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GDF11 does not improve the palmitate induced insulin resistance in C2C12

Y.-Y. JING, D. LI, F. WU, L.-L. GONG, R. LI

Department of Endocrinology, the First Affiliated Hospital of Chongqing Medical University, Chongqing, China

Abstract. – OBJECTIVE: GDF11 (Growth Differentiation factor 11) has been reported to rejuvenate skeletal muscle, heart and brain in aged mice, and the aged skeletal muscle is closely related to insulin resistance. We wondered whether GDF11 has an effect on skeletal muscle insulin resistance.

MATERIALS AND METHODS: High fat diet induced obese mice with insulin resistance were established *in vivo*. Palmitate-induced insulin resistance in C2C12 myotubes was established *in vitro*. The mRNA expression of GDF11, GLUT4, IRS-1 (insulin receptor substrate-1) and PGC-1α (peroxisome proliferator-activated receptor-gamma coactivator 1) were tested by reverse transcriptase-polymerase chain reaction (RT-PCR). The protein level of GDF11 and PGC-1α were detected by Western blot. The glucose uptake was measured by 2NBDG uptake assay.

RESULTS: In high fat diet induced obese mice, both serum level of GDF11 and the expression of GDF11 in skeletal muscle decreased. Similarly, the expression of GDF11 also reduced in palmitate treated C2C12 myotubes. *In vitro*, the glucose uptake and the expression of GLUT4, IRS-1 and PGC-1α significantly decreased after palmitate intervention, but GDF11 treatment did not reverse the reduction of glucose uptake and the expression of GLUT4, IRS-1 and PGC-1α in C2C12 myotubes.

CONCLUSIONS: We firstly confirmed that the expression of GDF11 decreased both in the skeletal muscle of obese mice and palmitate treated myotubes, but supplementation GDF11 does not ameliorate the palmitate-induced insulin resistance in C2C12 myotubes.

Key Words:
Skeletal muscle, Insulin resistance, GDF11, Glucose uptake, PGC-1α.

Introduction

Insulin resistance is crucial for the pathogenesis of type 2 diabetes. Insulin resistance occurs primarily within the muscle, liver and fat tissue. As skeletal muscle accounts for almost 80% of glucose utilization after insulin stimulation, skeletal muscle plays an important role in maintaining glucose homeostasis, defecting in muscle insulin sensitivity and function cause metabolic disease[1]. Muscle insulin resistance is mediated by mitochondrial deficiency or dysfunction[2]. Muscles in obese individuals with insulin resistant contain fewer mitochondria than those in normal individuals[3]. The reduction of mitochondrial content decreases the capacity of muscles to oxidize fatty acids, leading to intramuscular lipid accumulation and insulin resistance finally[2,4]. From the beginning of this sentence is divided into the next paragraph. Several noticeable investigations[5-7] showed that GDF11 levels in blood declined with age and its supplementation to reach young physiological range in old mice rejuvenated the feature and function of the age-related heart and skeletal muscle. It has been reported[8,9] that skeletal muscle aging is closely related to insulin resistance. Moreover, GDF11 treatment remodeled aged skeletal muscle fibers through enhancement mitochondrial function and increasing the expression of peroxisome proliferator activated receptor gamma coactivator-1α (PGC-1α)[7], which is a master regulator of mitochondrial. It has been shown that overexpression of PGC-1α in mouse skeletal muscle can improve glucose metabolism[10]. However, whether GDF11 has an effect on skeletal muscle insulin resistance is unknown. Considering that GDF11 reversed skeletal muscle aging and enhanced mitochondrial biogenesis, our study analyzed the effect of GDF11 on the skeletal muscle insulin resistance. In this work we aimed to investigate the level of GDF11 in obese mice with insulin resistance induced by high fat diet (HFD). Also, the *in vitro* effects of the addition of recombinant GDF11 to skeletal muscle cells on insulin resistance were determined.

