Puerarin ameliorates skeletal muscle atrophy in STZ-induced type 1 diabetic rats by enhancing Akt/mTOR while inhibiting autophagy signaling pathway

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May 5, 2020

Abstract

Background and Purpose: Puerarin is an important isoflavone component extracted from Pueraria lobate in traditional Chinese medicine. It has a wide range of pharmacological effects. Increasing evidence indicates that puerarin alleviates hyperglycemia and numerous related complications. In this study, we explored the effect of puerarin on skeletal muscle atrophy caused by type 1 diabetes in rats. Experimental Approach: Male Sprague Dawley (SD) rats with streptozotocin (STZ)-induced type 1 diabetes were used in this study. We measured skeletal muscle weight, size and strength together with the transformation of skeletal muscle types in type 1 diabetic rats. Skeletal muscle L6 cells were used for in vitro study. Key Results: Puerarin increased muscle tissue weights and improved muscle strength. An enhanced skeletal muscle cross-sectional area was accompanied by reduced mRNA expression of muscle atrophy marker genes, including F-box only protein 32 (Atrogin-1) and muscle-specific RING-finger 1(Murf-1), both in vitro and in vivo. The transformation from type I fibers (slow muscle) to type II fibers (fast muscle) was also observed after puerarin administration. In vitro studies suggested that puerarin upregulated Akt/mTOR but downregulated the LC3/p62 signaling pathway, eventually resulting in muscle hypertrophy. Conclusions and Implications: Our study observed that puerarin mitigated skeletal muscle atrophy in type 1 diabetic rats. Subsequently, we found that the related mechanisms closely involved the upregulation of protein synthesis via the Akt/mTOR signaling pathway. Whether this anti-diabetic muscle atrophy effect in mice applies to humans remains unknown.

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Short Running Title: Puerarin alleviates diabetic muscle atrophy

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Word count: 2956 words

Acknowledgements

This work was financially supported by the following foundations: the CAMS Initiative for Innovative Medicine (CAMS-I2M, 2016-I2M-3-007 and 2017-I2M-1-010), National Major Research Development Program of China (2018ZX09711001-012, 2018ZX09711001-003-005, and 2017YFG0112900) and the National Natural Science Foundation of China (81470159 and 81770847).

Conflict of interest

The authors declare that there are no conflicts of interest.

Author contributions:

L.Y., G.F.Q., X.Y.Y. and G.H.D. participated experimental design; L.Y. X.C. N.L. W.H.J. N.Q.W. B.Y.H. and X.Y.Y. performed the experiments; L.Y., X.C., N.L. and X.Y.Y analyzed the data; L.Y., X.Y.Y. and G.H.D. wrote the manuscript; and L.Y., L.Z., G.F.Q., X.Y.Y. and G.H.D. edited the manuscript.

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Conclusions and Implications: Our study observed that puerarin mitigated skeletal muscle atrophy in type 1 diabetic rats. Subsequently, we found that the related mechanisms closely involved the upregulation of protein synthesis via the Akt/mTOR signaling pathway. Whether this anti-diabetic muscle atrophy effect in mice applies to humans remains unknown.

Keywords

Puerarin; type 1 diabetes; skeletal muscle atrophy; rats; protein synthesis; muscle type transformation

Abbreviations

Atrogin-1, F-box only protein 32; AUC, area under the curve; COX, cytochrome oxidase; DM, differentiation medium; FoxO3a, forkhead box class O factor 3a; HG, high glucose; I, insulin; IPGTT, intraperitoneal glucose tolerance test; LA, lactic acid; M, mannitol; Mstn, myostatin; Murf-1, muscle-specific RING-finger 1; Myh1, myosin heavy chain IIx/d; Myh2, myosin heavy chain IIa; Myh4, myosin heavy chain IIb; Myh7, myosin heavy chain I; MyHC, myosin heavy chain; PA, pyruvic acid; SDH, succinate dehydrogenase; STZ, streptozotocin; T1D, type 1 diabetes; TA, tibialis anterior;

Introduction

Diabetes is one of the most challenging public health problems and affects approximately 425 million people worldwide, leading to poor health outcomes and high health care costs (Arneth, et al.,2019). Type 1 diabetes (T1D) is a major subtype of diabetes and is an autoimmune disease that is characterized by the destruction of islet β cells in the pancreas triggered by genetic and environmental factors (Zheng, et al.,2018).

