Jianpi Qinghua Formula Reduced Intramyocellular Lipids (IMCLs) by Inhibiting Excessive Activation of mTORC1

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Research

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Abstract

Background:IMCLs are an important factor in skeletal muscle insulin resistance. This study aimed to explore the effect of Jianpi Qinghua formula (JPQHF) on IMCLs and its mechanism, as well as the relationship between IMCLs and other skeletal muscle insulin sensitivity factors, thereby elucidating the mechanism by which JPQHF improves insulin sensitivity.

Methods: In an in vivo experiment, JPQHF and pioglitazone (PIO) were individually used to treat C57 mice with high-fat diet-induced obesity. In an in vitro experiment, JPQHF and rapamycin in serum were individually used to treat C2C12 cells induced with palmitic acid. The IMCLs of tissue and cells were subjected to oil red O staining. The RNA and protein expression of PPARγ, myogenin, mTORC1 and members of the PI3K/AKT pathway in skeletal muscle tissue and C2C12 cells was examined. Differences between the different intervention groups were determined.

Results: IMCLs were significantly increased in mice with obesity induced by a high-fat diet and the C2C12 cell line treated with palmitic acid compared to the corresponding controls. mTORC1 phosphorylation and PPARγ levels were also increased, and AKT phosphorylation and myogenin levels were decreased. Intervention with JPQHF reversed the above changes. In addition, the PPARγ level in C2C12 cells was reduced after intervention with rapamycin, an inhibitor of mTORC1. However, AKT phosphorylation and myogenin levels did not recover after rapamycin intervention.

Conclusion: IMCLs were significantly increased in obese C57 mice and palmitic acid-treated C2C12 cells. JPQHF reduced IMCLs both in vivo and in vitro. Mechanistically, this effect likely occurred through JPQHF-mediated inhibition of the overactivation of mTORC1 and a subsequent reduction in the expression of PPARγ. However, the function of JPQHF in elevating myogenin levels and the PI3K/AKT pathway may not be entirely dependent on mTORC1.

Background

Insulin resistance is the main pathological mechanism of type 2 diabetes. As skeletal muscle is the main tissue that consumes glucose, skeletal muscle insulin resistance is the key step of systemic insulin resistance.

Excess energy taken up through the diet is stored in adipose tissue. When the amount and volume of the adipose tissue increase to a threshold and lipids can no longer be stored in adipose tissue,lipids are transferred to unconventional adipose storage organs (such as the liver, heart and skeletal muscle). Ectopic fat is deposited outside and under the muscular fascia of skeletal muscle. Additionally, intramyocellular lipids (IMCLs) and extramyocellular lipids (EMCLs) are deposited under the fascia. IMCLs are an important cause of insulin resistance[1], and a reduction in IMCLs significantly improves insulin resistance[2].

In recent years, the mTOR pathway has been considered a regulator that controls energy utilization or synthetic storage and a key factor that aggravates insulin resistance. mTORC1 and mTORC2 are two types of mTOR proteins[3]. mTORC1 is part of an important pathway that promotes fat synthesis[4]. In obese patients and models of obesity through high-fat diet feeding, $p^{Ser2448}$mTOR was excessive activated. The downstream protein p70 S6K1 hyper-phosphorylates IRS-1 at Ser307/312/527/616/636, leading to insulin resistance[5]. Moreover, mTORC1 itself phosphorylates the Ser636/639 sites of IRS-1, thereby reducing IRS-1 activity[6]. mTORC2 induces glycogen synthesis; furthermore, overactivated mTORC1 causes insulin resistance due to the indirect inhibition of mTORC2[7].

Jianpi Qinghua Fang (JPQHF) means strengthen the spleen and removal phlegm-dampness and has been used for the treatment of diabetic insulin resistance for more than ten years in Department of Endocrinology of Shuhuang Hospital.Clinical studies have confirmed that JPQHF reduced postmeal blood glucose(2hPG) in patients with pre-diabetes[8].JPQHF reduced fasting blood glucose and HbA1c increases GLP-1, alleviated insulin resistance in patients with type 2 diabetes[9]and reduced their lipid metabolism index including TG, LDL-C and BMI[10].Animal studies confirmed that JPQHF improved hepatic insulin signaling pathway in STZ and high-fat diet induced type 2 diabetes rats ,reduced liver lipid ectopic deposition[11]and improved expression of GLUT4 in skeletal muscle cells[12]thereby improves glucose utilization.JPQHF reduced content of adipose tissue in insulin resistant mice induced by high-fat diet[13].Meanwhile Skeletal muscle oil red O staining of these mice revealed lower IMCLs in JPQHF intervening high-fat feeding group compared with high-fat feeding group.

In view of the the relationship between IMCL and insulin resistance of skeletal muscle. the purpose of this study was to investigate the effect of JPQHF on the IMCLs and the mechanism, there by explain effect on insulin resistance.

Methods

Animals

Seventy 4-week-old male C57 mice and twenty male Wistar rats weighting 200-300 g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (production licence No. SCXK, Shanghai 2017-0011). The animals were kept in the SPF environment of the Animal Center of Shanghai University of Traditional Chinese Medicine. An appropriate ambient temperature (20-23°C), humidity (50%-60%) and circadian rhythm (12/12-h light/dark cycle) and free access to drinking water and activities were provided. All experimental procedures were conducted according to the guidelines of the Animal Care and Ethics Committee of the Shanghai Traditional Chinese Medicine University (China). The C57 mice and Wistar rats were subjected to animal experiments and administered serum containing JPQHF.Deep anesthesia was implemented by intraperitoneal injection of 1% pentobarbital sodium at a dose of 50mg/kg body weigh, confirmed by stable breathing and no reaction to pinching tail.After collecting blood from the

ventriculus dexter (mice)and abdominal aorta(rats),mice or rats were executed by cervical dislocation under deep anesthesia.

Dosing

The C57 mice were adaptively fed for 1 week. Ten mice were randomly selected as the control group and fed a normal diet after being weighed. The other sixty mice were in the high-fat diet group (HD group) and treated with a diet containing 60% fat (Research Diet, 12492). The mice were fed a high-fat diet for 8 weeks and weighed once a week. After 8 weeks, the forty-five heaviest mice were chosen from the sixty mice in the HD group. A random number table was used to randomly select ten mice from these forty-five mice. An IPGTT was conducted with the selected 10 mice and 10 mice in the control group. The modelling criteria were as follows: (1) the body weight significantly differed between the HD group and control group and (2) the glucose tolerance of mice in the HD group was obviously impaired compared with that in the control group. After confirmation of successful modelling, 45 mice were randomly divided via SPSS26.0 into 3 groups of 15 mice each: the model group (fed a diet containing 60% fat and normal saline), JPQHF group (fed a diet containing 60% fat and JPQHF) and PIO group (fed a diet containing 60% fat and PIO). There was no significant difference in body weight among the 3 groups. Interventions were maintained for 4 weeks.

