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A natural compound jaceosidin ameliorates endoplasmic reticulum stress and insulin resistance via upregulation of SERCA2b

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ABSTRACT

Increased endoplasmic reticulum (ER) stress has emerged as a vital contributor to dysregulated glucose homeostasis, and impaired function of sarco-endoplasmic reticulum Ca^{2+}-ATPase 2b (SERCA2b) is one of the central mechanisms underlying ER stress. In this study, we reported that SERCA2b upregulation contributed to the amelioration of ER stress and insulin resistance by a small natural compound jaceosidin. In a model of differentiated C2C12 myotubes, jaceosidin-triggered SERCA2b upregulation enhanced insulin sensitivity and decreased ER stress. Moreover, the activity of Ca^{2+}-ATPase in thapsigargin-treated myotubes was also augmented by jaceosidin. Furthermore, jaceosidin significantly suppressed blood glucose levels, improved glucose tolerance and lowered body weight, but did not alter food intake in insulin-resistant obese mice. In addition, this compound markedly reduced lipid accumulation, suppressed the expression of lipogenic genes in liver and ameliorated liver injury. The ameliorative effects of jaceosidin were due to its ability to reduce ER stress via increasing the expression of SERCA2b in the muscles of obese mice. Taken together, jaceosidin could improve ER stress and attenuate insulin resistance via SERCA2b upregulation in mice skeletal muscles.

1. Introduction

The World Health Organization estimated that 171 million people worldwide had type II diabetes in 2000, and this number is projected to increase to 366 million by 2030 as a result of an uncontrolled increase in the incidence of obesity [1]. In recent years, more and more reports have concerned the relationship among endoplasmic reticulum (ER) stress, obesity and insulin resistance [1–3]. Obesity is consistent with the development of ER stress and the activation of unfolded protein response (UPR) in the liver, adipose tissue and brains of mice [3,4], which in turn contributes to the development of insulin resistance, type II diabetes, leptin resistance, and cardiovascular disease [1,5].

Sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is a Ca^{2+}-transport ATPase whose well-known function is reuptake of Ca^{2+} from the cytosol into the lumen of ER [6,7]. Thapsigargin, an inhibitor of SERCA [8], blocks reuptake of Ca^{2+} from the cytoplasm and creates a chaotic environment in the ER lumen, which leads to ER stress and subsequent initiation of the UPR [1,9]. In mammals, three different SERCA genes (*atp2a1-3*) lead to the generation of three different isoforms (SERCA1-3), each of which has at least two further subisoforms [10,11]. Among all the SERCA isoforms, SERCA2b has been most reported in diabetes. For instance, increased expression of SERCA2b greatly reduces ER stress in the liver, increases glucose tolerance, and establishes euglycemia in severely obese and diabetic mice [1,12,13]. Restoration of SERCA2b using gene therapy in the pancreas of diabetic patients could produce a beneficial effect [12]. However, drug candidates or chemicals that ameliorate insulin resistance via lightening ER stress or enhancing SERCA2b activity are still lacking.

Jaceosidin, a flavone isolated from the herb of *Artemisia vestita* wall, has been reported to have many biological activities [14–19]. Most importantly, it shows a potent hypoglycemic effect [20,21]. However, the mechanism underlying its regulation of blood glucose remains unclear. In the present study, we demonstrated for the first time that jaceosidin increases mRNA and protein levels

Abbreviations: ER, endoplasmic reticulum; SERCA2b, sarco-endoplasmic reticulum Ca^{2+}-ATPase 2b; IR-β, insulin receptor-β; IRS-1, insulin receptor substrate 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CHOP, C/EBP homologous protein; eIF, eukaryotic translation initiation factor; GRP, glucose-regulated protein; HFD, high fat diet; NCD, normal chow diet.

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of SERCA2b both in myotubes and in obese mice, which reduces ER stress, increases glucose tolerance and enhances insulin sensitivity.

2. Materials and methods

2.1. Materials

Jaceosidin was purchased from Shanghai Aobo Biotechnology Company (Shanghai, China). Anti-phospho-Akt^{Ser473}, anti-phospho-Akt^{Thr308}, anti-Akt, anti-phospho-eIF2α^{Ser51}, anti-SERCA2, anti-phospho-IR-β$^{Tyr1150/1151}$, anti-phospho-IRS-1^{Y986}, anti-PPAR antibodies and peroxide reagent were from Cell Signaling Technology (Beverly, MA, USA). Anti-GAPDH, anti-CHOP, anti-α-Tubulin, anti-phospho-PPAR-γ^{Ser112}, anti-GRP78, goat anti-mouse IgG, goat anti-rabbit IgG, donkey anti-goat IgG HRP conjugated antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Insulin and pioglitazone were from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin, fetal bovine serum (FBS) and horse serum (HS) were purchased from Invitrogen (Carlsbad, CA, USA). D-glucose was purchased from Sunshine Biotechnology (Nanjing, China). All other chemicals were purchased from Sigma Aldrich Corporation (St. Louis, MO, USA). For *in vitro* experiments, jaceosidin and pioglitazone were dissolved in DMSO to a concentration of 10 mM (stock solution); for *in vivo* experiments, jaceosidin and pioglitazone were dissolved in DMSO to a concentration of 100 mg/ml (stock solution), and stored at −20 °C.

2.2. Cell culture and cell differentiation

Murine myoblast cell line C2C12 was purchased from the Shanghai Institute of Cell Biology (Shanghai, China), maintained in DMEM, supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 in the air. For cell differentiation, C2C12 cells were allowed to grow in six-well plates to 50% confluence. Then cells were maintained in growth medium (DMEM supplemented with 10% FBS), and change medium every twenty-four hours until the cells were 100% confluent. Then myoblasts were induced differentiation into myotubes with differentiation medium (DM, DMEM supplemented with 2% horse serum) or differentiation medium with insulin (DMI, differentiation medium with 100 nM insulin). The mediums were changed every twelve hours. Forty-eight hours after differentiation, 0.1% DMSO, 1 μM and 10 μM jaceosidin and 10 μM pioglitazone were added to the myotubes, then the myotubes were differentiated for another twenty-four hours. Finally, the myotubes were stimulated with 100 nM insulin for ten minutes before collected for the following experiments [22,23].

2.3. Animals

Adult (age, 6 weeks) male C57BL6/J mice were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). The mice were fed with free access to high fat diet (HFD, 60 kcal% Fat) or normal chow diet (NCD, 10 kcal% fat) in plastic cages at 21 ± 2 °C and kept on a twelve-hour light-dark cycle. Body weight was measured every week. After fed for sixteen weeks, the mice were distributed into groups (n = 6) and were intraperitoneally injected with vehicle (PBS, NCD and HFD control), 2 mg/kg jaceosidin, 4 mg/kg jaceosidin or 4 mg/kg pioglitazone for two weeks, respectively.

Morbidly obese (ob/ob) male mice were purchased from the Model Animal Genetics Research Center of Nanjing University (Nanjing, China). The mice were distributed into two groups (n = 6) and treated with 4 mg/kg jaceosidin or vehicle control for 2 weeks.

Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of China, 2006) and the related ethical regulations of our university. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

2.4. Glucose tolerance test (GTT) and insulin tolerance test (ITT)

GTT and ITT were performed as described [2] with some modifications. Briefly, for GTT, mice were fasted overnight (7 p.m.−9 a.m.) and then injected with D-glucose (2 g/kg) intraperitoneally. Blood glucose levels were measured 0, 30, 60, 90 and 120 min after glucose administration. For ITT, mice were fasted for six hours (8 a.m.−2 p.m.) and injected intraperitoneally with insulin (1 IU/kg). Blood glucose levels were measured 0, 30, 60, 90 and 120 min after insulin administration.