Corresponding Author: Rong Li, MD; e-mail: rongli232006@163.com

Materials and Methods

Experiment Materials and Instruments

Low fat diet (LFD) and high fat diet (HFD) (Research Diets Inc., New Brunswick, NJ, USA); GDF11 (R&D Systems, Minneapolis, MN, USA); CCK-8 kit (Biotool, Jupiter, FL, USA); 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)-2-deoxyglucose (2NBDG) (Invitrogen, Carlsbad, CA, USA); radio immunoprecipitation assay (RIPA) lysis buffer, BCA Protein Assay kit, enhanced chemiluminescence (Beyotime, Shanghai, China); microplate reader (Thermo Scientific, Waltham, MA, USA); polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA); mouse monoclonal anti-GDF11 Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit polyclonal anti-PGC-1α (Abcam, Cambridge, MA, USA), anti-β-actin (Proteintech, Rosemont, IL, USA); Trizol reagent (TaKaRa, Otsu, Shiga, Japan); SYBR Green PCR kit (TTaKaRa, Otsu, Shiga); iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA).

Animals

Animal care and experimental procedures were performed with the approval of the Animal Care Committees of Chongqing Medical University. C57BL/6J male mice (n=14) were from the Animal Center of Chongqing Medical University. After one-week adaptive feeding, mice were randomly assigned to receive a low fat diet (LFD: 4.3% (w/w) fat content, 10% kcal) (LFD group, n=7) or a high fat diet (HFD: 34% (w/w) fat content, 60% kcal) (n=7) for 11 weeks. At the end of 11th week, mice in each group had several tests of body weight, glucose and insulin tolerance test. Blood and skeletal muscle were collected from each mouse for further assessment.

Intraperitoneal Glucose Tolerance Test (IPGTT) and Insulin Tolerance Test (ITT)

For the glucose tolerance test, mice fasted overnight were injected intraperitoneally with glucose solution (2 g/kg body weight). Blood glucose concentrations at 0, 15, 30, 60, and 120 min were measured by tail snipping method. For the insulin tolerance test, mice fasted for 6 h were injected intraperitoneally with human insulin (Humulin R, 0.75 unit/kg body weight). Blood glucose concentrations at 0, 15, 30, 60, and 120 min were measured by tail snipping method.

Cell Culture

C2C12 mouse myoblast cells were grown in Dulbecco's modified Eagle medium (DMEM) Gibco (Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) at 37°C in an atmosphere of 95% humidity and 5% CO_2. When grew confluency, cells were differentiated in media containing 2% horse serum Gibco (Grand Island, NY, USA) at 37°C in 5% CO2 for 6 days. Differentiation medium was changed every day. C2C12 myotubes were cultured in serum-deprived medium for 2 h before treatment with or without palmitate, which was conjugated to 2% bovine serum albumin (BSA) as described previously[11]. Then the cells were treated with or without GDF11 (50 ng/ml,100 ng/ml) for 24 h.

Cell Viability Assay

Cytotoxicity experiments were performed to determine the nontoxic dose of GDF11 (R&D Systems, Minneapolis, MN, USA). C2C12 myoblasts were seeded in 96-well plates at 5×103 cells per well in 100 µl of growth medium and cultured with or without different dose of GDF11 for 24 h. Then, CCK-8 kit (Biotool, Jupiter, FL, USA) was used to detect cell viability according to manufacturer's instructions. Relative cell viability of treatment was calculated as a percentage of the viability.

Glucose Uptake Measurement

Glucose uptake was assayed using 2-NBDG (Invitrogen, Carlsbad, CA, USA) as described earlier with slight modifications[12]. Briefly, C2C12 myotubes were treated as indicated, media were removed and cells were washed with Hank's Balanced Salt Solution (HBSS) buffer followed by stimulation with 100 nM insulin for 30 min. Then, cells were further incubated in HBSS buffer containing 100 mM 2-NBDG for 30 min at 37°C. The medium was then washed twice with pre-cold HBSS, followed by RIPA lysis buffer (Beyotime, Shanghai, China) on ice in the dark for 10 min. The cell homogenate was centrifuged at 14.000 g for 15 min at 4°C. Supernatants were prepared for the fluorescence and protein quantification. The fluorescence was measured using a microplate reader (Thermo Scientific, Waltham, MA, USA) at excitation and emission wavelengths of 485 nm and 535 nm, respectively. Fluorescence intensity was normalized to protein content in every sample.

