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Resveratrol ameliorates high-fat diet-induced insulin resistance and fatty acid oxidation *via* ATM-AMPK axis in skeletal muscle

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Abstract. – OBJECTIVE: Resveratrol (RSV) is a polyphenolic phytoalexin that exhibits diverse pharmacological actions, including its effect on the insulin resistance. However, the mechanism through which RSV improves insulin resistance is not fully understood yet. The aim of this study was to determine the mechanism through which RSV ameliorates insulin resistance in skeletal muscle of high-fat diet (HFD)-induced mouse model, as well as palmitic acid (PA) treated L6 cells, with a specific focus on the response of RSV on fatty acid oxidation.

MATERIALS AND METHODS: Male C57BL6/J mice were randomly divided into three groups: normal diet-fed mice (ND), the high-fat diet-fed mice (HFD), HFD supplemented with RSV (100 mg/kg body weight [BW]/day orally; n = 10). Fasting plasma glucose, insulin, total cholesterol, triglyceride (TG), and free fatty acid levels were determined. The intraperitoneal glucose tolerance test was used to measure blood glucose and area under the curve. The quantitative insulin sensitivity index was calculated to assess insulin resistance. Skeletal muscles were collected for histology study and protein expression measurement. L6 cells were cultured with PA and the glucose concentration in the culture medium, and the intracellular TG levels were tested. RSV, chloroquine, palmitoyltransferase and Ku-55933 were administered to differentiate L6 cells.

RESULTS: The HFD fed mice showed increased BW, hyperglycemia, and hyperlipidemia. The expressions of ataxia telangiectasia mutated (ATM), 5' adenosine monophosphate-activated protein kinase (AMPK), carnitine palmitoyltransferase 1, cytochrome oxidase subunit IV protein were significantly decreased in the skeletal muscles of HFD fed mice and PA-treated L6 cells. All these effects induced by HFD and PA were reversed by RSV treatment.

CONCLUSIONS: ATM is a key factor to improve HFD-induced lipid metabolism and insulin resistance in skeletal muscles. The effects of RSV on ameliorating HFD-induced abnormal lipid metabolism and insulin resistance mediated through ATM-AMPK pathway may due to its improvement in fatty acid oxidation efficiency and sequential reduction in ROS production in skeletal muscle. These results provide important theoretical evidence for the application of RSV in the prevention and treatment of diabetes mellitus and related metabolic diseases.

Key Words:
Insulin resistance, Resveratrol, ATM-AMPK, Skeletal muscle, Lipid metabolism.

Introduction

Obesity is one of the most prevalent metabolic diseases in the world. It is primarily driven by a high-fat diet (HFD) and sedentary life-style[1]. Obesity is an essential factor that increases the risks of type 2 diabetes through the induction of insulin resistance. It has been well studied that as less as one week of HFD can induce detectable insulin resistance in rodents, which may even precede the dramatic increase in body weight and fat mass[2]. Under normal physiological conditions, insulin promotes glucose uptake by insulin-sensitive tissues, such as skeletal muscle and adipose tissue. When insulin resistance occurs,

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sensitivity to insulin reduces in such tissues, leading to decreased clearance of circulating glucose, which is characterized by hyperglycemia and hyperinsulinemia under fasting conditions[3]. The 5' adenosine monophosphate-activated protein kinase (AMPK) is the primary energy sensor in most eukaryotic cells. Ataxia-telangiectasia mutated (ATM) is an AMPK kinase, which can phosphorylate AMPK and promote mitochondria biogenesis[4]. AMPK suppresses the synthesis of fatty acid and sterol and promotes the activation of carnitine palmitoyltransferase 1 (CPT1) for fatty acid oxidation in the mitochondria to release ATP[5]. Excessive ingestion of lipids increases circulating concentration of free fatty acids (FFA), triglycerides (TG), and total cholesterol (TC), which will be taken up and oxidized into ATP through over-activation of mitochondria. The sequential over-accumulation of ATP negatively represses AMPK activation to reduce the further production of ATP[4]. It is found that the abundance of mitochondria in the skeletal muscle is reduced in patients with insulin resistance, which leads to decreased capacity of fatty acid oxidation and sequential accumulation of intramuscular lipids[4].