The JPQHF, composed of 15 g of Dangshen, 15 g of Huangqi, 15 g of Shanyao, 15 g of Huangjing, 3 g of Huanglian, 9 g of Huangqin, 15 g of Gegen and 15 g of Guijianyu, was purchased from Shanghai Kangqiao Traditional Chinese Medicine Decoction Co., Ltd., and had passed quality inspection. A decoction was prepared according to standard procedure[11] and kept at -20°C. The intragastric dose of the decoction administered to the mice was 0.1 ml/10 g. Based on the drug dosing coefficient between mice and humans, the concentration of the decoction was 2.0961 g of crude drug /ml. PIO was dissolved in pure water to 0.6165 mg/ml to generate an effective concentration for dosing. The suspension was shaken well before each intragastric administration.

Preparation of serum containing JPQHF

Thirty Wistar rats were randomly divided into two groups (15/15): the JPQHF group, the rats in which were administered JPQHF decoction at the following dose, and the blank serum group, the rats in which were without intervention. JPQHF was prepared as described above. The final concentration of the decoction was 2.35 g crude drug/ml. The gavage dose was 1 ml/100 g body weight. The rats were intragastrically administered the decoction twice daily at 9 am and 5 pm. Blood was collected from the abdominal aorta on the 5th day at 1 hour after administration. The blood was centrifuged (2500 rpm, 5 min) after incubation at room temperature for 2 hours. Then, the serum was heated in a 56°C water bath for 30 min, stored at -80°C and filtered before each use.

Cell culture and treatment

C2C12 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and always cultured at 37°C in a humid 5% CO_2 incubator. The cells were cultured in 10% FCS-containing DMEM (4.5 g/L glucose) until reaching 80% confluence in a 25T culture flask for approximately 3 days. Then, the medium was changed to 2% horse serum-containing DMEM (4.5 g/L glucose), and the cells were incubated for an additional 2 days, with the medium changed to fresh medium every day.

The C2C12 cells were cultured in the above medium for 24 hours. The concentrations of BSA, a penicillin-streptomycin solution (100×), blank serum, JPQHF decoction, palmitic acid culture medium and rapamycin were 1%, 1%, 8%, 8%, 0.5 mmol/L and 100 nmol/L, respectively.

The intervention conditions for each group were as follows:

control (Con): BSA culture medium;

palmitic acid (PA): palmitic acid culture medium;

control+blank serum (con+BS): BSA cell culture medium+ blank serum;

palmitic acid+blank serum (PA+BS): palmitic acid culture medium+ blank serum;

palmitic acid+JPQHF serum (PA+JS): palmitic acid culture medium+JPQHF serum; palmitic acid+rapamycin+blank serum (PA+RA+BS): palmitic acid culture medium+rapamycin+blank serum;

control+rapamycin+blank serum (con+RA+BS): BSA culture medium+rapamycin+blank serum.

A penicillin-streptomycin solution (100×) was added to each group. C2C12 cells were cultured in the above media for 24 hours. The concentration of insulin was 100 nm/L, and insulin treatment was carried out for 20 min.

Oil red O staining of the mouse gastrocnemius muscle and C2C12 cells

The tissue samples were taken from the -80°C freezer and placed in a cooling rack. The tissue was quickly transferred to a pre-cooled frozen slicer and sliced to a thickness of 8 μm. After fixation in cold 10% formaldehyde for 10 min, the slices were rinsed with distilled water 3 times and dried it at room temperature for 5 min. The slices were then incubated in propylene glycol for 5 min and immersed in an oil red O solution preheated in an oven at 60°C for 8-10 min. Then, the sliced were differentiated in a 85% propylene glycol solution (100% pure propylene glycol diluted with distilled water) for 10 min and washed 2 times with distilled water. The slices were then dyed for 30 sec in Mayer's haematoxylin and washed

thoroughly with running water for 3 min. The slices were immersed in distilled water, observed under a microscope and photographed.

After different treatment interventions (including palmitic acid, rapamycin and serum containing JPQHF, but not insulin), C2C12 cells were stained with oil red O according to the instructions of a Solarbio Cell Oil Red O staining kit (#G1262).

GPO-PAP assay

Triglyceride levels in the skeletal muscle were measured using the GPO-PAP method performed according to the instructions of a kit (Nanjing Jiancheng Bioengineering Institute, A110-2-1).

PCR

Total RNA was extracted from mouse skeletal muscle with TRIzol (Biomaga, 7311). A two-step method was used to identify the relative expression of RNA in each group according to the instructions of the Takara Reverse Transcription Kit (RR036A) and DNA Amplification Kit (RR820A). The primers were synthesized by Sangon Biotech (Shanghai).Primer sequences were as follows: β-actin: forward (5′-3′) TTACTGCCCTGGCTCCTA, reverse (5′-3′) ACTCATCGTACTCCTGCTTG; Srebp-1c: forward (5′-3′) TGTCTGGGAAGGGAGCATAA, reverse (5′-3′) GCTGTTCTGTGGTTTGTTTACCT; PPAR-γ: forward (5′-3′) ATCGAGGACATCCAAGAC, reverse (5′-3′) CAATCTGCCTGAGGTCTG; Myod: forward (5′-3′) CGCGCTCCAACTGCTCTGAT, reverse (5′-3′) CAGCCGCACTCTTCCCT; and myogenin: forward (5′-3′) GCACTGGAGTTCGGTCCCAA, reverse (5′-3′) ATCCTCCACCGTGATGCTG.

Western blotting (WB)

Total protein was extracted from mouse skeletal muscle and cells by conventional methods. Proteins were quantified by the BCA method (Thermo, RH237552) and separated by SDS-PAGE. The concentration of the stacking gel was 4%, and the concentration of the separating gel was 10% except when the target proteins were mTOR and pmTOR, for which 8% gels were used. Then, the electrophoresed proteins were transferred to PVDF membranes, which were incubated overnight with primary antibody at 4°C. The following primary antibodies were used in this experiment: anti-PI3K (CST, #4249, 1:1000 dilution rate), anti-AKT (CST, #3063, 1:1000 dilution rate), anti-pAKT (CST, #4060, 1:1000 dilution rate), anti-mTOR (CST, #2983, 1:1000 dilution rate), anti-pmTOR (CST, #5536, 1:1000 dilution rate), anti-PPARγ (CST, #2435, 1:1000 dilution rate), anti-myogenin (Absin, #abs101516, 1:1000 dilution rate), and anti-GAPDH (CST, #2118, 1:2000 dilution rate). The next day, the membrane was washed 3 times in TBST

(1×). The membranes were then incubated with secondary antibody (BOSTER, BA1050, 1:5000) for 1 hour and washed 3 times in TBST (1×). Then, the blots were photographed and assessed by electrochemiluminescence (ECL).

Statistical analyses

Measured data were analysed with SPSS 26.0. Data with a normal distribution are expressed as the mean ± SD. Differences for which *P-value < 0.05 or **P < 0.01 were considered statistically significant.

Results

Eight weeks high-fat diet feeding caused obesity and insulin resistance in mice

The weights of mice from the HD group and control group during the modelling period are shown in fig. 1. The weights of mice in the HD group and control group in week 0 were no different (P > 0.05). Differences in weight appeared beginning in week 2 and became evident at week 4 (P < 0.05). At the end of the 8 week period, the weight of mice in the DH group was significantly higher than that of mice in the control group (P < 0.01) (fig. 1a).