Western Blot

Proteins were extracted from tissues and cells using lysis buffer for Western blot. Briefly, skeletal muscle and C2C12 myotubes were homogenized in ice-cold RIPA buffer containing 1 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The whole lysate was centrifuged at 14.000 rpm at 4°C for 15 min and the supernatant saved at -20°C for Western blot analysis. Protein concentration was determined using the bicinchoninic acid assay (BCA) Protein Assay kit (Beyotime, Shanghai, China). The harvested lysates of equal protein were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis, and transferred to polyvinylidene fluoride (PVDF) membranes, Millipore (Billerica, MA, USA). The membranes were blocked in 5% non-fat dry milk in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20, and then incubated with primary antibodies, mouse monoclonal anti-GDF11 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-PGC-1α (Abcam, Cambridge, MA, USA), anti-β-actin (Proteintech, Rosemont, IL, USA) in blocking buffer at 4°C overnight. Then membranes were washed three times, and incubated with second antibodies (goat anti-rabbit IgG, goat anti-mouse IgG) (Millipore, Billerica, MA, USA) in blocking buffer for 1 h at room temperature. Immuno-reactive proteins were visualized using enhanced chemiluminescence (Beyotime, Shanghai, China) and protein was compared to β-actin; the band intensities were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

Real-Time PCR

Total RNA was extracted from C2C12 myotubes or skeletal muscle using the Trizol reagent (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instruction. 1 μg of total RNA from each sample was reverse-transcribed to cDNA using a reverse transcription system (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's protocol. After cDNA synthesis, quantitative Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Each quantitative reaction was performed in triplicate. To normalize expression data, GAPDH was used as the internal control gene. Relative gene expression levels were calculated using the $2^{-\Delta\Delta}$Ct method. The primer sequences used for the polymerase chain reaction (PCR) amplification were showed in Table I.

Enzyme-linked Immunosorbent Assay (ELISA) Measurements

Venous blood samples were centrifuged for 15 min at 1500 g at 4°C, the separated serum was stored at -80°C until assay. GDF11 levels were measured with ELISA kits (Cloud-Clone Corp., Houston, TX, USA). Operations were strictly carried out following up kit instructions.

Table I. Sequences of primers for RT-PCR.

Primers	Sequences
GDF11	Forward 5′- CAGTGGACTTTGAGGCTTTTGG-3′ Reverse 5′- TGATTGGGGACATCTTGGTAGG-3′
Desmin	Forward 5′- CGACGCTGTGAACCAGGAGT-3′ Reverse 5′- GTAGTTGGCGAAGCGGTCAT-3′
Myogenin	Forward 5′- GCAATGCACTGGAGTTCGGT-3′ Reverse 5′- TCCTCCACCGTGATGCTGTC-3′
GLUT4	Forward 5′- CTGACCACAAACGATGACCCTC-3′ Reverse 5′- CTGACCACAAACGATGACCCTC-3′
IRS-1	Forward 5′- CTGACCACAAACGATGACCCTC-3′ Reverse 5′- CTGACCACAAACGATGACCCTC-3′
PGC-1α	Forward 5′- CTGACCACAAACGATGACCCTC-3′ Reverse 5′- TGCGGTTGTGTATGGGACTTCT-3′
GAPDH	Forward 5′- GACATCAAGAAGGTGGTGAAC-3′ Reverse 5′- GAAGGTGGAAGAGTGGGAGTT -3′