Resveratrol (RSV) is a polyphenolic phytoalexin found in grapes. RSV can exert a variety of biological effects, including anti-diabetes[6-8], anti-obesity[9,10], antioxidant[11-13], hypoglycemic[14], hypolipidemic[15], anti-inflammatory[16,17], anti-cancer[18,19], anti-platelet aggregation[20], cardiovascular protection[21], and extend lifespan in diet-induced obese rodents[22]. Mice that lack either AMPK-α1 or AMPK-α2 subunit showed no insulin sensitivity improvements upon RSV treatment[23], indicating a role of AMPK in such insulin sensitivity regulation. However, the specific mechanism is not known yet. Therefore, the aims of this study were to 1) confirm the effects of RSV on HFD-induced lipid accumulation and insulin resistance both *in vivo* and *in vitro*, and 2) determine if such effects were exerted through ATM-AMPK pathway.

Materials and Methods

Reagents

RSV, palmitic acid, and dimethyl sulfoxide were purchased from Sigma-Aldrich (Shanghai, China). Palmitic acid was dissolved in 50% ethanol and diluted with medium containing 10% FFA-free bovine serum albumin. Anti-β-actin, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-AMPK, anti-p-AMPK (Thr172), anti-cytochrome oxidase subunit IV (COXIV) antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-CPT1 antibody was purchased from Abcam (Cambridge, UK). Bicinchoninic acid protein quantification kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). TG, FFA, total cholesterol (TC) assay kit were purchased from Nanjing Jiancheng Biotechnology Research Institute (Nanjing, China). DMEM medium and trypsin were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from ScienCell (Carlsbad, CA, USA). Mouse insulin enzyme-linked immunosorbent assay (ELISA) kit was purchased from ALPCO (Salem, NH, USA). Chloroquine (Chq) and Ku55933 (Ku) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

C57BL6/J male mice, 5-6 weeks of age, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in facility on 12 h light/dark cycles under room temperature (22 ± 2°C) with relative humidity 40%-60%. All mice had free access to feed and water. Mice were fed with a normal diet (ND) or a high-fat diet (HFD) with saline or RSV (100 mg/kg feed/d) for 8 weeks. The ND (D12450J; 348 kcal/100 g diet) consisted of 20% calories from protein (19.7% from casein, 0.3% from L-cystine), 10% calories from fat (4.4% from lard, 5.5% from soybean oil), 70% calories from carbohydrate (50% from corn starch, 12.3% from maltodextrin, 6.8% from sucrose). The HFD (D12492; 524 kcal / 100 g diet) consisted of 20% calories from protein (19.7% from casein, 0.3% from L-cystine), 60% calories from fat (54.4% from lard, 5.5% from soybean oil), 20% calories from carbohydrate (12.3% from maltodextrin, 6.8% from sucrose).

Before sample collections, the mice (n = 10 per treatment group) were fasted overnight, weighed, and anesthetized with 1% pentobarbital sodium (60 mg/kg body weight [BW]). All procedures were approved by the Animal Ethics Committee of Hebei Medical University and implemented in accordance with the Hebei Provincial Laboratory Animal Management Regulations.

Lipid Profile and Fasting Insulin

Blood samples from the heart were clotted under room temperature for 30 min and then cen-

trifuged at 1000 ´ g for 10 min under 4°C. Serum TG, FFA, TC, and fasting insulin level were measured by corresponded commercial kits.

Cell Culture and Differentiation

L6 myotubes were cultured in DMEM (25 mmol/L glucose) supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C in a 5% CO_2 incubator. L6 myotubes cells were differentiated with 2% serum for 5 days and then kept in the regular culture medium for 24 h for control group or treated with 200 µmol/L palmitic acid (PA) in serum-free conditioned medium for 24 h. Meanwhile, 150 µmol/L RSV, 100 µmol/L Chq, or 10 µmol/L Ku were given to the corresponded PA-treated cell groups for 3 h, 3 h, and 1 h, respectively. For cell TG content measurement, 20 µmol/L RSV was applied to PA-treated cells for 24 h. Three replicates were used in each treatment group.

Intra-Peritoneal Glucose Tolerance Test (IPGTT)

After 12 h fasting, mice were intra-peritoneally injected with 2 g glucose/kg BW. Blood was collected from the tail vein before glucose injection and at 30, 60, and 120 min post-injection. Glucose area under the curve (AUC-G) was calculated as: AUC-G = 0.25 × fasting plasma glucose + 0.5 × 30 min G + 0.75 × 60 min G + 0.5 × 120 min. The quantitative insulin sensitivity index (QUICKI) value is equal to the sum of the logarithms of fasting plasma glucose (FPG) and fasting insulin (FINS).