The IPGTT (1.5 g glucose per 1 kg weight) was conducted in 10 mice from the HD group and 10 mice from the control group and showed that 60 min and 120 min after glucose loading, a clear difference in blood glucose between the two groups was observed (P < 0.05 and 0.01, respectively), and the AUC for the HD group was significantly higher than that for the control group (P < 0.05) (fig. 1b). This suggested that a high-fat diet damaged glucose tolerance and caused insulin resistance in mice.

JPQHF had little effect on body weight

Body weight over the 4-week intervention period is shown in **fig.** 1c. Body weight in the control group was significantly lower than that in the three intervention groups (P<0.01), and there was no significant difference in body weight between the three intervention groups (P>0.05).

JPQHF reduced systemic insulin resistance

After 4 weeks of intervention, to verity the effect of JPQHF on insulin sensitivity, the IPGTT (1.5 g of glucose per 1 kg weight) and IPITT (0.5 IU insulin per 1 kg weight) were conducted, and blood glucose levels at 0 min, 30 min, 60 min, and 120 min are shown in fig. 1d and e. JPQHF reduced blood glucose levels at 30 min, 60 min, and 120 min compared with those of the model group in the IPGTT and IPITT (P<0.01 and 0.05, respectively).

JPQHF reduced IMCLs and improved myogenic differentiation and the insulin signalpathway

Oil red O staining of the gastrocnemius muscle and triglyceride levels of the 4 groups are shown in fig. 2**.** The IMCL and triglyceride content were obviously higher in the model group than in the control group

(P<0.01). JPQHF obviously decreased the IMAT and triglyceride content compared with that in the model group (P<0.01). According to the results of PCR, at the mRNA level, a high-fat diet increased REBP1c and PPARγ and reduced myod and myogenin (P<0.01). Furthermore, JPQHF regulated PPARγ and myogenin levels (P<0.01), but had little effect on SREBP1c or myod levels (fig. 3 a, b, c, d). At the protein level, a high-fat diet also increased PPARγ and reduced myogenin levels. JPQHF regulated the expression of PPARγ and myogenin, as proven by WB (fig. 3 e, f, g). Proteins in the insulin signalling pathway, including total PI3K, total AKT and pAKT, were decreased in the model group, but JPQHF enhanced the expression of these proteins (fig. 4).

JPQHF restrained excessive activation of the mTOR signalling pathway

To detect the probable target of JPQHF in muscle, the total mTOR and $p^{Ser2448}$mTOR protein levels were detected by WB (fig. 4). The protein level of pmTOR was higher in the model group than in the control group (P<0.01) and lower in the JPQHF group than in the model group (P<0.01).

Effect of JPQHF in the C2C12 cell line

To investigate whether JPQHF affects muscle IMCLs and the insulin signalling pathway via the inhibition of mTORC1, the C2C12 cell line was treated with palmitic acid and then treated with serum containing JPQHF supplemented with rapamycin. Palmitic acid is a common agent used to generate insulin resistance models that causes fatty acid-induced lipid deposition. Rapamycin is a common inhibitor of mTORC1.

In this experiment, when insulin was absent (fig. 6 a, c, e), oil red O staining of C2C12 cells showed an increased tendency towards lipid deposition in the palmitic acid treatment group. Both rapamycin and JPQHF reversed this tendency (fig. 5). Simultaneously, the PI3K/AKT protein was almost not expressed in each group. Palmitic acid significantly increased the relative protein expression levels of pMTOR/mTOR and pMTOR (PA group vs. CON group, PA+BS group vs. CON +BS group, P < 0.01). Both JPQHF and rapamycin restrained the changes to the above two parameters (PA+JS group vs. PA+BS group, PA+RA+BS group vs. PA+BS group, P < 0.01). PPARγ protein expression was significantly higher in the palmitic acid treatment group (PA group vs. CON group, PA+BS group vs. CON+BS group, P < 0.01). Both JPQHF and rapamycin suppressed PPARγ expression (PA+JS group vs. PA+BS group, PA+RA+BS group vs. PA+BS group, P < 0.01). Palmitic acid also suppressed the protein level of myogenin (PA group vs. CON group, PA+BS group vs. CON +BS group, P < 0.01), while JPQHF enhanced myogenin level (PA+JS group vs. PA+BS group, P < 0.01), but rapamycin did not (PA+RA+BS group vs. PA+BS group, P < 0.01).

Palmitic acid suppressed the total PI3K and total AKT levels and decreased the pAKT/AKT ratio under insulin stimulation (PA group vs. CON group, PA+BS group vs. CON +BS group, P < 0.01) (fig. 7a, c), and the protein expression of pmTOR and pmTOR/mTOR ratio were also inhibited in the palmitic acid treatment group (PA group vs. CON group, PA+BS group vs. CON +BS group, P < 0.01). The total PI3K and total AKT protein levels and pAKT/AKT ratio were higher in the JPQHF group than in the palmitic acid treatment group (PA+JS group vs. PA+BS group, P < 0.01), and the protein expression of pmTOR and

pmTOR/mTOR ratio were also elevated in the JPQHF group (PA+JS group vs. PA+BS group, P < 0.01). As mTORC1 is downstream of PI3K/AKT, in the JPQHF group, the protein levels of pmTOR and the pmTOR/mTOR ratio were directly proportional to insulin signalling. These findings suggest that under insulin conditions, mTOR is mainly stimulated by pAKT. Furthermore, as JPQHF elevated the PI3K/AKT protein ratio, pmTOR was also elevated.

Discussion

IMCLs are an important factor of insulin resistance in skeletal muscle. Triglycerides are a source of lipid intermediate metabolites (such as fatty acids, diglycerides, and lipoacyl-CoA, ceramides). Fatty acids inhibit intracellular glucose transport[14] and intracellular glucose utilization[15], thereby reducing glucose utilization. Diglycerides and fatty acyl-CoA participate in PKC pathway activation. Then, the phosphorylation of serine and threonine residues on the insulin receptor influences tyrosine phosphorylation, thus affecting the binding of insulin receptors and substrates and interfering with the insulin signalling pathway[16]. In vitro muscle tube intervention with palmitic acid revealed the close relationship between the accumulation of ceramide and insulin resistance[17]. IMCLs have been shown to be significantly increased in people with insulin resistance[18] and animal models[14], and insulin resistance was reversed when IMCLs were exhausted without the loss of body weight after the intake of a high-fat diet was controlled[19].

The mTOR pathway has received increasing attention in terms of energy metabolism. The mTOR protein is the catalytic subunit of two distinct complexes known as mTOR complex 1 (mTORC1) and mTORC2. The mechanism of mTORC1 activation and proteins downstream of mTORC1 that promote lipid synthesis are not entirely clear. It has been suggested that 4EBP1 inhibits PPARγ translation by influencing nuclear translocation. The activation of mTORC1 promoted PPARγ RNA translation by restraining 4EBP1[20]. mTORC1 phosphorylates its downstream factor S6K1, and the downstream protein eIF promotes sterol responsive element binding protein (SREBP) aggregation in the nucleus, therefore promoting lipid synthesis. Overactivation of mTORC1 in the skeletal muscle of high-fat diet-fed C57 mice was accompanied by decreased skeletal muscle content and increased lipid deposition[21]. mTORC1 inhibits autophagy, and the overactivation of mTORC1 strongly suppressed autophagy, thereby affecting protein renewal and reducing the skeletal muscle content[22]. Overactivated mTORC1 also hyper-phosphorylates Ser307/31/527/616/636 on IRS-1 via the downstream proteinS6K1, resulting in insulin resistance[5]. Moreover, mTORC1 itself phosphorylates the Ser636/639 residues of IRS-1, thereby reducing IRS-1 activity[5].