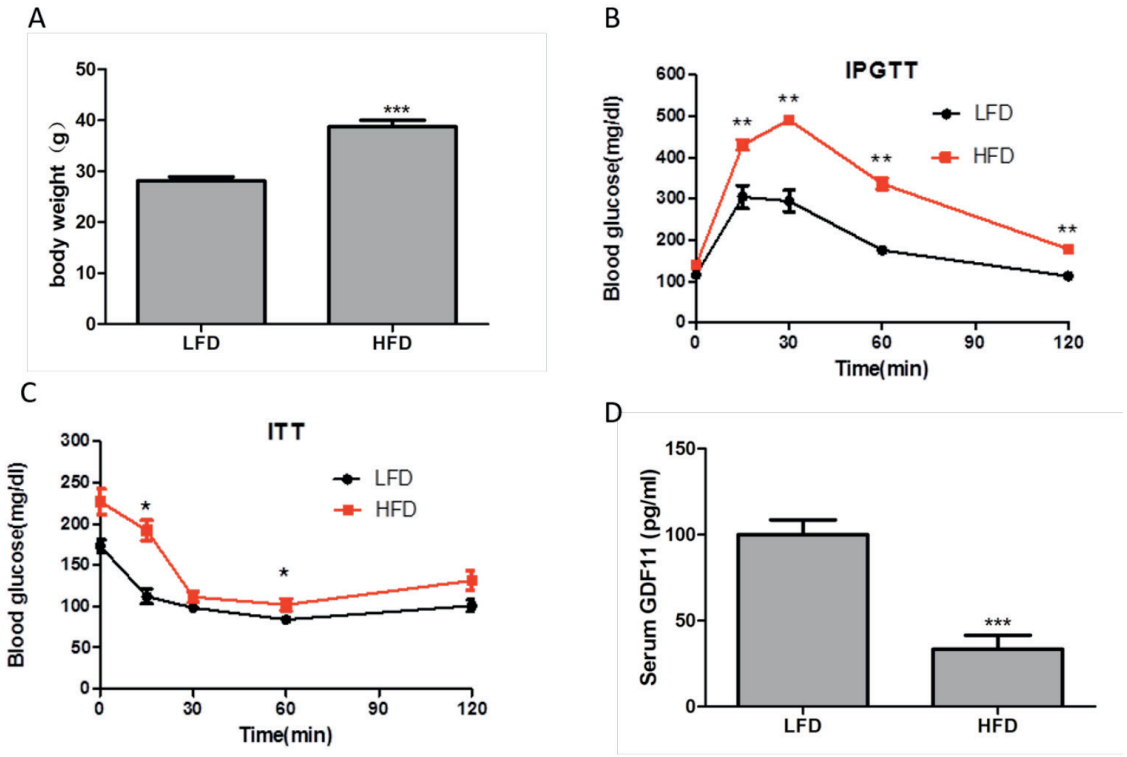

Figure 1. The changes of GDF11 serum level were observed in HFD induced obese mice with insulin resistance. Notes: ***A,*** Body weight; ***B,*** IPGTT; ***C,*** ITT; ***D,*** serum level of GDF11. Each value is presented as mean ± SEM, n=7; $^{*}p<0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$ vs. LFD.

Statistical Analysis

Statistical analyses were performed by using GraphPad Prism 5 software (La Jolla, CA, USA). Student's *t*-test was used for the two group comparisons. To validate one-way ANOVA, post hoc test by Newman-Keuls's for multiple comparisons was used. Results are expressed as means ± standard error (SEM). *p*-value less than 0.05 were considered statistically significant.

Results

Reduced GDF11 Levels in Serum were Showed in Obese Mice with Insulin ReSistance

Compared with LFD group, mice in HFD group showed higher levels of body weight ($p<0.001$; Figure 1A). IPGTT showed that the fasting blood glucose of HFD mice was much higher than that of LFD mice from 15 min ($p<0.01$; Figure 1B). ITT showed that systemic insulin sensitivity decreased in HFD fed mice ($p<0.05$; Figure 1C). Importantly, compared with LFD group, GDF11 in serum in HFD group was significantly lower ($p<0.001$; Figure 1D).

The Expression of GDF11 was Down-Regulated in Skeletal Muscle of Obese mice and in Palmitate Treated C2C12 Myotubes

In vivo, compared with LFD group, the mRNA and protein level of GDF11 were down-regulated in skeletal muscle in HFD group ($p<0.05$; Figure 2 A-B). In vitro, six days after the differentiation induction, myoblasts gradually fused to myotubes. The mRNA level of myotube marker genes desmin and myogenin significantly increased in differentiation group compared with undifferentiation group ($p<0.05$, $p<0.01$; Figure 2 C-D). Similar to the results *in vivo*, the mRNA level of GDF11 was lessened in palmitate treated C2C12 myotubes ($p<0.001$; Figure 2E).

GDF11 did Not Improve the Palmitate-induced Insulin Resistance in C2C12 Myotubes

To assess the non-cytotoxic concentration of GDF11, the viability of C2C12 cells were tested at different concentrations of GDF11 varied from 0 to 100 ng/ml using CCK8 assay. There were no significant effects of different GDF11 concentrations on the