2.5. Biochemical analyses

Glucose level in whole blood from tail vein was measured using a glucose meter (LifeScan, Inc., Milpitas, CA, USA). Plasma insulin level was assayed using a Mouse High Range Insulin ELISA Kit (American Laboratory Products Company, Windham, USA). Plasma activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using commercial kits (Bio-Clinical System, Gyeonggi-do, South Korea). The concentrations of triglyceride (TG) and cholesterol were measured with enzymatic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacture's protocols.

2.6. Reverse transcription and quantitative real-time polymerase chain reaction (Q-PCR)

Total RNA was extracted from cells or animal tissues using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The reverse transcription with 1 μg of total RNA was conducted under the following conditions: 42 °C for 20 min, and 99 °C for 5 min. Gene expression was then analyzed with SsoFast™ EvaGreen® Supermix (Bio-Rad) on CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) and normalized with 18S ribosome RNA (*rn18s*) level. The primer sequences were as follows: *rn18 s* forward, AGTCCCTGCCCTTTGTACACA, *rn18 s* reverse, CGATCCGAGGGCCTCACTA; *hspa5* forward, ACTTGGGGACCACCTATTCCT, *hspa5* reverse, ATCGCCAATCAGACGCTCC; *dnajb9* forward, CTCCACAGTCAGTTTTCGTCTT, *dnajb9* reverse, GGCCTTTTTGATTTGTCGCTC; *pdia3* forward, CGCCTCCGATGTGTTGGAA, *pdia3* reverse, CAGTGCAATCCACCTTTGCTAA; *serca2b* forward, TGGAACAACCCGGTAAAGAGT, *serca2b* reverse, CACCAGGGGCATAATGAGCAG; *fasn* forward, GGAGGTGGTGATAGCCGGTAT, *fasn* reverse, TGGGTAATCCATAGAGCCCAG; *scd1* forward, TTCTTGCGATACACTCTGGTGC, *scd1* reverse, CGGGATTGAATGTTCTTGTCGT; *acc1* forward, ATTGGGCACCCCAGAGCTA, *acc1* reverse, CCCGCTCCTTCAACTTGCT; *srebp1c* forward, GCGGTTGGCACAGAGCTT, *srebp1c* reverse, GGACTTGCTCCTGCCATCAG.

2.7. Western blot

Total protein extraction from tissues and cells were performed as described [1]. Western blotting was performed as published [2] with some modifications. In brief, proteins were resolved by SDS-PAGE and electrotransferred proteins onto polyvinylidene fluoride membranes. Then, the membranes were blocked in 5% bovine serum albumin (BSA) at room temperature for 1 h and incubated with primary antibody at 4 °C overnight, followed by incubation with secondary antibody at room temperature for 1 h. After extensive washing, the membrane was developed using a chemiluminescence assay system (Beverly, MA, USA) and exposed

to films (Kodak) for appropriate time periods. The densitometry of immunoblots was quantified with Image J software (NIH).

2.8. Analysis of in vivo insulin receptor signaling

The *in vivo* insulin receptor signaling was analyzed as published [2] with minor modifications. The mice were fasted for six hours (9 a.m.−3 p.m.) before experiments. After anesthetization of mice with pentobarbital sodium, insulin (0.25 IU/kg) or PBS were injected to mice through the portal vein. Five minutes after, livers or muscles from right leg were excised, flash frozen in liquid nitrogen and stored in −80 °C until processing.

2.9. Statistical analysis

Data are expressed as means ± SD of three independent experiments and each experiment includes triplicate sets *in vitro* and of six animals of per group *in vivo*. Statistically evaluated by Student's *t*-test was performed when only two value sets were compared, and one-way ANOVA followed by Dunnett's test when the data involved three or more groups. $P < 0.05$ was considered to be significant.

3. Results

3.1. Insulin sensitivity is rescued by jaceosidin in insulin-resistant myotubes in C2C12 cells

To conveniently examine if insulin resistance in myotubes is indeed associated with ER stress, which may be regulated by the small compound jaceosidin, we established an *in vitro* model with C2C12 cells as previously described [24]. The molecular structure of jaceosidin is shown in Fig. 1A. As shown in Fig. 1B, undifferentiated C2C12 myoblast cells grew independently and in a dispersed fashion. When differentiation medium was changed for three days, the cells began to merge and to create myotubes. After being given a low level of insulin (100 nM) stimulation, more intensive myotubes appeared in the cells. Myotubes, which were fused by many muscle cells together, were lined with clusters of nuclei inside in immunofluorescent photos (Fig. 1C).

Activation of insulin receptor signaling by single insulin stimulation was observed in C2C12 myotubes and characterized with the phosphorylation of insulin receptor (IR)-β, insulin receptor substrate 1 (IRS-1) and Akt (Fig. 1D). However, insulin-resistant myotubes showed an impaired ability to phosphorylate these proteins. In this model, we observed that jaceosidin dramatically increased the phosphorylation of IR-β, IRS-1 and Akt when stimulated by 100 nM insulin for ten minutes in insulin-resistant myotubes (Fig. 1D–E). As a drug with hypoglycemic action to treat diabetes, pioglitazone was used as a positive control. It also increased the phosphorylation level of Akt but showed no notable effects on phosphorylated IR-β or IRS-1 (Fig. 1D–E).

3.2. SERCA2b is upregulated and ER stress is reduced by jaceosidin in insulin-resistant myotubes

We then investigated whether the attenuation of insulin resistance by jaceosidin was related with the expression and function of SERCA2b in myotubes. The insulin-resistant myotubes (2% HS + 100 nM insulin) showed a depressed Ca^{2+}-ATPase activity and reduced SERCA2b protein (Fig. 2A–C). Jaceosidin could