T1D patients have many complications involving heart, kidney, and other tissues. Type 1 diabetic subjects display a dramatic loss of muscle, causing severe skeletal muscle atrophy as the disease progresses (Sala and Zorzano,2015). Skeletal muscle is a major target tissue of diabetic damage (Krause, et al.,2011). Skeletal muscle atrophy is a pathological condition defined as a reduction in muscle mass caused by excessive protein degradation (Hong, et al.,2019). Skeletal muscle atrophy disrupts quality of life and increases mortality and morbidity (Powers, et al.,2016).

An increasing number of studies indicate that the balance between protein synthesis and degradation is responsible for muscle atrophy (Hoffman and Nader,2004). Pathways involved in this process include the mammalian target of rapamycin (mTOR) signaling pathway (Laplante and Sabatini,2012), which is a crucial component of the anabolic machinery for protein synthesis, and the ubiquitin-proteasome pathway (Milan, et al.,2015), which is responsible for the turnover of the majority of soluble and myofibrillar muscle proteins, such as myosin heavy chain (MyHC), via transcriptional activation of a set of E3 ligase-encoding genes, e.g., muscle RING-finger 1 (Murf1) and F-box only protein 32 (Atrogin-1) (Chen, et al.,2019). In addition, autophagy also exerts a vital role in the degradation of skeletal muscle as a consequence of an ordered transcriptional program involving a battery of genes, e.g., LC3 and p62 (Llano-Diez, et al.,2019). Although our understanding of the molecular mechanisms involved in skeletal muscle atrophy has substantially progressed in recent decades, no effective drug has come into the market to date. Thus, there is an urgent unmet need for the development of novel drugs to combat skeletal muscle atrophy.

In traditional Chinese medicine, *Pueraria lobata* is a medical and edible plant that is widely distributed in eastern and southern Asia (Chen, et al.,2018). Puerarin is one of its major bioactive components. Puerarin is an isoflavone compound with a polyphenol structure. Puerarin has documented therapeutic effects on diabetes and diabetic complications (Hsu, et al.,2003; Wu, et al.,2013; Chen, et al.,2018; Yang, et al.,2019; Yin, et al.,2019). Puerarin exerts a hypoglycemic effect (Tanaka, et al.,2016), improves insulin resistance (Chen, et al.,2018), and protects islet cells (Rojas, et al.,2018) in diabetes patients. In addition, puerarin also has beneficial effects on diabetic complications, especially diabetic cardiovascular complications (Pan, et al.,2009; Li, et al.,2016), diabetic nephropathy (Li, et al.,2017), and diabetic retinopathy (Ren, et al.,2000; Zhu, et al.,2014).

In this study, we hypothesis that puerarin may exert protective effects on skeletal muscle atrophy provoked by type 1 diabetes. We aim to contribute to the research and development of drugs for diabetic skeletal muscle atrophy.

Methods

2.1 Materials

Puerarin (HPLC, 98%) was provided as a lyophilized powder by the Institute of Materia Medica, Chinese Academy of Medical Sciences (Beijing, China). Streptozotocin (STZ) was purchased from Sigma (St. Louis, MO, USA). Blood glucose testing was performed using an Accu-Chek Active meter from Roche (Basel, Switzerland). Lactic acid (LA) and pyruvic acid (PA) content kits as well as succinate dehydrogenase (SDH) and cytochrome oxidase (COX) staining kits were purchased from Solarbio (Beijing, China). SuperScript III reverse transcriptase and TRIzol isolation reagent were obtained from Invitrogen (Carlsbad, CA, USA). Direct-zol RNA kits were obtained from ZYMO research (Irvine, CA, USA). SsoFast EvaGreen® Supermix was obtained from Bio-Rad (Hercules, CA, USA). RIPA buffer, 5× loading buffer, and enhanced chemiluminescence kits were purchased from Applygen (Beijing, China). Protease inhibitor cocktail and phosphatase inhibitor cocktail were obtained from CWbio (Jiangsu, China). Protein concentrations were quantified using a BCA assay kit purchased from Thermo Fisher Scientific (Rockford, IL, USA). Antibodies used in this study are listed in Table 1.

2.2 Animal care

Male Sprague-Dawley (SD) rats (160-190 g) were obtained from Beijing HuaFuKang Bioscience Co., Ltd. (Beijing, China). The rats were housed under a 12-hour light/dark cycle at a temperature of 22 ± 3°C and a humidity of 55 ± 5%. Rats were given free access to food and water for seven days before the experiment. All animal procedures were approved by the animal care and use committee of the Institute of Materia Medica, Chinese Academy of Medical Sciences and were performed in strict accordance with research guidelines for the care and use of laboratory animals.