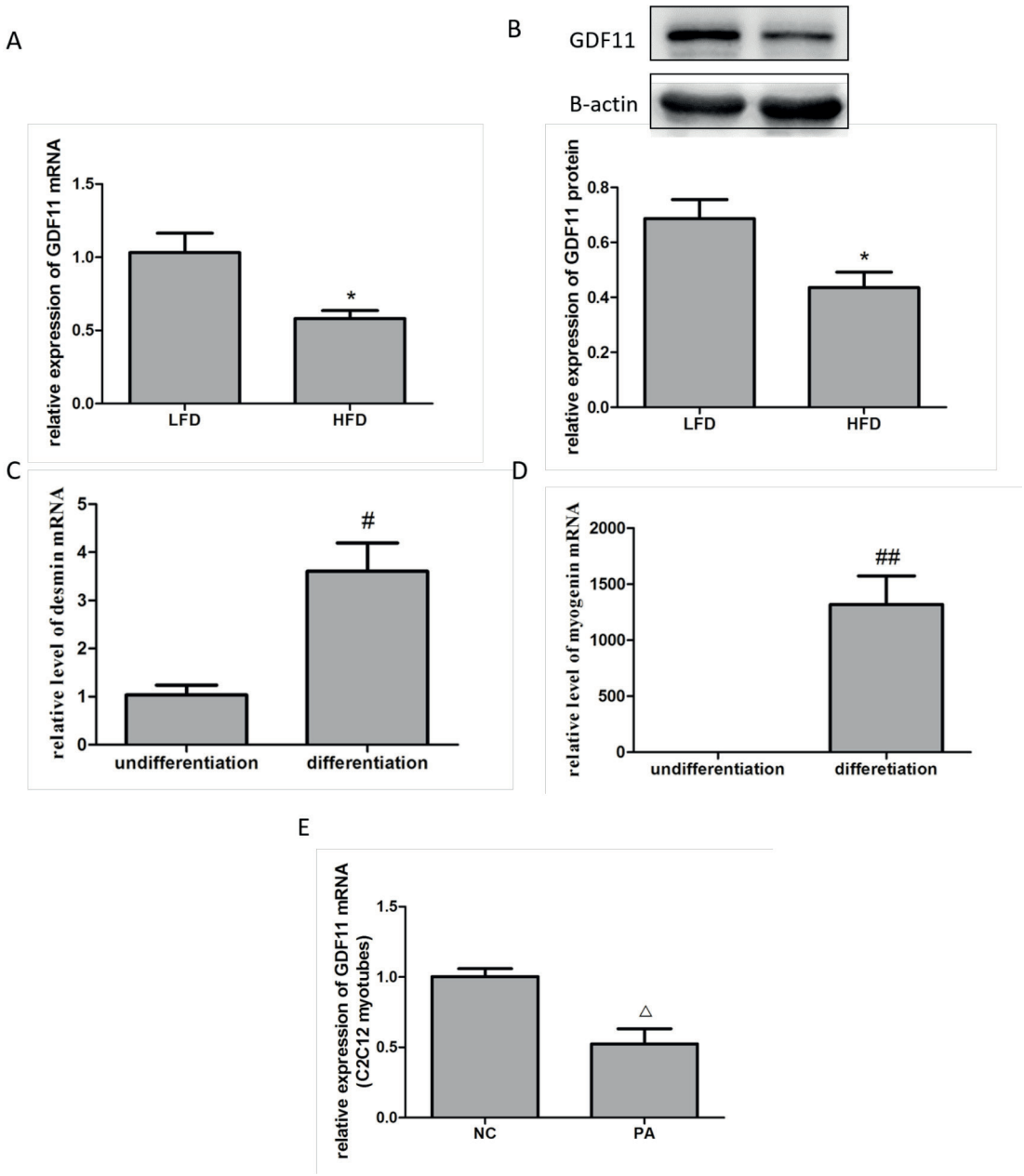

Figure 2. The expression of GDF11 in skeletal muscle in HFD mice and in C2C12 myotubes treated by palmitate were observed. Notes: ***A,*** mRNA level of GDF11 in mice; ***B,*** protein level of GDF11 in mice; ***C,*** mRNA level of desmin; ***D,*** mRNA level of myogenin; E, mRNA level of GDF11 in C2C12. Each value is presented as mean ± SEM, n=3. $^{*}p<0.05$ vs. LFD; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. undifferentiation; $^{\Delta}p<0.05$ vs. NC. NC: normal control; PA: palmitate.

cell viability ($p>0.05$; Figure 3A), and the concentrations were used for the followed research. The 2-NBDG-uptake assay showed that insulin stimulated glucose uptake was significantly reduced by palmitate treatment compared with control group ($p<0.01$). However, supplementation of GDF11 did not reverse the decreased glucose uptake ($p>0.05$; Figure 3B). The expression of GLUT4 (glucose transporter 4) and IRS-1 (insulin receptor substrate 1) were also significantly down-regulated by palmitate treatment ($p<0.001$), but GDF11 did not up-regulate the expression of GLUT4 and IRS-1 ($p>0.05$; Figure 3 C-D).