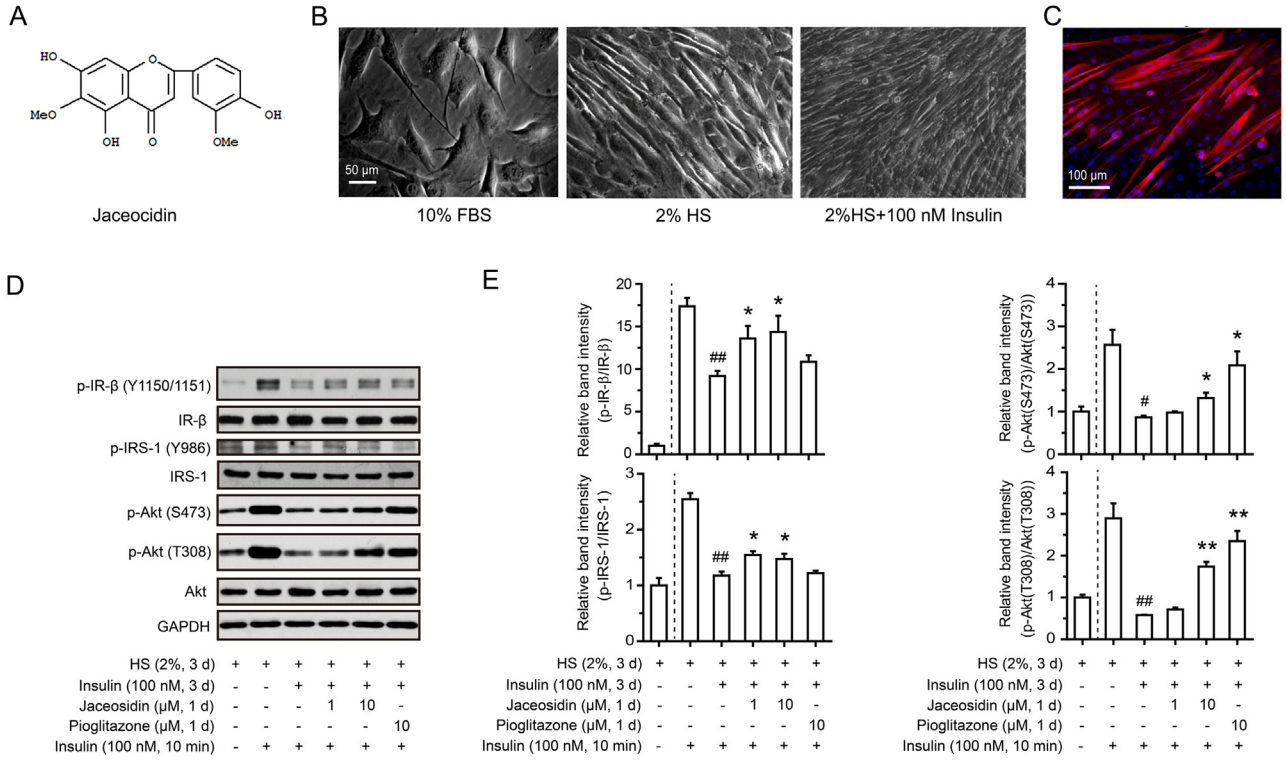

Fig. 1. Insulin sensitivity is ameliorated by jaceosidin in insulin-resistant myotubes in C2C12 cells.
Mouse myoblast C2C12 cells were differentiated either in DM (differentiation medium, 2% horse serum (HF)) or in DMI (DM + 100 nM insulin) for 2 days. Then, the cells were treated with 1 μM jaceosidin, 10 μM jaceosidin or 10 μM pioglitazone for another day. Finally, the cells were stimulated with 100 nM insulin for 10 min and collected for measurement. (A) Chemical structures of jaceosidin. (B) Representative images of undifferentiated C2C12 cells (top panel, 10% FBS), differentiated C2C12 myotubes (middle panel, 2% HS) and insulin-resistant myotubes (basal panel, 2% HS + 100 nM insulin). (C) Cytoskeleton and nucleus in proliferated and differentiated cells by immunofluorescence microscopy. (D) Phospho-IR-β, phospho-IRS-1, phospho-Akt^{Ser473} and phospho-Akt^{Thr308} were determined by western blot. (E) Data summary of (D) are expressed as a histogram of mean ± SD of three independent experiments. #$P < 0.05$, ##$P < 0.01$ *vs.* DM + insulin challenged; *$P < 0.05$, **$P < 0.01$ *vs.* DM I+ insulin challenged.

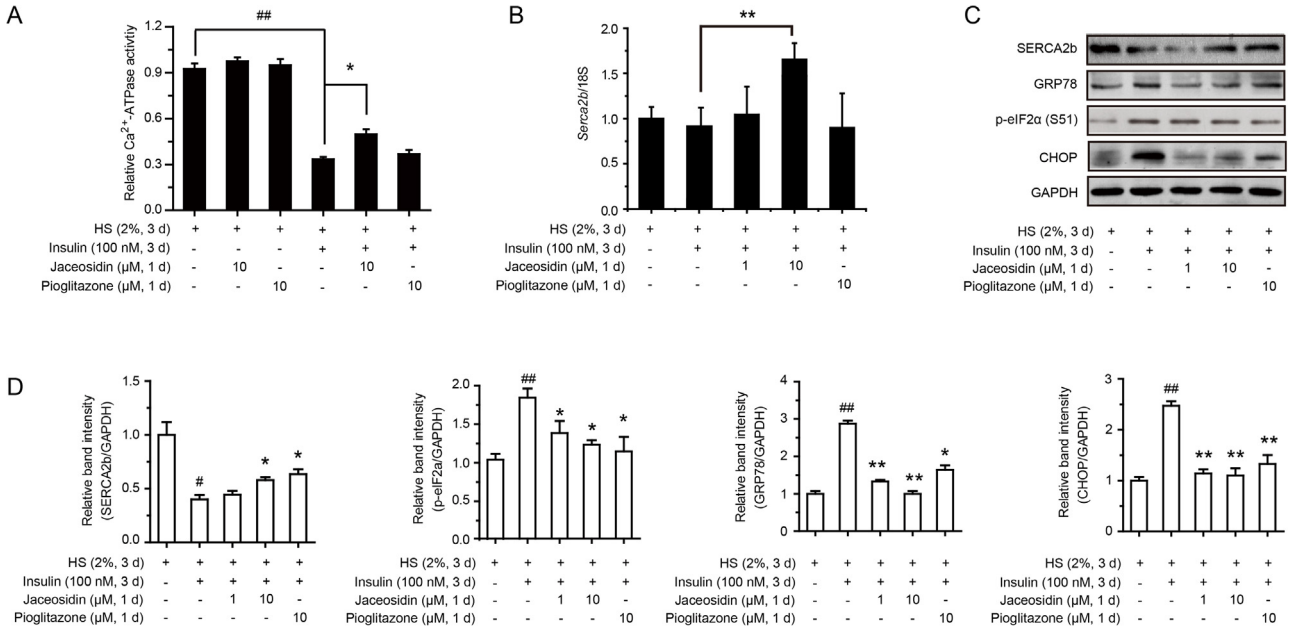

Fig. 2. Endoplasmic reticulum stress in insulin-resistant myotubes is inhibited by jaceosidin in C2C12 cells.
(A) Relative Ca^{2+}-ATPase activity in C2C12 cells was determined. (B) Transcription of SERCA2b in C2C12 cells was determined by real-time PCR and normalized with *rn18S*. (C) The protein level of SERCA2b, GRP778, CHOP and phosphorylated eIF2α were determined by western blot. (D) Data summary of (C) are expressed as a histogram of mean ± SD of three independent experiments. Error bars are the means ± SD of the three independent experiments. #$P < 0.05$, ##$P < 0.01$ *vs.* DM + insulin challenged; * $P < 0.05$, ** $P < 0.01$ *vs.* DM I+ insulin challenged.

significantly increase the mRNA transcription and protein expression of SERCA2b, as well as enhanced its Ca^{2+}-ATPase activity (Fig. 2A–C). As downstream of SERCA2b dysfunction, CHOP, GRP78 and phosphorylated eIF2α were all expressed highly in a state of ER stress [25,26]. It was found that jaceosidin could markedly reduce these signals (Fig. 2C–D).

If jaceosidin could increase the protein levels of SERCA2, this compound would increase the resistance of C2C12 myotubes to ER stress. Therefore, C2C12 myotubes were incubated with 100 nM thapsigargin, the SERCA inhibitor, for 6 h, and then treated with various concentrations of jaceosidin for another 18 h. As expected, expression of CHOP and phosphorylation of eIF2α increased obviously in thapsigargin-treated myotubes, whereas 10 μM of jaceosidin almost abolished these effects (Fig. 3A). We further observed that the activity of Ca^{2+}-ATPase was significantly reduced in thapsigargin-treated myotubes ($P < 0.01$), and 10 μM of jaceosidin markedly increased the activity of Ca^{2+}-ATPase (Fig. 3C). In comparison, thapsigargin-treated myotubes were incubated with different concentrations of pioglitazone for 18 h. Interestingly, pioglitazone did not alter the state of ER stress and the activity of Ca^{2+}-ATPase (Fig. 3B and D), which instead brought out the unique effect of jaceosidin.