2.2 Type 1 diabetic animal modeling and drug treatments

Briefly, 30 rats were fed adaptively for 1 week, and then 6 rats were randomly divided into the normal control group (control). The remaining rats were injected intraperitoneally with 65 mg kg^{-1}streptozotocin (STZ). Fasting blood glucose was tested using blood obtained from the tail tip after 1 week of STZ injection. In addition, 20 rats with fasting blood glucose levels greater than 16.5 mmol L^{-1} were chosen as type 1 diabetic rats and randomly divided into two groups: T1D control group (model, N=10) and puerarin administration group (puerarin, N=10). The administration group was treated with orally with 100 mg $kg^{-1}day^{-1}$ puerarin, while the normal control group and the diabetic model group were treated with a corresponding dose of normal saline.

2.3 Animal muscle strength detection

The grip strength test was employed to determine foreleg tensile force using a grip strength meter provided by Beijing Zhishuduobao Biological Technology (Beijing, China) per the manufacturer's instruction. In brief, rats were positioned to grasp the metal bar with forelegs only and then pulled horizontally until letting go. Pulls were repeated thrice with three rounds in sequence in rats. For repetitive pulls, rats were given a 30-second pause in between the pulls; between rounds, rats were given a 30-min rest period. The maximum grip strength of the 9 pulls was used for analysis.

The inclined plane test was employed using a modified Rivlin method (Rivlin and Tator,1977). The rats were positioned on a 2-mm thick, 14-cm wide and 24-cm long plate with a rubber mat loaded on a rectangular wooden board. The rat was placed on the mat with its head up, and its body was parallel to the longitudinal axis of the inclined plane. The angle between the sloping plate and the ground was 90 degrees. The maximum persistent duration on the sloping plate was recorded, and the test was repeated thrice.

The wire-hanging test was performed with a 3 mm-diameter wire. The wire was suspended at a height of 0.5 m. Rats hung from the center of the wire by exclusively using forelegs to grab the wire. The time until

the rat fell from the wire was recorded. The test was performed thrice in succession, and the average value was calculated. Values for voluntarily jumps were not included in the calculation.

2.4 Histological analysis

Skeletal muscle tissues were fixed in 10% formalin. Transverse paraffin sections of 5 μm in thickness were subjected to hematoxylin-eosin (HE) staining. For succinate dehydrogenase (SDH) and cytochrome oxidase (COX) staining, gastrocnemius muscle samples were obtained and flash frozen in liquid nitrogen. Then, 10-μm thick frozen sections were subject to staining according to the instructions of SDH and COX staining kits (Solarbio, Beijing, China).

2.5 Quantitative real-time PCR

Total RNA was isolated using TRIzol isolation reagent (Invitrogen, USA) and then further purified with Direct-zol RNA kits (ZYMO Research, USA). First-strand cDNA was synthesized using 1.5 μg of total RNA with a reverse transcription reaction mix that included SuperScript III reverse transcriptase (Invitrogen, USA) and Oligo-dT17 as primers. Gene expression was detected using SsoFast EvaGreen® Supermix (Bio-Rad, USA) on a CFX-96 real-time PCR System (Bio-Rad, USA) with gene-specific primer pairs (Table S1). The results were quantified after normalization with TBP (Radonić, et al.,2004).

2.6 Cell culture and differentiation

L6 rat myoblasts were purchased from ATCC (Manassas, Virginia, USA) and cultured in DMEM with 10% FBS serum, 100 I.U. mL^{-1} penicillin and 100 μg mL^{-1}streptomycin (Invitrogen, USA). The differentiation of myoblasts into myotubes was achieved by incubating confluent myoblasts with differentiating media (2% FBS serum, 100 I.U. mL^{-1}penicillin and 100 μg mL^{-1} streptomycin) for six days.

2.7 Western Blot analysis

Protein samples from gastrocnemius muscle and cells were lysed in ice-cold RIPA buffer (Applygen, Beijing, China) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (CWbio, Jiangsu, China). Lysates were clarified by centrifugation (12,000 × g for 15 min at 4°C), and the concentration was quantified using a BCA assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Samples were mixed with 5 × loading buffer (Applygen, Beijing, China), and proteins were then denatured at 100°C for 10 min. For immunoblotting, equal amounts of protein were fractionated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. Then, membranes were blocked with 5% bovine serum albumin (BSA) for 2 h at room temperature. Membranes were incubated with primary antibodies (Table S2) followed by a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, MA, USA). Immunoreactive bands were detected by enhanced chemiluminescence (Applygen, Beijing, China). Semi-quantitative grayscale intensity was measured using Gel-pro 3.1 (Media Cybernetics, Silver Spring, MD, USA).