Therefore, it is thought that inhibiting overactivated mTORC1 would reduce lipid deposition and its negative feedback on the insulin pathway, subsequently alleviating insulin resistance. Studies have attempted to block the mTORC1/S6K1 pathway with low-dose rapamycin to inhibit mTORC1 in diabetic patients, but the long-term effect was not satisfactory. Although the activation of PI3K was continuously stimulated through negative feedback, when the insulin signal was blocked, there was no change in

insulin-mediated glucose uptake[23]. This may be because when rapamycin was used to inhibit mTORC1 for a long time, mTOR protein levels in the blood declined, and then the level of mTORC2 declined[7]. Since mTORC2 directly activates IRS/PI3K/AKT signalling, inhibiting mTORC2 will also reduce the physiological response to insulin.

JPQHF was summarized by the nationally famous Chinese medical doctor Professor Cai Gan who, based on books on traditional Chinese medicine and clinical experience, has used JPQHF to treat patients for more than 10 years with obvious effects. Clinical studies confirmed that JPQHF relieved insulin resistance in patients with pre-diabetes, increased GLP-1 in patients with type 2 diabetes[24]and improved endothelial function in patients with metabolic syndrome[25]. Basic research also verified that JPQHF alleviated insulin resistance in the diabetic rat liver by reducing gluconeogenesis and increasing glycogen synthesis[11].Previous studies have confirmed that JPQHF increased the GLUT4 protein level in skeletal muscle and increased glucose transport, thus reducing insulin resistance in skeletal muscle.

The mechanisms of traditional Chinese medicines are multitargeted because of their complex structures and compositions. To explore other targets of JPQHF in insulin resistance in the skeletal muscle, this study involved both animal experiments and cellular experiments. PIO was selected as a positive control agent for JPQHF to improve systemic glucose tolerance. However, during the process of experimental exploration, PIO was found to be an activator of PPAR with an influence on IMCLs, PPARγ and their relationship with the PI3K/AKT pathway. Rapamycin was used to treat the positive control group in the cellular experiment to investigate the possible effects of JPQHF on mTORC1 and the downstream protein PPAR.

In vivo experiments have confirmed that compared to controls, C57 mice with obesity induced by a high-fat diet had obvious systemic insulin resistance, a significantly increased IMCL content, impairment of the PI3K/AKT insulin signalling pathway in skeletal muscle, reduced levels of the lipid synthesis marker PPAR and myogenin at the mRNA and protein levels and significantly increased mTORC1 activation.

After JPQHF intervention, the insulin resistance of the obese C57 mice was significantly reduced, accompanied by a significantly decreased IMCL content, restoration of the PI3K/AKT pathway and reversal of changes in the expression of mTORC1, PPAR and myogenin. To further verify whether JPQHF is effective by inhibiting the activity of mTORC1, palmitic acid was used to treat the C2C12 cell line, and rapamycin was used as a positive control agent for in vitro experiments. The results confirmed that JPQHF reduced the IMCL content, increased the PI3K/AKT pathway, inhibited mTORC1 and PPAR and increased myogenin. Moreover, a comparative study with rapamycin further demonstrated that the reductions in IMCLs and PPAR were achieved by inhibiting the activity of mTORC1. However, the function of JPQHF in improving the activity of the PI3K/AKT pathway and myogenin may not be completely mediated by mTORC1. Another possible explanation is that rapamycin at the concentration used in this study inhibited both mTORC1 and mTORC1, therefore affecting the activity of PI3K/AKT. In summary, more experiments are needed. The findings also demonstrated that the mechanisms of traditional Chinese medicines, especially Chinese herbal compounds, are multitargeted.

Conclusion

JPQHF reduced insulin resistance in C57 mice with high-fat diet-induced obesity. The mechanisms of this effect include a reduced IMCL content, increased PI3K/AKT insulin signalling pathway activation and the enhanced myogenic differentiation of skeletal muscle. JPQHF inhibited the expression of lipid-generating factors in skeletal muscle by inhibiting overactivated mTORC1 to reduce the IMCL content.

Abbreviations

Intramyocellular lipids:IMCLs;pioglitazone :PIO;extramyocellular lipids:EMCLs;

mammalian target of rapamycin:mTOR;mTOR complex:mTORC;peroxisome proliferator-activated receptor:PPAR;S6 Kinase 1:S6K1;Jianpi Qinghua formula:JPQHF;glucagon like peptide 1:GLP-1;intraperitoneal glucose tolerance test :IPGTT;glucose transporter type 4:GLUT4;intraperitoneal insulin tolerance test:IPITT.

Declarations

Ethics approval and consent to participate

All experimental procedures were conducted according to the guidelines of the Animal Care and Ethics Committee of the Shanghai Traditional Chinese Medicine University (China).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HL and JX were the organizers and leaders of this study.

XH completed the experiment and write the paper together.

QC and YL participated in the completion of this study.

All authors have read and approved the manuscript.

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References

1. Samuel VT, Shulman GI. Mechanisms for insulin resistance: common threads and missing links. CELL. 2012;148(5):852-871.
2. Kelly D, Vega R, Sessions H, et al. MODULATORS OF MYOCYTE LIPID ACCUMULATION AND INSULIN RESISTANCE AND METHODS OF USE THEREOF.
3. Saxton RA, Sabatini DM.mTOR Signaling in Growth, Metabolism, and Disease.CELL.2017;169(2):361-371.
4. Caron A, Richard D, Laplante M.The Roles of mTOR Complexes in Lipid Metabolism. ANNU REV NUTR.2015;35:321-348.
5. Shah OJ, Hunter T.Turnover of the active fraction of IRS1 involves raptor-mTOR- and S6K1-dependent serine phosphorylation in cell culture models of tuberous sclerosis. MOL CELL BIOL.2006;26(17):6425-6434.
6. Tzatsos A.Raptor binds the SAIN (Shc and IRS-1 NPXY binding) domain of insulin receptor substrate-1 (IRS-1) and regulates the phosphorylation of IRS-1 at Ser-636/639 by mTOR. J BIOL CHEM.2009;284(34):22525-22534.
7. Lamming DW, Ye L, Katajisto P et al.Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity.Science(New York,N.Y.) 2012;335(6076):1638-1643.
8. Zhu YH,Zhang XT,Lu H.Clinical observation of small samples of Chinese herbal medicine intervention in residents with low glucose tolerance for two years.Journal of Practical Diabetology,2010;6(02):47-48.
9. Yao Z.Clinical Observation on the treatment of 60 cases of Type 2 Diabetes with dampness-heat syndrome of spleen deficiency by "Jianpi Qinghua Prescription".Shijiazhuang, Hebei, China.2006;567-572.
10. Gong F,Chen QG,Han X,et al.Effects of Jianpi Qinghua Formula on glycolipid metabolism indexes and body mass in patients of type 2 diabetes mellitus with syndrome of deficiency of both qi and yin.Shanghai Journal of Traditional Chinese Medicine.2020;54(S1):55-