3.3. Hyperglycemia and hyperinsulinemia in obese mice is significantly improved by jaceosidin

To examine the effects of jaceosidin in insulin-resistant mice, we chose the high fat diet (HFD) model that mostly complies with the clinical pathogenesis of type II diabetes. In addition, since males are more sensitive than females to the high-fat diet [27], male C57 mice, 6 weeks old, were divided into a normal control group (normal chow diet, NCD) and a diabetes model group (HFD). The body weight and food intake of mice were recorded. The other conditions of the two groups of mice were consistent. After 16 weeks, the HFD-treated mice demonstrated significantly increased body weight and were much more obese than the normal control group. Then, the obese mice were distributed into groups and were intraperitoneally injected with a vehicle, 2 mg/kg jaceosidin, 4 mg/kg jaceosidin or 4 mg/kg pioglitazone for two weeks. The day the mice were given drug treatment was marked as Day 0. After two weeks, jaceosidin markedly suppressed the average body weight of the mice fed a HFD ($P < 0.01$), whereas pioglitazone seldom affected the body weight (Fig. 4A–B). It should be noted that the suppressive effect of jaceosidin on body weight was not due to a potential toxicity because this compound did not affect the food intake of mice (Fig. 4C). On the 14th day of drug administration, we measured blood triglycerides and cholesterol and observed significantly higher levels of triglycerides and cholesterol in obese mice (Fig. 4D–E). Moreover, unlike pioglitazone, jaceosidin significantly reduced the blood triglycerides and cholesterol in HFD-treated mice ($P < 0.01$, Fig. 4D–E).

Glucose tolerance and insulin resistance are two important indicators in the measurement of insulin sensitivity. Therefore, the mice were given GTT and ITT, and the area under the curve (AUC) was calculated. It was found that the glucose sensitivity of the HFD group was significantly decreased because the body could not metabolize high doses of glucose promptly to maintain glucose homeostasis (Fig. 4F). At the same time, the body's sensitivity to insulin was also abnormal because the insulin injected in HFD-treated mice did not have an effective hypoglycemic function (Fig. 4G), which indicated severe insulin resistance. However, compared with pioglitazone, jaceosidin (4 mg/kg) significantly improved glucose tolerance and insulin sensitivity in obese mice ($P < 0.05$, Fig. 4F–G). Moreover, basal blood glucose concentrations (Fig. 4H) and serum insulin concentrations (Fig. 4I) were also reduced significantly in obese mice after administration of 2 mg/kg or 4 mg/kg jaceosidin ($P < 0.05$).

To determine whether the effects of jaceosidin could be seen in another model of obesity, we treated ob/ob mice with 4 mg/kg of jaceosidin or vehicle control. After 2 weeks of treatment, jaceosidin produced marked improvements in body weight (Fig. 4J), glucose tolerance (Fig. 4K) and insulin sensitivity (Fig. 4L) in ob/ob mice.

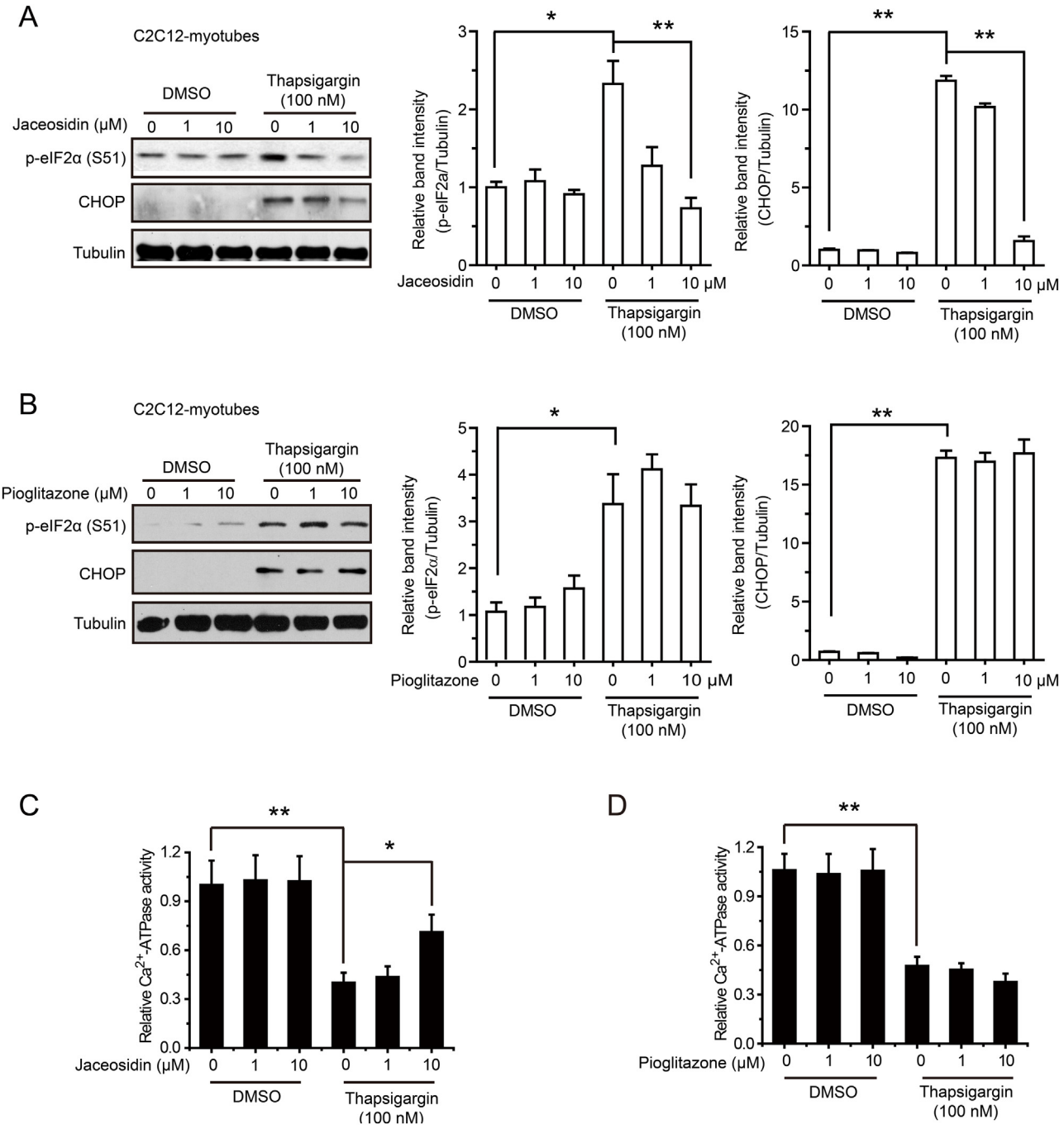

Fig. 3. Thapsigargin-induced endoplasmic reticulum stress is alleviated by jaceosidin in C2C12 cells.
C2C12 cells were differentiated in DM for 2 days, and then incubated with 0.1% DMSO or 100 nM thapsigargin for 6 h, followed by incubation with different concentrations of jaceosidin or pioglitazone for another 18 h. The myotubes were collected. (A) The expression of CHOP and phosphorylation of eIF2α were determined by western blot after jaceosidin treatment. (B) The expression of CHOP and phosphorylation of eIF2α were determined by western blot after pioglitazone treatment. Tubulin was used as a loading control. (C) Relative Ca^{2+}-ATPase activity was determined after jaceosidin treatment. (D) Relative Ca^{2+}-ATPase activity was determined after pioglitazone treatment. * $P < 0.05$, ** $P < 0.01$.