2.8 Immunofluorescence analysis

To determine the diameter of myotubes in vitro, L6 myotubes were fixed with 4% paraformaldehyde for 30 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 15 min, and then blocked with 5% BSA in PBST for 1 h at room temperature. Myotubes were incubated with an anti-MyHC antibody (MF-20, 1:100, DSHB) diluted in 5% BSA overnight at 4°C. After washing in wash buffer, myotubes were incubated with Goat Anti-Mouse IgG H&L (Alexa Fluor® 488, ab150113, 1:1000, Abcam) for 1 h at room temperature. Nuclei were stained with DAPI (1:1000, Sigma). Images were captured by a high-content screening machine (Cellomics, Thermo Fisher Scientific, USA), and the diameter of the myotubes was measured by ImageJ software (NIH, USA).

2.9 Data and statistical analysis

The exact group size (n) for each experimental group/condition is provided, and "n" refers to independent values not replicates. Results are expressed as the means ± SEM. Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, Inc. CA, USA). For statistical comparisons, unpaired two-tailed Student's t test or one-way ANOVA or Chi-square (or Fisher's exact) test was used as appropriate. Differences were considered significant at P[?]0.05.

1.

Results

Effect of puerarin on general indicators of type 1 diabetic rats induced by STZ

Body weights of rats were assessed every week during the 8-week treatment (Figure 1a). Compared with those in the normal control group, body weights in the diabetic control group significantly decreased. However, 100 mg kg^{-1}puerarin treatment had no pronounced effect on body weights of T1D rats with the exception of values recorded in the 3^{rd} week. Puerarin also has no significant effect on food intake (Figure 1b). Fasting blood glucose levels did not change significantly under 100 mg kg^{-1} puerarin treatment (Figure 1c). Glucose tolerance was assessed using the intraperitoneal glucose tolerance test (IPGTT) at week 8 of treatment. The results showed that 100 mg kg^{-1} puerarin decreased blood glucose levels after intraperitoneal injection of glucose at 60 min (Figure 1d). However, there was no notable decrease in the area under the curve (AUC) of IPGTT in the puerarin administration group compared with the T1D control group (Figure 1e). T1D modeling increased both blood lactic acid and pyruvic acid levels in rats, while 100 mg kg^{-1} puerarin treatment did not show a significant effect (Figure 1f, 1g).

3.2 Puerarin improved skeletal muscle weight, strength and fiber area in type 1 diabetic rats

Skeletal muscle atrophy is characterized by reductions in muscle mass and fiber size; thus, we explored the effect of puerarin on skeletal muscle function and structure. Skeletal muscle strength is a main indicator for muscle function. After one month, foreleg tensile force was reduced in type 1 diabetic rats (Figure. 2a). Conversely, no change in time on the inclined plane and hanging wire was observed in the model group, while puerarin treatment extended time both on the inclined plane and hanging wire (Figure 2b, 2c). After two months of drug administration, our results showed an impressive decrease in skeletal muscle strength in type 1 diabetic rats, including foreleg tensile force and time on the inclined plane and hanging wire. Simultaneously, this change was reversed by puerarin treatment as revealed by a marked improvement in foreleg tensile force (Figure 2a) and time on the inclined plane (Figure 2b) and hanging wire (Figure 2c).

Our data showed that the muscle weights, including the soleus muscle that predominantly contains slow-twitch fibers, the fast-twitch muscle tibialis anterior (TA) and mixed-type gastrocnemius muscle, were remarkably decreased in T1D rats compared with normal controls. After 8 weeks of puerarin administration, weights of the soleus, TA and gastrocnemius muscles significantly increased (Figure 2d, 2f, 2h). In addition, muscle tissue indexes were also increased after puerarin treatment compared with those of diabetic controls (Figure 2e, 2g, 2i).

Then, hematoxylin-eosin (HE) staining was also performed to clarify the effect of puerarin on muscle fiber size (Figure 3a). In the present study, we found that the cross-sectional areas of the soleus, TA and gastrocnemius muscles all significantly shrank; however, after two months of puerarin treatment, the cross-sectional areas of the three muscle types strikingly increased (Figure 3b-d). Consistent with the muscle fiber size results, the frequency distributions of muscle fiber areas for different muscle fibers yielded similar results (Figure 3e-g).

