscle atrophy in diabetes patients.

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References
Luo M, Liu Z, Hao H, Lu T, Chen M, Lei M, Verfaillie CM,