3.4. Fat accumulation and lipogenic gene expression is decreased by jaceosidin in the livers of obese mice

Excessive accumulation of hepatic lipids is closely associated with insulin resistance in humans. The liver mass of the HFD group was heavier than the NCD group, and jaceosidin reduced the increased liver mass in the obese mice (Fig. 5A–B). This reduction was confirmed by biochemical analyses that demonstrated significantly reduced hepatic triglyceride levels in jaceosidin-treated obese mice (Fig. 5C). To assess whether this suppression could be due to liver damage, we measured plasma ALT and AST. It was found that the HFD indeed induced hepatic injury with increased serum transaminases. Compared with the control group, jaceosidin reduced the serum levels of ALT and AST in a dose-dependent manner (Fig. 5E–F). In the following study, we examined H&E-stained sections taken from the liver of obese mice, and noted a marked decrease in a number of lipid droplets in the liver of obese mice administered 4 mg/kg jaceosidin compared with the HFD control group (Fig. 5D). Finally, the expression profile of lipogenic genes showed that mRNA levels for stearoyl-CoA

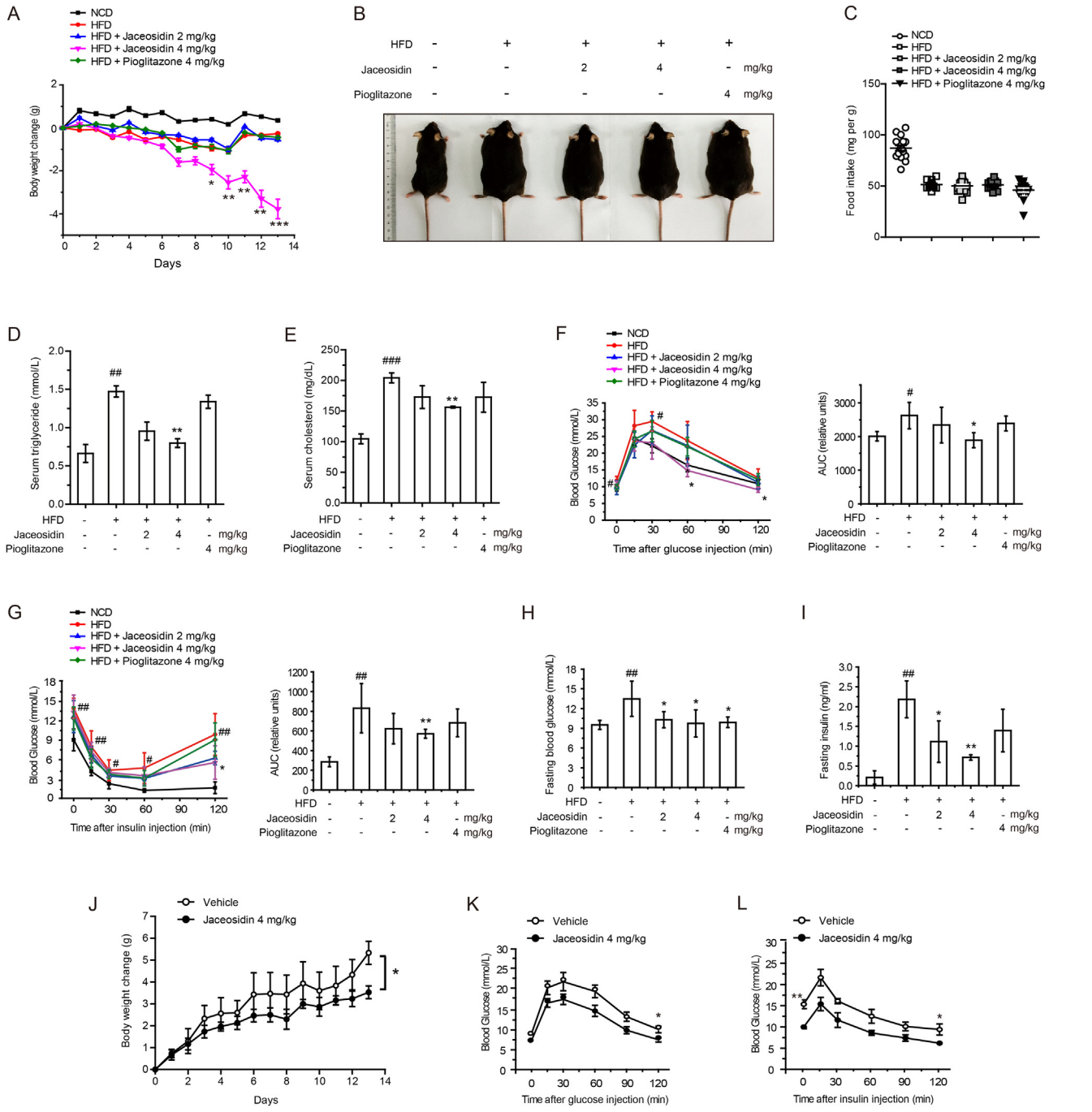

Fig. 4. Jaceosidin improves glucose homeostasis in obese mice.
C57BL6/J mice were either fed a HFD or a normal NCD for 16 weeks. The mice fed a HFD were distributed into 4 groups randomly, and the day was marked as experimental day 0. Then, the mice were injected intraperitoneally with vehicle (0.8% DMSO in PBS, NCD and HFD), 2 mg/kg jaceosidin, 4 mg/kg jaceosidin or 4 mg/kg pioglitazone once a day (n = 6). (A) Body weight change of the mice treated with vehicle (0.8% DMSO in PBS, NCD and HFD), 2 mg/kg jaceosidin, 4 mg/kg jaceosidin or 4 mg/kg pioglitazone (n = 6). (B) One representative photo of six mice in every group was shown. (C) Food intake over 24 h on day 14 of drug treatment. (D-E) Level of serum triglyceride and cholesterol after 14 days of treatment was measured. Error bars are the means ± SD of the six mice. (F) Glucose tolerance test on day 14 (left panel) and area under the curve (AUC, right panel). (G) Insulin tolerance test on day 16 (left panel) and AUC (right panel). (H) Basal blood glucose levels were measured on day 18. (I) On day 20, peripheral blood was collected from the tail vein, and basal insulin levels were measured by ELISA. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, *vs.* NCD; * $P < 0.05$, ** $P < 0.01$ *vs.* HFD. (J) Body weight change of *ob*/*ob* mice treated with 4 mg/kg jaceosidin or vehicle control for 2 weeks. (K-L) Glucose tolerance test and insulin tolerance test of *ob*/*ob* mice treated with 4 mg/kg jaceosidin or vehicle control for 2 weeks. Error bars are the means ± SD of the six mice. * $P < 0.05$, ** $P < 0.01$ *vs.* ob/ob vehicle-treated control.

desaturase-1 (SCD1), fatty acid synthase (FASn), acetyl co-A carboxylase 1 (ACC1) and sterol regulatory element binding protein 1c (SREBP1c) were dramatically reduced (Fig. 5G). Comparably, 4 mg/kg of pioglitazone had no effects on the mRNA level of ACC1 or SCD1 (Fig. 5G).