3.3 Puerarin downregulated muscle atrophic markers in T1D rats

The occurrence and development of muscle atrophy were accompanied by significant changes in muscle atrophic factors, such as F-box only protein 32 (Atrogin-1) and muscle-specific RING-finger 1 (Murf-1) (Cohen, et al.,2015; Lee, et al.,2018). We measured the expression of Atrogin-1, Murf-1 and myosin heavy chain (MyHC) mRNA, which represents the amount of muscle fibers. Atrogin-1 and Murf-1 were prominently upregulated, and MyHC was downregulated in T1D rats. On the other hand, puerarin administration distinctly reversed this trend as evidenced by the downregulation of Atrogin-1 and Murf-1 and the upregulation of MyHC (Figure 4a-c). In parallel with changes in mRNA levels, changes in levels of Atrogin-1, Murf-1 and MyHC protein also yielded the same results (Figure 4d-g). Moreover, mRNA expression of myostatin (Mstn), which specifically acts as a negative regulator of skeletal muscle growth, was also decreased in the gastrocnemius (Figure 4c).

3.4 Puerarin promoted the transformation from slow-twitch muscle to fast-twitch muscle in T1D rats

We investigated the effect of puerarin on the change in skeletal muscle fiber types and the preferred metabolic types. Our study found that T1D rats exhibited a tendency to transform muscle from a glycolytic type (fast or type II fibers) to oxidative type (slow or type I fibers). After 8 weeks of puerarin treatment, this trend was attenuated (Figure 5a) as demonstrated by a dramatic increase in integrate optical density and the ratio of type I fibers to type II fibers in the model group. However, these parameters decreased in the group administered puerarin (Figure 5b-e). More specifically, puerarin increased the muscle fiber area of fast-twitch glycolytic type IIB fibers but reduced the area of slow oxidative type I fibers (Figure 5f, 5g). In addition, the expression of Myh7, Myh2, Myh4 and Myh1, which encode myosin isoforms MyHC-I, MyHC-IIa, MyHC-IIb and MyHC-IIx/d, respectively, were also analyzed, and the results confirmed that the production both of fast-twitch and slow-twitch fibers was increased in the puerarin-treated group compared with the T1D model group (Figure 5h).

3.5 Puerarin ameliorated muscle atrophy induced by high glucose in L6 myotubes

We also explored the effect of puerarin in L6 myotubes under high glucose stimulation *in vitro* . We used a dose-response (D-glucose, 0, 50, 100 mM) and time-response (D-glucose, 0, 6, 12, 24, 48, 72 h) model to investigate the effect of glucose concentration on the expression of muscle atrophic markers, including Atrogin-1 and Murf-1, in myotubes. As shown in Figure 6a, b, 100 mM glucose resulted in a time-dependent increase the expression of muscle atrophic markers (Atrogin-1 and Murf-1), and significant differences were noted at 24-72 h (Figure 6c, 6d). We then used 100 mM glucose and a 48-h incubation for subsequent experiments. Using an equal concentration of mannitol as a hyperosmotic control and 100 nM insulin as a protective control, we treated L6 myotubes with 10, 100, 1000 μM puerarin with or without 100 mM glucose for 48 h. Here, cells incubated in 100 mM glucose for 48 h also demonstrated upregulated myostatin (Mstn) expression and downregulated myosin heavy chain (MyHC) expression. Puerarin did not affect the expression of muscle atrophic markers in normal L6 myotubes but obviously downregulated the expression of Atrogin-1, Murf-1 and Mstn (Figure 6e-g). Puerarin enhanced MyHC expression (Figure 6h) in a dose-dependent manner.

Consistent with the results of mRNA expression, Western Blot analysis showed that puerarin markedly inhibited Atrogin-1 and Murf-1 and improved MyHC expression (Figure 7a-d). To further determine the effect of puerarin on the phenotype of L6 myotubes, MyHC immunofluorescence staining was performed (Figure 7e). The results showed that 100 mM glucose reduced the myotube area. When treated with puerarin, the area of myotubes under 100 mM glucose stimulation increased significantly (Figure 7f). In addition, the analysis of frequency distribution of myotube area yielded similar results (Figure 7g).