Skeletal Muscle Morphology

The soleus muscle was retained and fixed by 2.5% glutaraldehyde. Skeletal muscle lipid deposition was observed by transmission electron microscopy.

TG Concentration in Skeletal Muscle

Skeletal muscle tissue was taken out with the same weight after mice were sacrificed by draining blood from the heart and grinded in PBS. The suspension was centrifugated at 4,000 ´ g for 20 min. TG concentration was measured by commercial ELISA kits. The results were normalized by the amount of protein.

Western Blot Analysis

The proteins were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Co., Ltd. MA, USA), blocked with 5% skim milk/TBST buffer (5 mmol/L Tris–HCl, pH 7.6, 136 mmol/L NaCl, 0.05% Tween-20), immunoblotted with primary, and secondary antibody. Antibody-antigen complexes visualized with enhanced chemiluminescence (ECL) Western Blotting Detection System and analyzed quantitatively by densitometry with Image-Pro Plus 6.0 (IPP 6.0) software.

Statistical Analysis

All data processing was performed using the Statistical Product and Service Solution (SPSS) 21.0 software (IBM Corp., Armonk, NY, USA) and the results were expressed as mean ± SEM. LSD *t*-test and rank sum test were used for comparisons between the two groups. One-way ANOVA was used to compare multiple groups with the post-hoc comparisons done by Tukey's test. Kruskal-Wallis H test analysis was used for non-parametric data with the post-hoc analyses done by Bonferroni's correction. The significant level was set at $p < 0.05$.

Results

Resveratrol Ameliorated the Effect of HFD on Insulin Resistance

The 8-week feeding of high fat diet significantly increased BW ($p < 0.01$; Figure 1A), plasma TG ($p < 0.01$; Figure 1B), TC ($p < 0.01$; Figure 1C), FFA ($p < 0.01$; Figure 1D), FPG ($p < 0.01$; Figure 1E), insulin ($p < 0.05$; Figure 1F), plasma glucose after IPGTT ($p < 0.01$; Figure 1G), and AUC-G ($p < 0.01$; Figure 1H) compared to the ND group. These results together with the decreased QUICKI ($p < 0.01$; Figure 1I), indicated that HFD successfully induced insulin resistance in mice. Supplementation of 100 mg/kg feed/d RSV significantly reversed such adverse impacts of HFD, leading to an increased insulin sensitivity compared to the mice fed with only HFD ($p < 0.05$).

Lipid Accumulation Induced by HFD in Muscle Was Reversed by Resveratrol

To further investigate the effect of RSV on skeletal muscle, we collected the soleus muscles at the end of the 8-week feeding experiment and determined TG content, morphology, and the expression of proteins related to insulin signaling and fatty acid oxidation in these mus-