11. Qiu Yan, Chen Qingguang, Li Junyan et al.Effects of Jianpi Qinghua Formula on synthesis of hepatic glycogen of type 2 diabetic rats.China Journal of Traditional Chinese Medicine and Pharmacy.2019; 34(02):594-597.
12. Junfei Xu. JPQ downregulates the P38MAPK signal pathway in skeletal muscle of diabetic rats and increases the insulin sensitivity of Skeletal Muscle. IntJ Clin Exp Med.2019;5(12):5130-5137.
13. Gong F,Chen QG,Lu H. Effects and mechanism of Jianpi Qinghua Recipe on body fat of diet induced obesity mice based on the role of IKKε in metabolism. Shanghai Journal of Traditional Chinese Medicine.2017;51(06):79-83.
14. Turner N, Cooney GJ, Kraegen EW et al.Fatty acid metabolism, energy expenditure and insulin resistance in muscle. J ENDOCRINOL. 2014; 220(2):T61-T79.
15. Petersen KF, Dufour S, Befroy D et al.Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. The New England journal of medicine.2004;350(7):664-671.
16. Hulver MW, Dohm GL.The molecular mechanism linking muscle fat accumulation to insulin resistance.The Proceedings of the Nutrition Society.2004;63(2):375-380.
17. Chavez JA, Siddique MM, Wang ST et al.Ceramides and glucosylceramides are independent antagonists of insulin signaling. J BIOL CHEM.2014; 289(2):723-734.
18. Hong BS, Li Y, Lai S et al. Ectopic Fat Deposition on Insulin Sensitivity: Correlation of Hepatocellular Lipid Content and M Value. J DIABETES RES.2016;2016:3684831.
19. Greco AV, Mingrone G, Giancaterini A et al.Insulin resistance in morbid obesity: reversal with intramyocellular fat depletion. DIABETES.2002;51(1):144-151.
20. Carnevalli LS, Masuda K, Frigerio F et al. S6K1 plays a critical role in early adipocyte differentiation. DEV CELL.2010;18(5):763-774.
21. Dungan CM, Li J, Williamson DL.Caloric Restriction Normalizes Obesity-Induced Alterations on Regulators of Skeletal Muscle Growth Signaling. LIPIDS.2016;51(8):905-912.
22. Ge Y, Yoon M, Chen J.Raptor and Rheb negatively regulate skeletal myogenesis through suppression of insulin receptor substrate 1 (IRS1). The Journal of biological chemistry.2011; 286(41):35675-35682.
23. Veilleux A, Houde VP, Bellmann K et al. Chronic inhibition of the mTORC1/S6K1 pathway increases insulin-induced PI3K activity but inhibits Akt2 and glucose transport stimulation in 3T3-L1 adipocytes. Molecular endocrinology (Baltimore, Md.).2010;24(4):766-778.
24. Tao Feng, Zhu Yunhua, Yao Zheng et al.Effect of Jianpi Qinghua prescription on glP-1 expression In diabetic rats.Beijing,China;2008-3.
25. Yao Z, Xu XT, TAO F et al.Clinical study on the improvement of endothelial function in patients with metabolic syndrome by Jianpi Qinghua prescription.Shanghai Journal of Traditional Chinese Medicine.2009;43(01):27-29

Figures

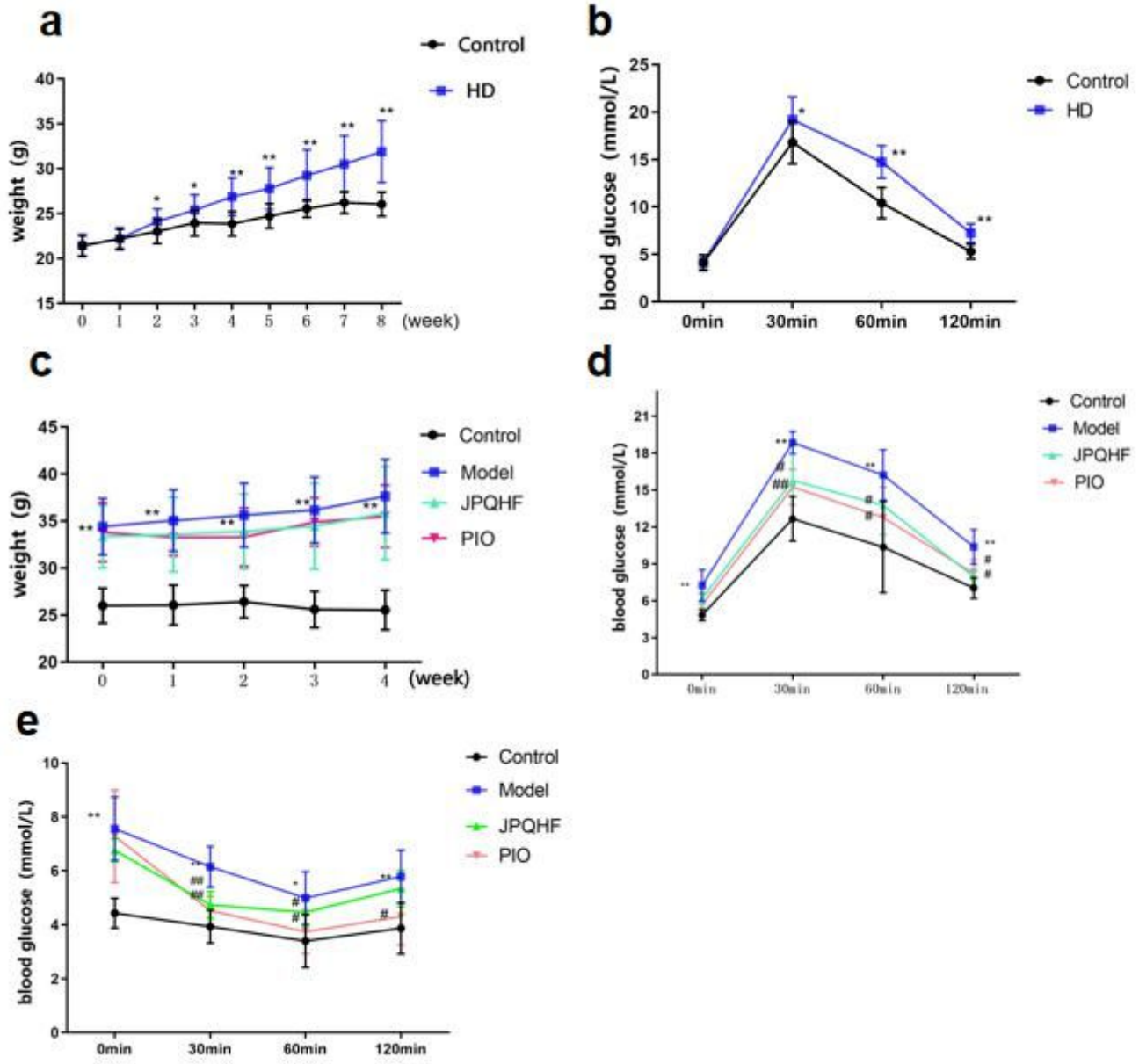

Figure 1

Effects of JPQHF on body weight and glucose tolerance in obese mice. (a) weight of mice during 8 weeks of molding.n=10 in control group and n=45 in HD group. (b) result of IPGTT after molding. n=10 in control group and HD group.(c) weight of mice during 4 weeks of treatment.n=10 in each group.(d) result of IPGTT after treatment. n=10 in each group.(e) result of IPITT after treatment. n=10 in each group.* P<0.05 compared with control group,** P<0.01 compared with control group,# P<0.05 compared with model group,##P<0.01 compared with model group