GDF11 did not Increase the PGC-1α Expression in Palmitate Treated C2C12 Myotubes

Compared with control group, the mRNA and protein expression of PGC-1α were significantly

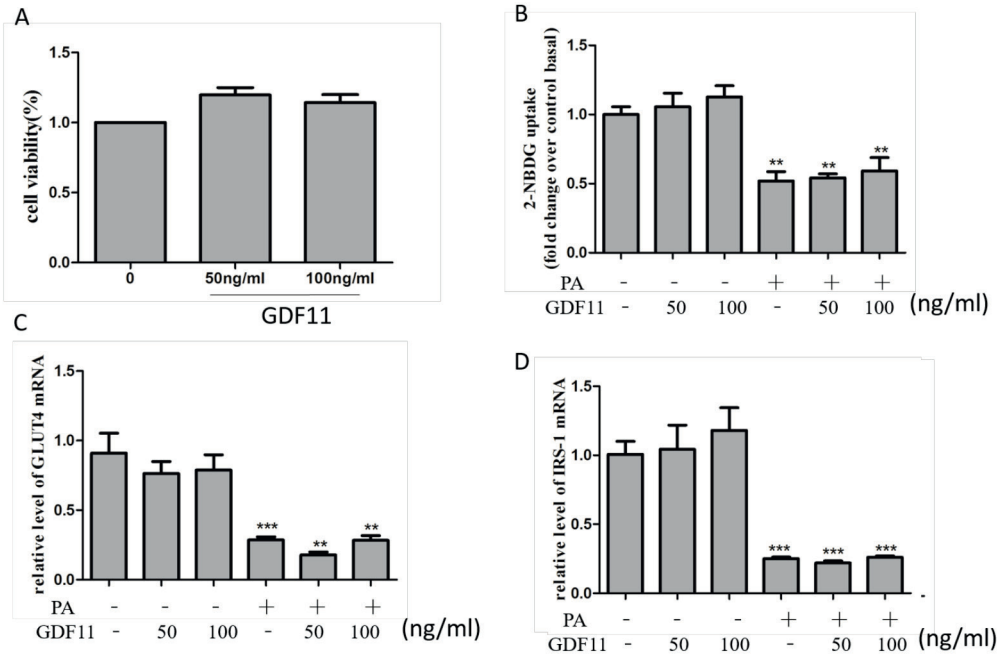

Figure 3. Effect of GDF11 on palmitate-induced insulin resistance in C2C12 myotubes. Notes: ***A,*** cell viability; ***B,*** glucose uptake; ***C,*** the mRNA expression of GLUT4; ***D,*** the mRNA expression of IRS-1. Each value is presented as mean ± SEM, n=3. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. control.

reduced by palmitate ($p<0.01$). However, supplementation GDF11 did not improve the reduction of PGC-1α ($p>0.05$; Figure 4 A-B).

Discussion

GDF11 has been suggested as a powerful anti-aging candidate with a broad effect on some tissues, including skeletal muscle, cardiac and brain[5,7,13]. Especially, because GDF11 improved the function of aged mouse skeletal muscle, our study focused on its effect on skeletal muscle insulin resistance. To our knowledge, we first found that both circulating levels and the skeletal muscle expression of GDF11 decreased in HFD-induced obese mice *in vivo*. Also, the mRNA level of GDF11 decreased in C2C12 myotubes treated by palmitate *in vitro*.