3.5. Jaceosidin recovered insulin sensitivity via improving ER stress in obese mice

To examine how jaceosidin could enhance insulin sensitivity in obese mice, we injected insulin (0.25 IU/kg) from the portal vein

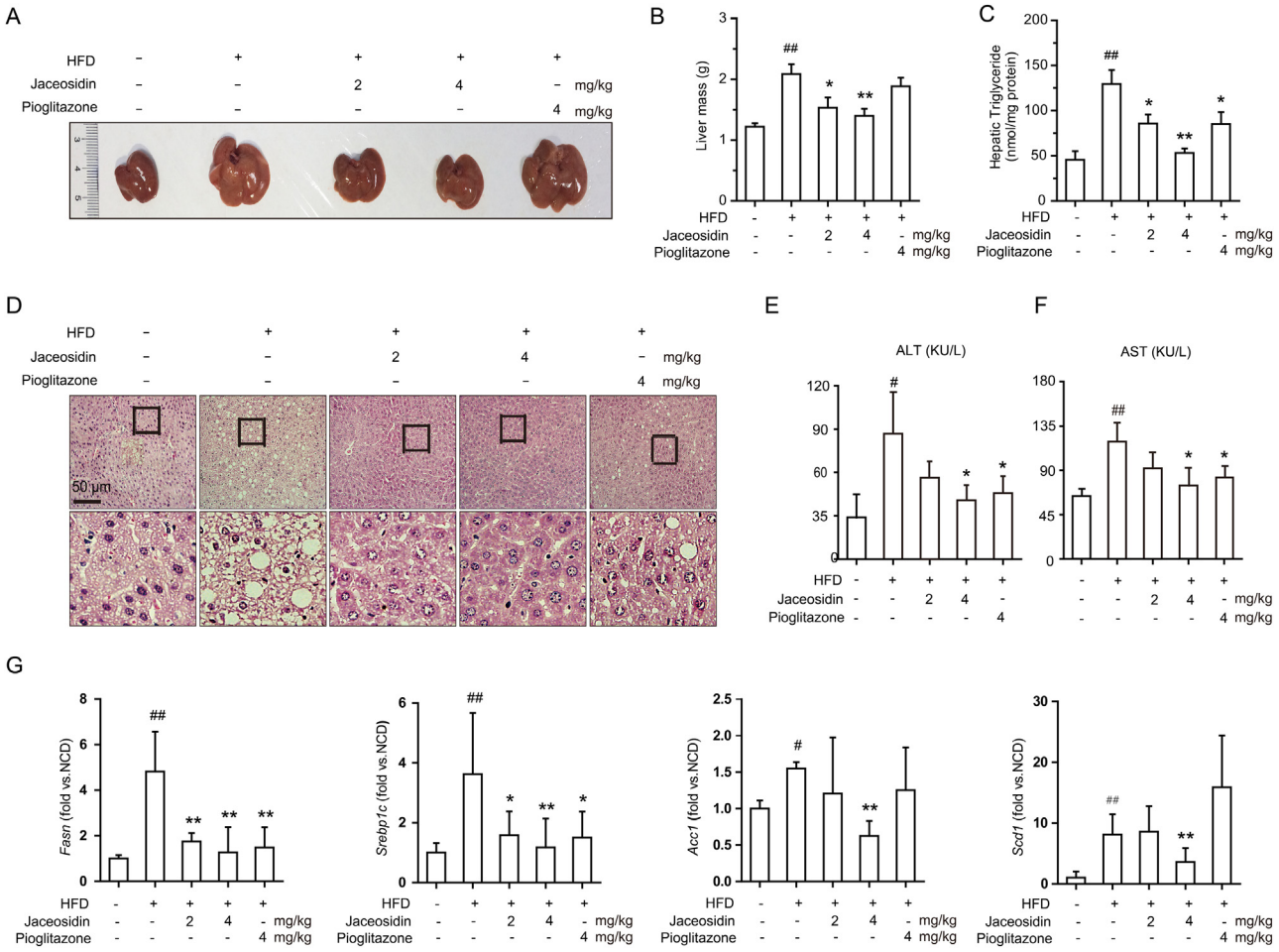

Fig. 5. Fat accumulation and lipogenic gene expression are mitigated by jaceosidin in the livers of obese mice. (A) One photo was acquired randomly of the mouse livers from each group. (B) Liver mass was measured. (C) Level of liver triglyceride was measured. (D) Paraffin sections of the liver were stained with hematoxylin and eosin and one representative photo of the six mice in every group is shown. (E-F) Levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured. (G) Transcription level of FASn, SCD1, SREBP1c and ACC1 in mice livers was determined by real-time PCR and normalized with *rn18S*. Error bars are the means ± SD of the six mice. # $P < 0.05$, ## $P < 0.01$ *vs.* NCD; * $P < 0.05$,** $P < 0.01$, *vs.* HFD.

and detected the phosphorylation changes of protein kinase B (Akt) in mouse muscle from their right legs. Inspiringly, five minutes after insulin injection, phosphorylation of Akt increased significantly in NCD-treated mice ($P < 0.01$), whereas the insulin-stimulated phosphorylation of Akt was notably impaired by HFD treatment (Fig. 6A). However, Akt phosphorylation could be significantly increased ($P < 0.01$) in mice administered 2 mg/kg or 4 mg/kg jaceosidin (Fig. 6A).

Furthermore, the levels of CHOP and phosphorylated eIF2α were significantly reduced by jaceosidin ($P < 0.01$, Fig. 6B). Jaceosidin also increased the transcriptional level of *PDIA3*, *HSPA5* and *DNAJB9*, which were ER chaperones and played important roles in ER folding capacity [2,28,29], whereas pioglitazone showed almost no effect on these genes (Fig. 6C). We also assessed the effect of jaceosidin-mediated SERCA2b activation on ER stress by evaluating the expression and transcription of SERCA2b. We observed that the protein level of SERCA2b in obese mice was significantly up-regulated after administration of 2 mg/kg or 4 mg/kg jaceosidin (Fig. 6D). Likewise, jaceosidin treatment markedly reversed the transcriptional level of SERCA2b (Fig. 6E). These data further validated that SERCA2b activated by jaceosidin played a vital role in the treatment of insulin resistance in metabolic disorders in mice.

4. Discussion

In the present study, we have reported that jaceosidin, a natural compound, significantly increased insulin sensitivity both *in vitro* and *in vivo* via an upregulation of SERCA2b. SERCA2b, the most widespread of all SERCA isoforms, is a housekeeping variant present in the ER of nearly all cell types, including skeletal muscle, smooth muscle and non-muscle tissues [30]. Of all the organs, skeletal muscles are considered the primary sites of insulin-dependent glucose disposal; thus, resistance of the skeletal muscles to insulin-dependent glucose uptake may be an early step in the development of type II diabetes [31–34]. Therefore, drugs or agents that regulate SERCA2b activity and lighten ER stress in skeletal muscle may effectively improve glucose homeostasis.