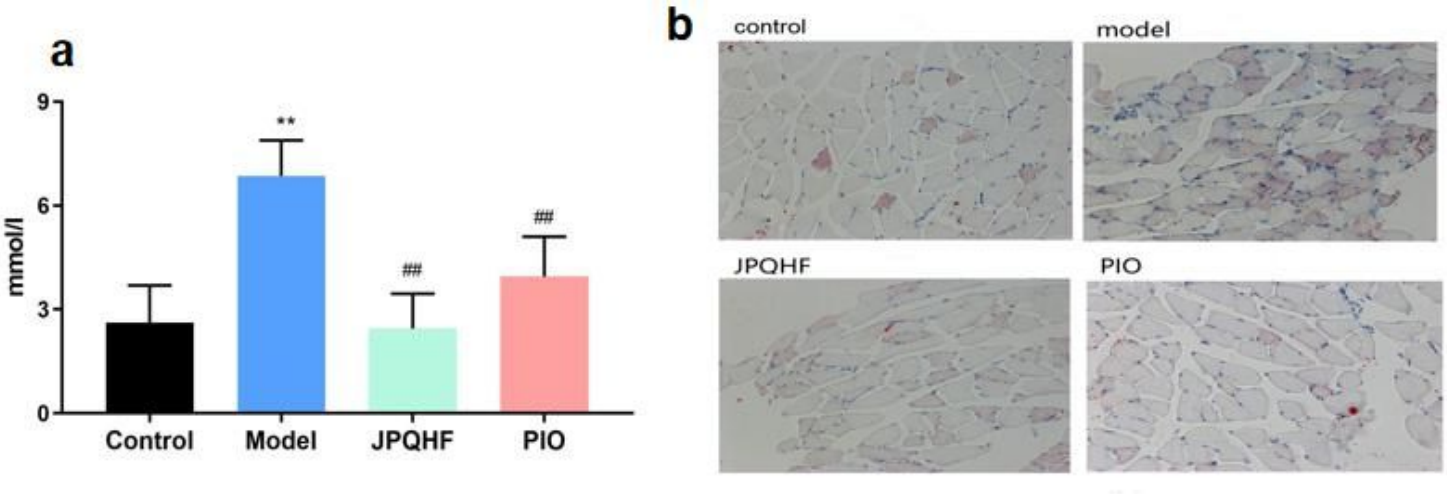

Figure 2

Effect of JPQHF on IMCL. (a)level of triglyceride in gastrocnemius of mice after treatmnet detected by GPO-PAP.　b) lipid deposition in gastrocnemius cells after treatment detected by Oil red O staining. (×200)** P<0.01 compared with control group,##P<0.01 compared with model group

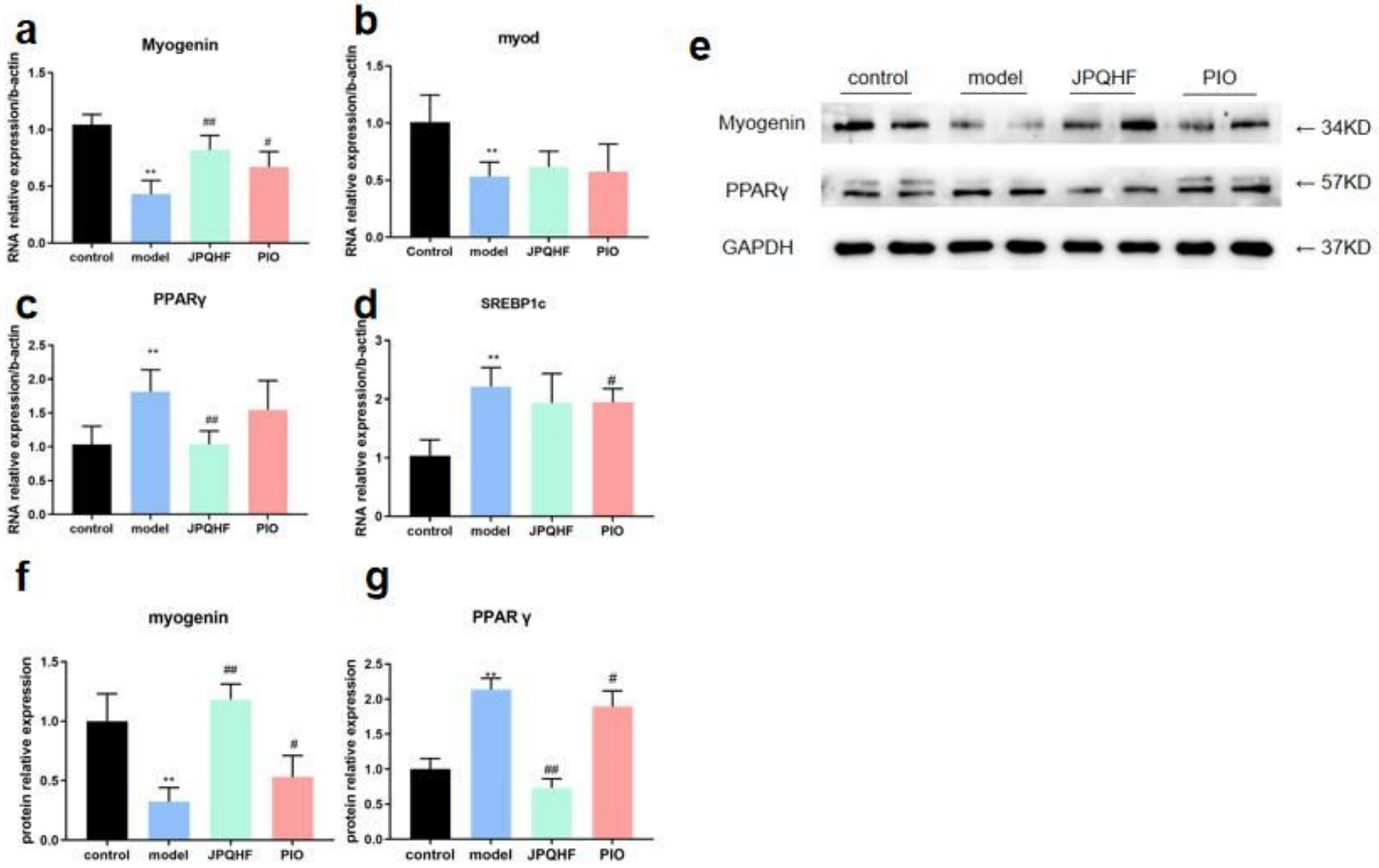

Figure 3

Relative expression levels of RNA and proteins of adipogenesis and myogenic differentiation markers in c57 mice gastrocnemius. (a)RNA relative expression level of myogenin. (b)RNA relative expression level of myod. (c)RNA relative expression level of PPARγ.(d)RNA relative expression level of SREPB1c. (e)Myogenin and PPARγ protein deteceted by Western blot.(f)Protein relative expression level of myogenin.(g)Protein relative expression level of PPARγ.** P<0.01 compared with control group,# P<0.05 compared with model group,##P<0.01 compared with model group.