3.6 Puerarin attenuated autophagy and upregulated Akt/mTOR signaling in L6 myotubes

Muscle atrophy is characterized by an imbalance in protein synthesis and degradation. To explore the mechanism of puerarin, we assessed the autophagy pathway, which plays a vital role in protein degradation (Llano-Diez, et al.,2019), and the Akt/mTOR pathway, which is closely associated with protein synthesis (Laplante and Sabatini,2012; Ogasawara, et al.,2016; Yoon,2017). Our results revealed that 100 mM glucose promoted the autophagy signaling pathway as determined by upregulated phosphorylation of ULK1, p62 expression and the ratio change of LC3II to LC3I. Administration of puerarin remarkably restrained these changes, reflecting an inhibitive effect on the autophagy pathway (Figure 7a-d). In addition, phosphorylation of Akt, mTOR, and its downstream targets, the 70 kDa ribosomal S6 kinase (p70S6K) and eIF4E-binding protein 1 (4EBP1), was downregulated by 100 mM glucose in L6 myotubes. Phosphorylation of Forkhead box class O factor 3a (FoxO3a), a main transcription factor regulating Atrogin-1 expression (Sandri, et al.,2004), was also significantly decreased under 100 mM glucose incubation in L6 myotubes. Notably, phosphorylation of Akt, mTOR, p70S6K, 4EBP1 and FoxO3a was increased after puerarin treatment (Figure 7e-j).

Discussion

In this paper, we demonstrated that puerarin (100 mg kg^{-1}) administration could significantly improve skeletal muscle atrophy in type 1 diabetic rats. This robust effect in puerarin-treated rats was manifested by increased skeletal strength and weights (soleus, TA and gastrocnemius), enlarged muscle fiber size, downregulated muscle atrophic markers (Atrogin-1 and Murf-1) and increased MyHC levels. Puerarin also promoted the transformation from slow-twitch muscle to fast-twitch muscle. Subsequently, we found that the anti-diabetic muscle atrophic effect of puerarin was closely related to attenuation of autophagy and upregulation of Akt/mTOR signaling.

In this study, we first demonstrated that puerarin administration could significantly ameliorate diabetic skeletal muscle atrophy. This effect is consistent with a previous report that the extraction of the plant from which puerarin is derived, namely, Radix *Pueraria lobata* (RP), prevented the skeletal muscle atrophy induced by a high-fat diet (HFD) in mice (Jung, et al.,2017). Additionally, one study reported that puerarin reduced triceps surae atrophy in mice with sciatic nerve injury (Wu, et al.,2014). Our study did not find that puerarin (100 mg kg^{-1}) obviously changed the animal's body weight compared with the diabetic model group. Considering that puerarin can promote the oxidation of fatty acids (Kim, et al.,2016; Cheung, et al.,2017; Oh, et al.,2019) and prevent the accumulation of intramyocellular lipids in diabetic rats (Chen, et al.,2018), we hypothesize that puerarin increases the weights of skeletal muscle while reducing fat content, resulting in almost no observed change in body weight.

Muscle fiber types can be roughly divided into fast and slow muscles, which represent glycolytic metabolism and oxidative metabolism, respectively (Schiaffino,2018). Muscle fiber types can transform from slow-twitch (oxidative type) to fast-twitch (glycolytic type) or vice versa (Zhang, et al.,2019), which represents a change in the type of muscle metabolism. There are few reports about changes in skeletal muscle fiber types caused by type 1 diabetes. In this paper, we reveal a transformation in muscle fiber types in type 1 diabetic rats from fast to slow type. Two months of diabetic conditions in rat were necessary and sufficient to cause muscle atrophy.

Studies have found that puerarin regulates activities of the mitochondrial respiratory chain complex in diabetic nephropathy (Liang, et al.,2019). In addition, puerarin could exert a role of mitochondrial protection (Chen, et al.,2018; Wang, et al.,2018, 2018). Additionally, puerarin has been reported to increase mitochondrial biogenesis in C2C12 cells (Jung, et al.,2017). This evidence indicated that puerarin could regulate energy metabolism in many different cells and tissues. Consistent with these reports, in our study, we stained for succinate dehydrogenase (SDH) and cytochrome oxidase (COX), which are aerobic metabolic enzymes that reflect aerobic metabolism levels in tissues (Özkök, et al.,2015). Our results suggest that puerarin promotes muscle metabolism in a glycolytic manner under diabetic conditions as evidenced by promoting muscle fibers to transform from slow-twitch muscle to fast-twitch muscle.