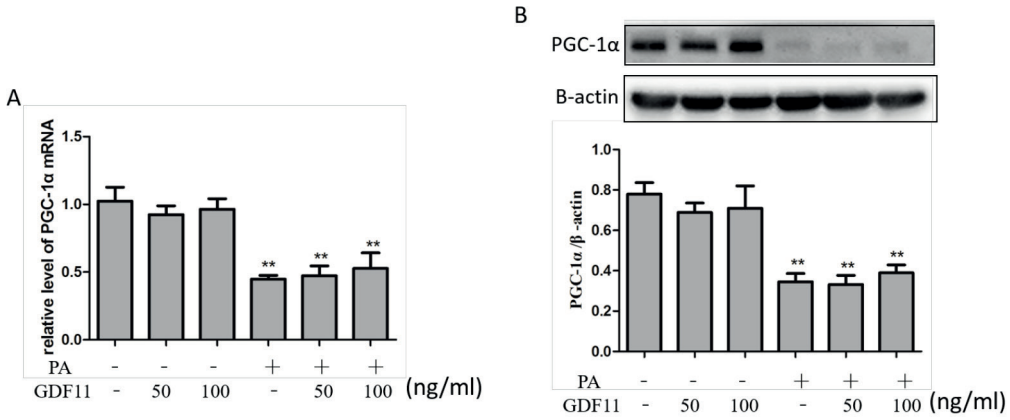

Figure 4. Effect of GDF11 on the mRNA and protein expression of PGC-1α in C2C12 myotubes. Notes: ***A,*** the mRNA expression of PGC-1α; ***B,*** the protein expression of PGC-1α. Each value is presented as mean ± SEM, n=3. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. control.

However, GDF11 treatment did not alter the palmitate-induced insulin resistance in C2C12 myotubes. Our results were in line with a recent report, which showed a reduction of GDF11 levels in ZMPSTE24-deficient mice (a premature mice model) when comparing with the wild-type littermates, but GDF11 administration does not extend the lifespan of premature-aged mice[14].

GDF11 is widely expressed in skeletal muscle, pancreas, kidney, heart and other tissues[15].

In this work, we aimed to investigate whether GDF11 haS an effect on skeletal muscle insulin resistance. To test the hypothesis, obesity with insulin resistance model was established. Numerous studies[16] reveal that rats with high-fat diet lead to insulin resistance and impaired glucose metabolism. *In vivo*, we observed that GDF11 in serum and skeletal muscle significantly decreased in obese mice with insulin resistance. The expression of GDF11 also decreased in myotubes treated by palmitate *in vitro*. A previous study[17] reported that cytokines, such as irisin, which expressed and released by muscle fibers, decreased in obese individuals with insulin resistance, and protein supplementation could improve insulin resistance. These phenomena allowed us to speculate whether supplementation GDF11 can reverse the skeletal muscle insulin resistance.

We subsequently evaluated whether GDF11 could improve palmitate-induced insulin resistance in C2C12 myotubes. Insulin-mediated glucose uptake was significantly reduced by palmitate in myotubes, which indicated that insulin resistance model was successfully established *in vitro* as previous study reported[11]. Meanwhile, the expression of GLUT4 and IRS-1 were down-regulated by palmitate treatment. However, supplementation GDF11 did not reverse the reduction of glucose uptake and the expression of GLUT4 and IRS-1. The concentration of GDF11 used in our paper was referred to previous researches[7,18,19]. The results are not consistent with our initial hypothesis, there may be the following reasons. Firstly, the decreased expression of GDF11 in skeletal muscle may be due to reduced muscle mass in obese mice with insulin resistance. Secondly, additional GDF11 may act on other organs rather than skeletal muscle. Poggioli et al[20] reported that GDF11 administration significantly induced reduction in adipose tissue in old mice, whereas skeletal muscle tissue remained unchanged, which suggested a potential indirect effect of exogenous GDF11 on cardiac tissue. Our study indicated that GDF11 did not have a direct effect on insulin resistance *in vitro*, but whether an indirect effect of GDF11 administration on mice with insulin resistance in vivo was unclear.

However, subsequent investigations questioned the anti-aging effects of GDF11. Smith et al[21] reported that GDF11 treatment induced hypertrophy instead of rescuing age-related hypertrophy. Egerman et al[22] discovered that GDF11 protein in blood and skeletal muscle of rats increased with age, and GDF11 reduced skeletal muscle regeneration by inhibiting myoblast differentiation. Egerman et al[22] proposed that the detection method of GDF11 protein in Sinha et al[7] study was difficult to distinguish the GDF11 protein from the GDF8 (myostatin) protein. Myostatin, a member of TGF-β superfamily, is highly homologous with GDF11 at the protein level[20]. To date, the endogenous GDF11 level in mouse serum has been a major unsolved question due to limitations in the detection technology[23].

Conclusions

We observed that serum level and skeletal muscle expression of GDF11 were reduced in obese mice with insulin resistance. Besides, the mRNA expression of GDF11 decreased in palmitate-induced insulin resistance in C2C12 myotubes. Whereas GDF11 treatment was not sufficient to improve the insulin resistance in C2C12 myotubes, our research provides a reference for the function of GDF11, but more researches are needed to explore the role of GDF11.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

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