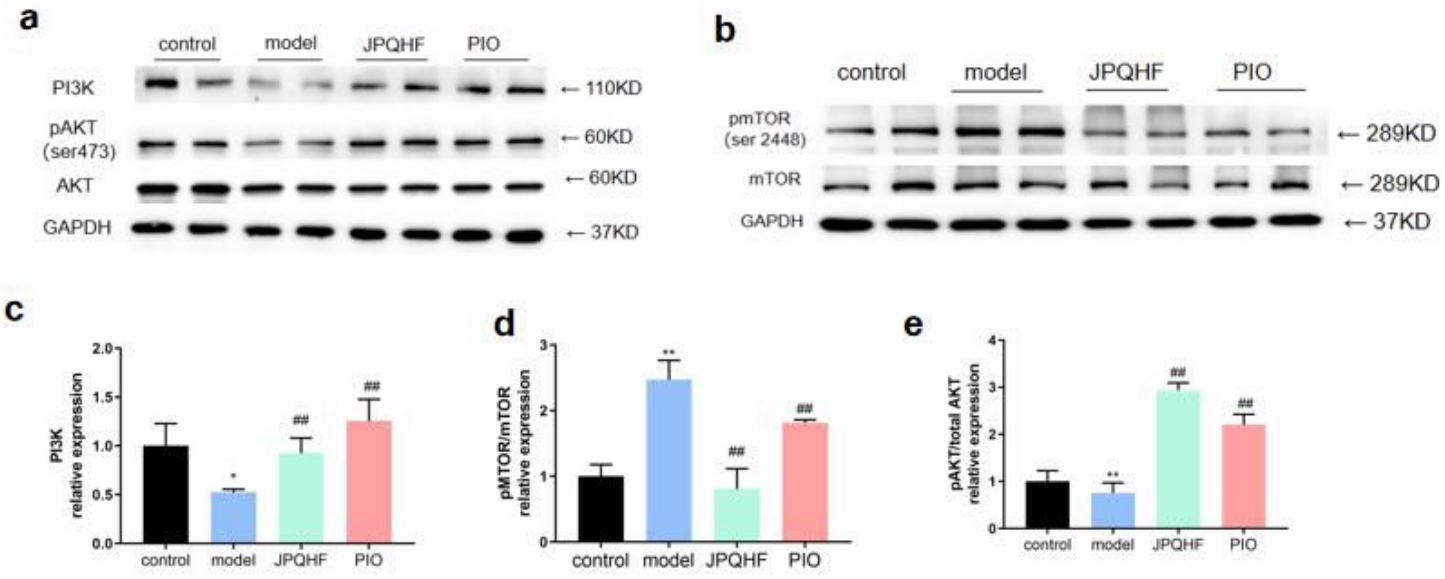

Figure 4

Protein expression of PI3K/AKT signal pathway and mTOR in c57 mice gastrocnemius .(a)Protein expression of total PI3K,pAKT and total AKT deteceted by Western blot .(b)Protein expression of pmTOR and total mTOR deteceted by Western blot .(c)Relative protein level of PI3K compared with GAPDH. (d)Relative protein level of pmTOR compared with total mTOR.(e)Relative protein level of pAKT compared with total AKT.* P<0.05 compared with control group,** P<0.01 compared with control group,##P<0.01 compared with model group.

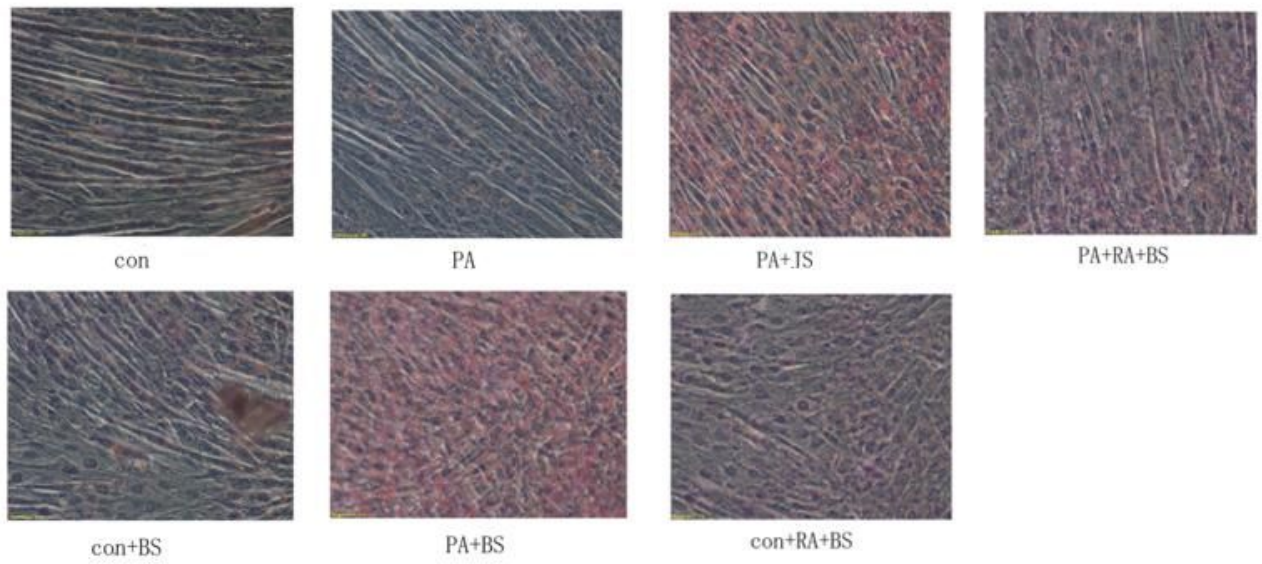

Figure 5

Effect of JPQHF on c2c12 cells.Lipid deposition in c2c12 cells after treatment detected by Oil red O staining(×200).