Previous studies reported that several factors are involved in skeletal muscle mass regulation, including Akt/mTOR signaling (Laplante and Sabatini,2012; Ogasawara, et al.,2016; Yoon,2017) and autophagy signaling (Cid-Díaz, et al.,2017; Jiao and Demontis,2017; Pratiwi, et al.,2018). In addition, the forkhead box (FOX) protein family has been implicated as a key regulator of muscle loss under various conditions, such as diabetes and sepsis (Kang, et al.,2017). Other reports showed that puerarin could upregulate phosphorylation of Akt and mTOR and inhibit autophagy, exerting an antifibrotic effect in atrial fibroblasts (Xu, et al.,2019). However, it was also reported that puerarin induced apoptosis in HPV-positive HeLa cervical cancer cells by inhibiting PI3K/Akt/mTOR signaling (Jia, et al.,2019). Moreover, puerarin suppresses autophagy to protect the rat brain against ischemia/reperfusion injury (Wang, et al.,2018), prevents the progression of experimental hypoxia-induced pulmonary hypertension (Zhang, et al.,2019), and provides a neuroprotective effect against transient cerebral ischemia at the ischemic penumbra in neurons (Hongyun, et al.,2017). In the present study, we clarified that puerarin alleviated muscle atrophy by increasing protein synthesis via the Akt/mTOR signaling pathway while inhibiting the autophagy signaling pathway. In addition, the phosphorylation of transcriptional factor FoxO3a was downregulated by puerarin, which is consistent with a previous report that FoxO3a played a regulatory role in autophagy and the ubiquitin-proteasome system during muscle atrophy (Sandri, et al.,2004).

Considering all the therapeutic effects we identified in our study, it is theoretically possible that puerarin may serve as a potential therapeutic agent targeting diabetic skeletal muscle atrophy. In addition, whether puerarin can be utilized in other types of atrophy remains unknown. Study on key regulatory factors and the target of puerarin should be performed in the future.

Taken together, our study reports for the first time that puerarin can ameliorate skeletal muscle atrophy in T1D animals. This finding not only provides a therapeutic indication for puerarin but also facilitates drug development for diabetic muscle atrophy.

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Tables

Table S1. Primers used for real-time PCR

Table S2. Antibodies used in the Western Blot assay

Figure legends

Figure 1. Effect of puerarin on general indicators of STZ-induced type 1 diabetic rats. Rats were treated with 100 mg kg^{-1} day^{-1}puerarin intragastrically for two months. (a) Change in body weight was detected every week. (b) Food intake at the 8^{th} week. (c) Fasting blood glucose at the 8^{th} week. (d) Blood-glucose curve of IPGTT. (e) AUC of IPGTT. (f) LA content in serum. (g) PA content in serum. a, d, e, Data are represented as the means ± SEM (control, n = 6; model, n = 10; puerarin, n = 8). Statistical analysis tested by one-way ANOVA with Dunnett's multiple comparisons test analysis. b, f, g, Data are represented as the means ± SEM (n = 5). Statistical analysis tested by one-way ANOVA with Dunnett's multiple comparisons test analysis. c, Data are represented as the means ± SEM (control, n = 6; model, n = 10; puerarin, n = 10). Statistical analysis tested by one-way ANOVA with Dunnett's multiple comparisons test analysis. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. the control group. * $p < 0.05$ vs. the model group.

Figure 2. Puerarin increased skeletal muscle strength and weight in type 1 diabetic rats. a-c, Effect of 100 mg kg^{-1} day^{-1} puerarin on muscle strength. (a) Foreleg tensile force. (b) Time on inclined plane. (c) Time on hanging wire. d-i, Muscle weights and muscle tissue indexes (muscle weight/body weight). (d) Soleus weight. (e) Soleus tissue index. (f) TA weight. (g) TA tissue index. (h) Gastrocnemius weight. (i) Gastrocnemius tissue index. Data presented are individual values with means ± SEM from n=5 rats for each group. Statistical analysis tested by one-way ANOVA with Dunnett's multiple comparisons test analysis. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. the control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

and vs. the model group.

Figure 3. Puerarin improved skeletal muscle fiber area in type 1 diabetic rats. (a) Representative images of H&E staining of soleus, TA and gastrocnemius muscle (400 x magnification). (b) Cross-sectional area of soleus muscle. (c) Cross-sectional area of TA muscle. (d) Cross-sectional area of gastrocnemius muscle. e-g, Frequency distribution of cross-sectional area of three types of muscle fibers in the (e) soleus, (f) TA, and (g) gastrocnemius muscles. b-d, Values shown are the means ± SEM from 3 fields, n=5. Statistical analysis tested by one-way ANOVA with Dunnett's multiple comparisons test. e-g, Statistical analysis tested by Chi-square (and Fisher's exact) test. ### $p < 0.001$ vs. the control group. *** $p < 0.001$ and vs. the model group.

Figure 4. Puerarin downregulated the expression of muscle atrophic markers in type 1 diabetic rats. a-c, Total mRNA extracted from the soleus, TA and gastrocnemius muscles was used for real-time PCR analysis. (a) Atrogin-1, Murf-1 and MyHC expression in the soleus muscle. (b) Atrogin-1, Murf-1 and MyHC expression in the TA muscle. (c) Atrogin-1, Murf-1, MyHC and Mstn expression in the gastrocnemius muscle. d-g, Total protein extracted from the gastrocnemius muscle was used for Western Blot analysis. (d-g) Immunoblot analysis of Atrogin-1, Murf-1 and MyHC of the gastrocnemius muscle. Data are represented as the means ± SEM (n=5). Statistical analysis tested by one-way ANOVA with Dunnett's multiple comparisons test analysis. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. the control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and vs. the model group.