We demonstrated that jaceosidin administration increased the phosphorylation of insulin receptor β subunits and its downstream IRS-1/Akt signaling, implying an attenuation of insulin resistance in myotubes (insulin 100 nM, 3d) (Fig. 1). SERCA2b expression and Ca^{2+}-ATPase activity in insulin-resistant myotubes showed a significant suppression, which was markedly rescued by both jaceosidin and pioglitazone administration (Fig. 2A and C). Enhancement of SERCA2b by jaceosidin not only re-established ER

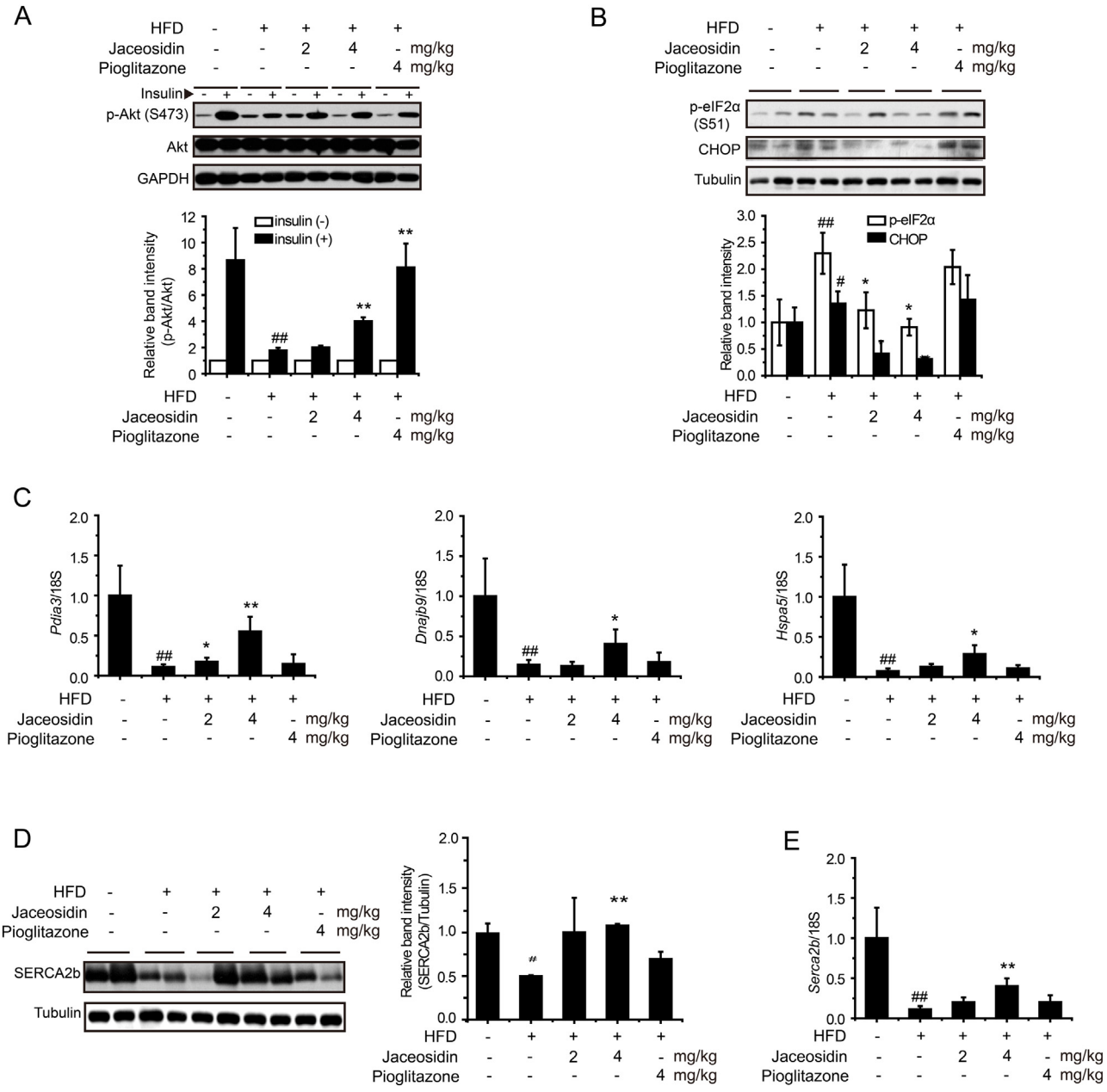

Fig. 6. Endoplasmic reticulum stress is suppressed by jaceosidin in the skeletal muscles of obese mice.
C57BL6/J mice were either fed a HFD or a normal NCD for 16 weeks. Then, the HFD-treated mice were injected intraperitoneally with vehicle, 2 mg/kg jaceosidin, 4 mg/kg jaceosidin or 4 mg/kg pioglitazone once a day continuously for 20 days. On day 20, mice were euthanized, and skeletal muscles were taken for Western Blot or realtime PCR assay. (A) The expression and phosphorylation of Akt in the skeletal muscles was determined (top panel). Relative band intensity was normalized with total Akt (basal panel). To examine the insulin sensitivity in obese mice, half of the animals in each group were injected with insulin (0.25 IU/kg) from the portal vein five minutes before sacrifice. (B) The expression of CHOP and phosphorylation of eIF2α in the skeletal muscles was determined (top panel). The samples were from mice without insulin injection. Each group includes two representative bands. Relative band intensity was normalized with α-tubulin (basal panel). (C) Transcription of PDIA3 (left panel), DNAJB9 (middle panel) and HSPA5 (right panel) were determined by real-time PCR and normalized with *rn18S*. (D) Expression of SERCA2b was determined by Western blot (top panel). The samples were from mice without insulin injection. Each group includes two representative bands. The relative band intensity was normalized with α-tubulin (basal panel). (E) Transcription of SERCA2b were determined by real-time PCR and normalized with *rn18S*. Images are representative of the six mice. Error bars are the means ± SD of the three mice. ## $P < 0.01$ *vs.* NCD; * $P < 0.05$, ** $P < 0.01$ *vs.* HFD.

Ca^{2+} levels but also alleviated ER stress. As a result of prolonged ER stress or UPR malfunction, GRP78, a molecular chaperone required for ER integrity and stress-induced autophagy, was enhanced. The markers of ER stress, eIF2 phosphorylation at the α-subunit serine 51 and CHOP expression, were also increased. Jaceosidin showed a significant inhibition of the overexpression of GRP78, phosphor-eIF2 and CHOP (Fig. 2A–B).

To further investigate whether SERCA2b was involved in the regulation of ER stress by jaceosidin, we used a myotube model stimulated with thapsigargin, a non-competitive inhibitor of SERCA. It was shown that jaceosidin increased the resistance of myotubes to thapsigargin-induced ER stress (Fig. 3A) and enhanced the activity of SERCA in thapsigargin-treated myotubes (Fig. 3C). In comparison, the same concentrations of pioglitazone

did not alter the state of ER stress or the activity of Ca^{2+}-ATPase in thapsigargin-treated myotubes (Fig. 3B and D). We know that pioglitazone is an agonist of peroxisome proliferator-activated receptor (PPAR)-γ [35,36]. We have found that the activity of PPAR-γ was significantly suppressed in insulin-resistant myotubes, and pioglitazone almost rescued the activity of PPAR-γ to a normal level, whereas jaceosidin did not alter the activity of PPAR-γ at all (data not shown). These findings indicate that jaceosidin improves glucose homeostasis in a distinct way from pioglitazone.