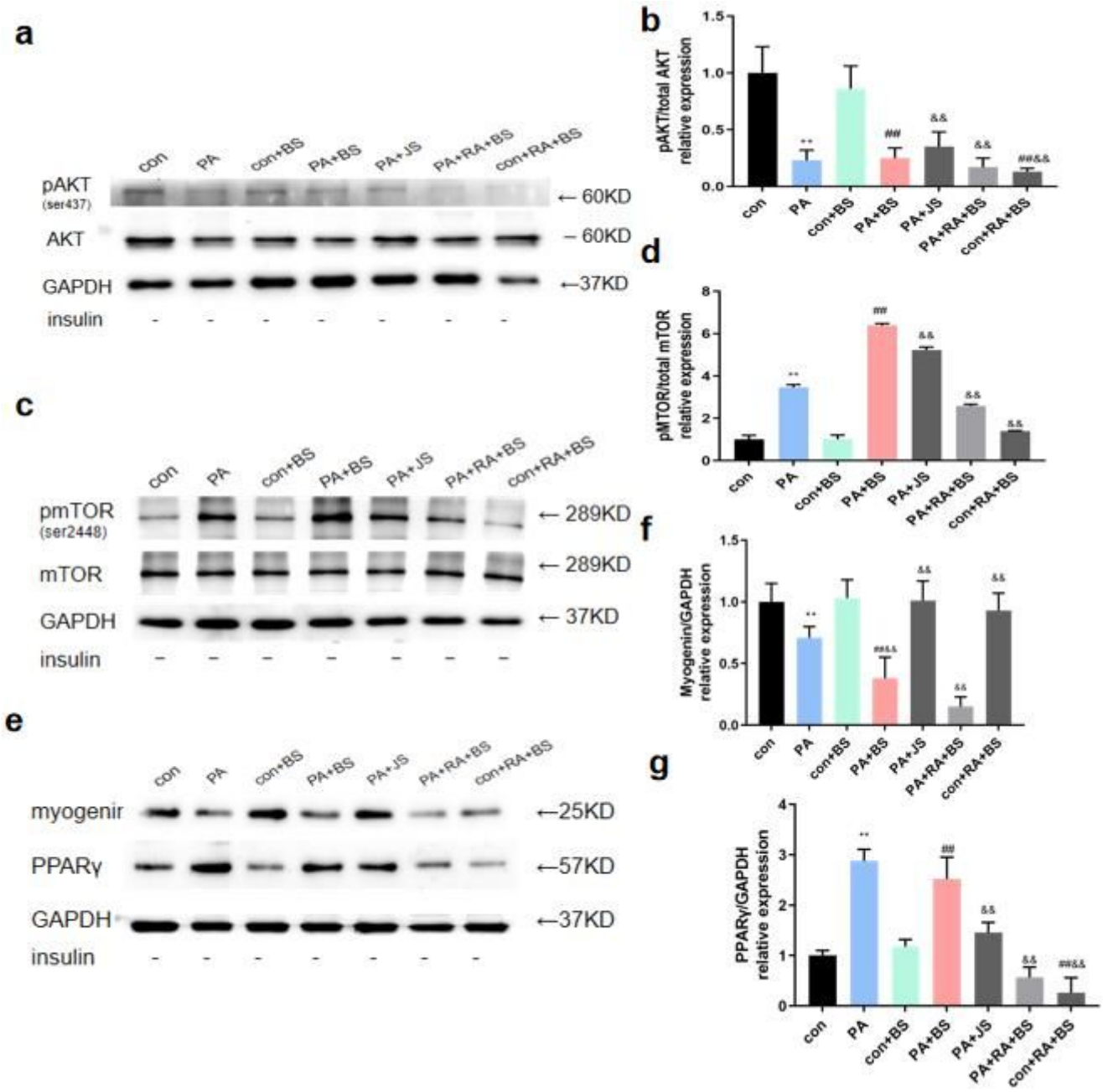

Figure 6

Protein expression in c2c12 cells when insulin is absent .(a)Protein expression of pAKT and total AKT deteceted by Western blot and relative expression level of pAKT/AKT(b).(c)Protein expression of pmTOR and total mTOR deteceted by Western blot and relative expression level of pmTOR/mTOR(d) .(e)Protein expression of myogenin and PPARγ deteceted by Western blot and relative expression level of myogenin and PPARγ.**P<0.01 compared with CON group,##P<0.01 compared with CON+BS group,&&P<0.01 compared with PA+BS group

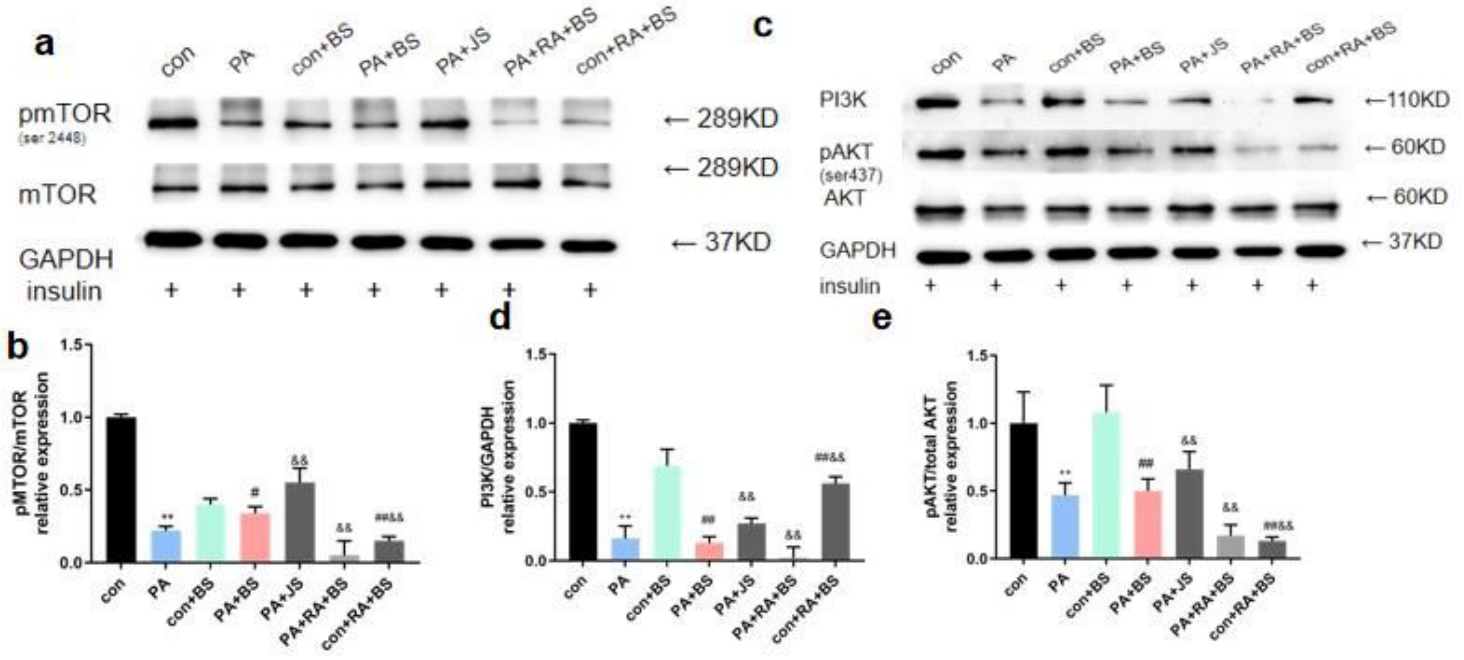

Figure 7

Protein expression in c2c12 cells when insulin is added into culture medium for 20min before collected. (a)Protein expression of pmTOR and total mTOR deteceted by Western blot and relative expression level of pmTOR/mTOR(b) .(c)Protein expression of PI3K,pAKT and AKT deteceted by Western blot and relative expression level of PI3K and pAKT/AKT(d).**P<0.01 compared with CON group,##P<0.01 compared with CON+BS group,&&P<0.01 compared with PA+BS group.

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