Figure 5. Effect of puerarin on muscle fiber phenotype in type 1 diabetic rats. Frozen sections of gastrocnemius muscle samples were subject to SDH and COX staining. (a) Representative images of SDH (above) and COX (below) staining in the gastrocnemius muscle. (b) Integrated optical density analysis of SDH staining. (c) Muscle fiber number ratio (type I/type II) in SDH staining. (d) Integrate optical density analysis of COX staining. (e) Muscle fiber number ratio (type I/type II) in COX staining. (f) Analysis of muscle fiber area of type I, type IIA and type IIB in SDH staining. (g) Analysis of muscle fiber area of type I, type IIA and type IIB in COX staining. (h) Myh1, Myh2, Myh4 and Myh7 expression in the gastrocnemius muscle. b-e, Values shown are means ± SEM from n=5. Statistical analysis tested by one-way ANOVA with Dunnett's multiple comparisons test analysis. f, g, Values shown are means ± SEM from 4 fields (n=6). h, Values shown are means ± SEM from n=5. Statistical analysis tested by one-way ANOVA with Dunnett's multiple comparisons test analysis. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. the control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and vs. the model group.

Figure 6. Puerarin decreased muscle atrophic markers in L6 myotubes. a-d, Fully differentiated L6 rat myotubes were treated with D-glucose. (a, b) Dose-response effect of high glucose (0, 50, 100 mM) for 48 h on the expression of muscle atrophic markers (a) Atrogin-1 and (b) Murf-1 gene. (c, d) Time-response effect of 100 mM glucose (0 h, 6 h, 12 h, 24 h, 48 h, 72 h) on (c) Atrogin-1 and (d) Murf-1 gene expression. e-h, 10, 100, 1000 μM puerarin or 100 nM insulin or 100 mM mannitol were used to treat fully differentiated L6 rat myotubes with or without 100 mM glucose for 48 h, and total mRNA was extracted for real-time PCR analysis. (e) Expression of Atrogin-1. (f) Expression of Murf-1. (g) Expression of Mstn. (h) Expression of MyHC. Data are represented as the means ± SEM from n=3 experiments. Statistical analysis tested by one-way ANOVA with Dunnett's multiple comparisons test analysis. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. DM group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and vs. HG group.

Figure 7. Puerarin ameliorated muscle atrophy induced by high glucose in L6 myotubes. a-d, Fully differentiated L6 myotubes were treated with 10, 100, 1000 μM puerarin or 100 nM insulin with or without 100 mM glucose for 48 h, and total protein was extracted for Western Blot analysis. (a-d) Immunoblot analysis of Atrogin-1, Murf-1 and MyHC. e-g, Immunofluorescence staining for MyHC (100 x magnification) in L6 rat myotubes treated with 10, 100, 1000 μM puerarin or 100 nM insulin with or without 100 mM glucose for 48 h. (e) Representative images of immunofluorescence staining for MyHC. (f) Myotube average area analysis of L6 rat myotubes. (g) Frequency distribution of myotube area. b-d, Data are represented as the means ± SEM from n=3 experiments. Statistical analysis tested by one-way ANOVA with Dunnett's multiple comparisons test analysis. f, Values shown are means ± SEM from 5 fields (n=3). Statistical analysis

tested by one-way ANOVA with Dunnett's multiple comparisons test analysis. g, Statistical analysis tested by Chi-square (and Fisher's exact) test. ## $p < 0.01$ and ### $p < 0.001$ vs. DM group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. the HG group.

Figure 8. Puerarin attenuated autophagy but upregulated Akt/mTOR signaling in L6 myotubes. Fully differentiated L6 myotubes were treated with 10, 100, or 1000 μM puerarin or 100 nM insulin with or without 100 mM glucose for 48 h, and total protein was extracted for Western Blot analysis. (a-d) Immunoblot analysis of phosphorylation of ULK1, p62 and LC3II/I. (e-j) Immunoblot analysis of phosphorylation of Akt, FoxO3a, mTOR, p70S6K and 4EBP1. Data are represented as the means ± SEM from n=3 experiments. Statistical analysis tested by one-way ANOVA with Dunnett's multiple comparisons test analysis. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. DM group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and vs. HG group.

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