Then, we investigated the effects of jaceosidin *in vivo* in a model of type II diabetes caused by obesity. Jaceosidin was found to improve glucose homeostasis and insulin resistance in obese mice. Compared with pioglitazone, 4 mg/kg of jaceosidin had a better effect on the improvement of glucose tolerance and insulin sensitivity (Fig. 4F-I). Jaceosidin administration also reduced blood triglycerides and cholesterol of mice treated with a HFD, which means an improvement in lipid metabolism and a suppression of insulin resistance (Fig. 4D–E). These effects also implied that jaceosidin had a different mechanism from pioglitazone, whose main adverse effects had been reported to be weight gain with adipose accumulation [37]. Alternatively, treatment with jaceosidin could significantly alleviate glucose tolerance and insulin sensitivity in ob/ob mice, an animal model of type II diabetes (Fig. 4J–L). Because leptin deficiency is recognized to have a major role in the pathogenesis of obesity in ob/ob mice, we may hypothesize that upregulation of SERCA2b in skeletal muscles can ameliorate the metabolic syndrome caused by leptin signal impairment.

Furthermore, jaceosidin administration also reduced liver mass in the HFD-treated mice (Fig. 5A–B). It has been reported that excessive fat accumulation is one of the major causes of ER stress in the liver [38–41], and overexpression of SERCA2b decreases fat accumulation in the liver of ob/ob mice [1]. Jaceosidin inhibited the hepatic level of triglycerides, attenuated hepatic lipid accumulation and protected livers from injury (Fig. 5C–F). Jaceosidin also significantly decreased the mRNA level of various lipogenic genes (FASn, SCD1, ACC1, SREBP1c) in the livers of obese mice (Fig. 5G).

Because jaceosidin exerted marked effects improving insulin-resistance with a unique mechanism of SERCA2b enhancement in myotubes cultured *in vitro*, we then assayed the insulin sensitivity in skeletal muscles in the obese mice. Compared with the lean mice, phosphorylation of Akt was reduced in obese mice. However, 4 mg/kg of jaceosidin significantly reversed this down-regulation (Fig. 6A), which implied that jaceosidin enhanced the insulin signaling in skeletal muscles. Our following study indicated that protein levels of phosphor-eIF2α and CHOP were significantly higher than in obese mice, and 4 mg/kg of jaceosidin significantly down-regulated the levels of these two proteins (Fig. 6B). Jaceosidin also up-regulated the suppressed mRNA level of ER chaperones (HSPA5, PDIA3, DNAJB9) in mouse skeletal muscles (Fig. 6C), hinting that jaceosidin improved ER folding capacity and then reduced ER stress in obese mice. It should be noted that the mRNA and protein levels of SERCA2b were both reduced in the skeletal muscles of obese mice and significantly increased by jaceosidin.

In all, as illustrated in Fig. 7, SERCA2b upregulation and enhancement of Ca^{2+}-ATPase activity by jaceosidin are responsible for the improvement of ER stress and insulin resistance. The mechanisms of insulin resisitance are complex and involve many factors at the levels of receptors, ion channels and cellular networks. Although we cannot rule out other sites of action and other mechanisms of jaceosidin that might directly or indirectly lead to the observed effects, e.g., the inhibition on inflammation [14], the efficacy of jaceosidin in upregulation of SERCA2b may provides important clues, at least in part, in elucidating its effects on insulin resistance.

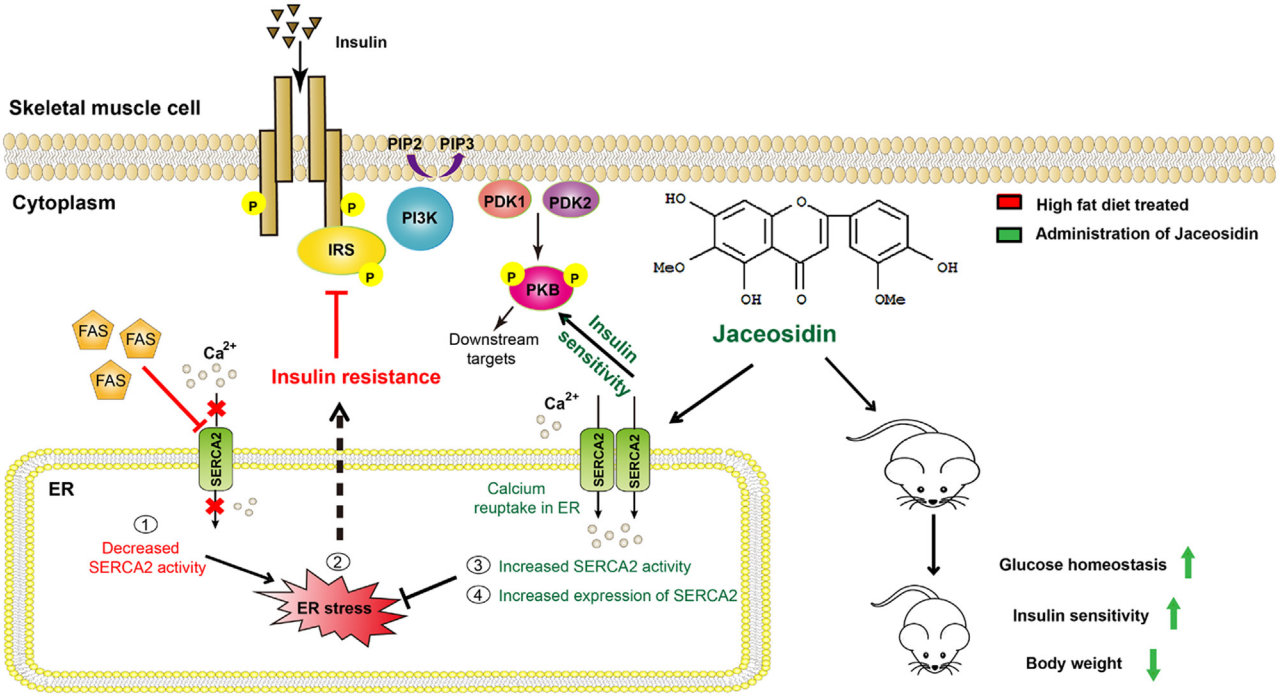

Fig. 7. Illustration for the mechanism underlying the amelioration of insulin sensitivity and glucose homeostasis by a natural compound jaceosidin.
When insulin-resistant myotubes and obese mice are administrated with jaceosidin, SERCA2b activity is upregulated to increase Ca^{2+} in endoplasmic reticulum, to lessen misfolded proteins and ER stress, thus potentiating the insulin sensitivity.

Jaceocidin is a natural flavonoid which demonstrates little toxicity both *in vitro* and *in vivo*. Jaceosidin of 100 μM showed no significant cytotoxicity in C2C12 cells. Single dose up to 10 g/kg jaceosidin was given to male and female mice intragastrically and no obvious abnormal reaction or animal death was observed. Therefore, jaceosidin may be a safe option to be used clinically.

On the other hand, the importance of SERCA in other diseases has also been emphasized. For example, mutations and altered expression levels of SERCA isoforms have been identified in various cancers [42]. Several studies have identified SERCA as a therapeutic target in Cancer [43,44]. Therefore, the observation that *in vivo* administration of jaceosidin alleviates ER stress via SERCA2b upregulation has important therapeutic implications for the treatment of many other diseases in humans.

Conflict of interest

The authors state no conflict of interest.

Author Contributions

Participated in research design: Qiang Xu, Xuefeng Wu

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Contributed new reagents or analytic tools: Xudong Wu, Yan Shen, Yang Sun

Performed data analysis: Zijun Ouyang, Wanshuai Li, Xingqi Wang, Xuefeng Wu

Wrote or contributed to the writing of the manuscript: Qiang Xu, Xuefeng Wu

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