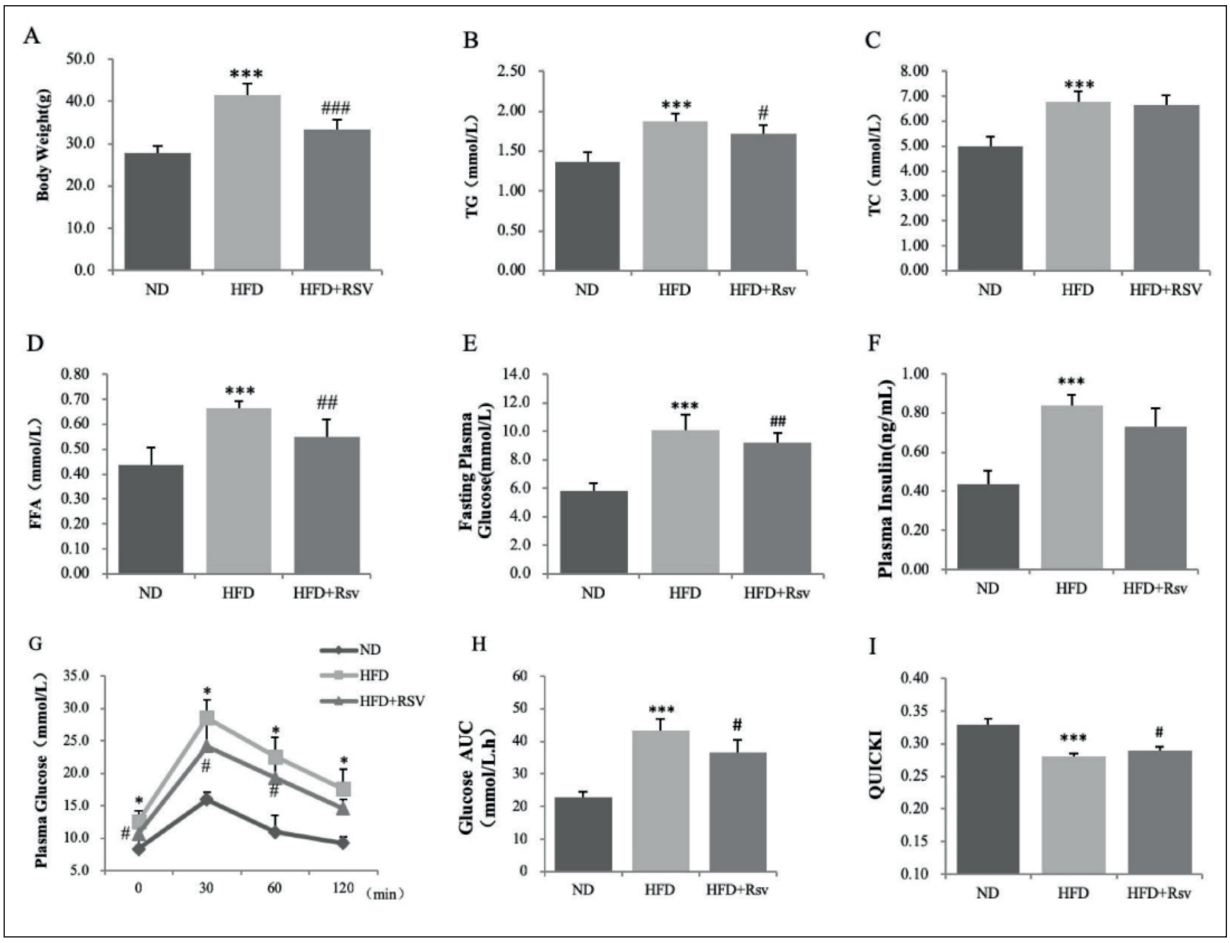

Figure 1. Effects of HFD and resveratrol on (**A**) body weight, (**B**) plasma triglycerides (TG), (**C**) total cholesterol (TC), (**D**) free fatty acids (FFA), (**E**) fasting plasma glucose (FPG), (**F**) plasma insulin (FINS), (**G**) plasma glucose before and 30, 60 and 120 min after IPGTT, (**H**) glucose area under the curve (AUC-G), and (**I**) quantitative insulin sensitivity index (QUICKI) in C57BL6/J mice feeding normal diet (ND), high-fat diet (HFD), or HFD with 100 mg/kg feed/d resveratrol (RSV) for 8 weeks (n = 10). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus ND group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus HFD group. All results are presented as mean ± SEM.

cle samples. Data showed that HFD resulted in higher TG concentration ($p < 0.01$; Figure 2A) and increased lipid accumulation with enlarged lipid droplets, as well as swelling mitochondria (Figure 2B-2D), in skeletal muscle compared to the indexes in the ND-fed mice. Meanwhile, HFD-fed mice showed decreased expression of ATM ($p < 0.05$; Figure 2E), p-AMPK ($p < 0.05$; Figure 2F), COXIV ($p < 0.05$; Figure 2G), and CPT1 ($p < 0.05$; Figure 2H). Supplementation of 100 mg/kg feed/d RSV in HFD reduced TG content ($p < 0.05$) and increased the expression of ATM ($p < 0.05$), p-AMPK ($p < 0.05$), COXIV ($p < 0.05$), and CPT1 ($p < 0.05$) in the skeletal muscle of the mice compared to those only fed with HFD.

Resveratrol Reversed the Effect of Palmitic Acid on L6 Cells

In addition to the *in vivo* experiments, we also adopted an *in vitro* insulin-resistant model by supplementing palmitic acid to L6 myotubes, which is a well-established method[24], to determine the mechanism through which RSV exerts its effect on insulin resistance. Compared to the L6 cells maintained in the regular culture medium for 24 h post-differentiation (Figure 3A), those treated with 200 μmol/L PA had more and larger lipid droplets (Figure 3B). Meanwhile, the remaining glucose concentration in the medium, which is an indirect indicator of insulin resistance as it reflects glucose uptake capability, was increased after 12 h of PA treatment ($p < 0.05$ at

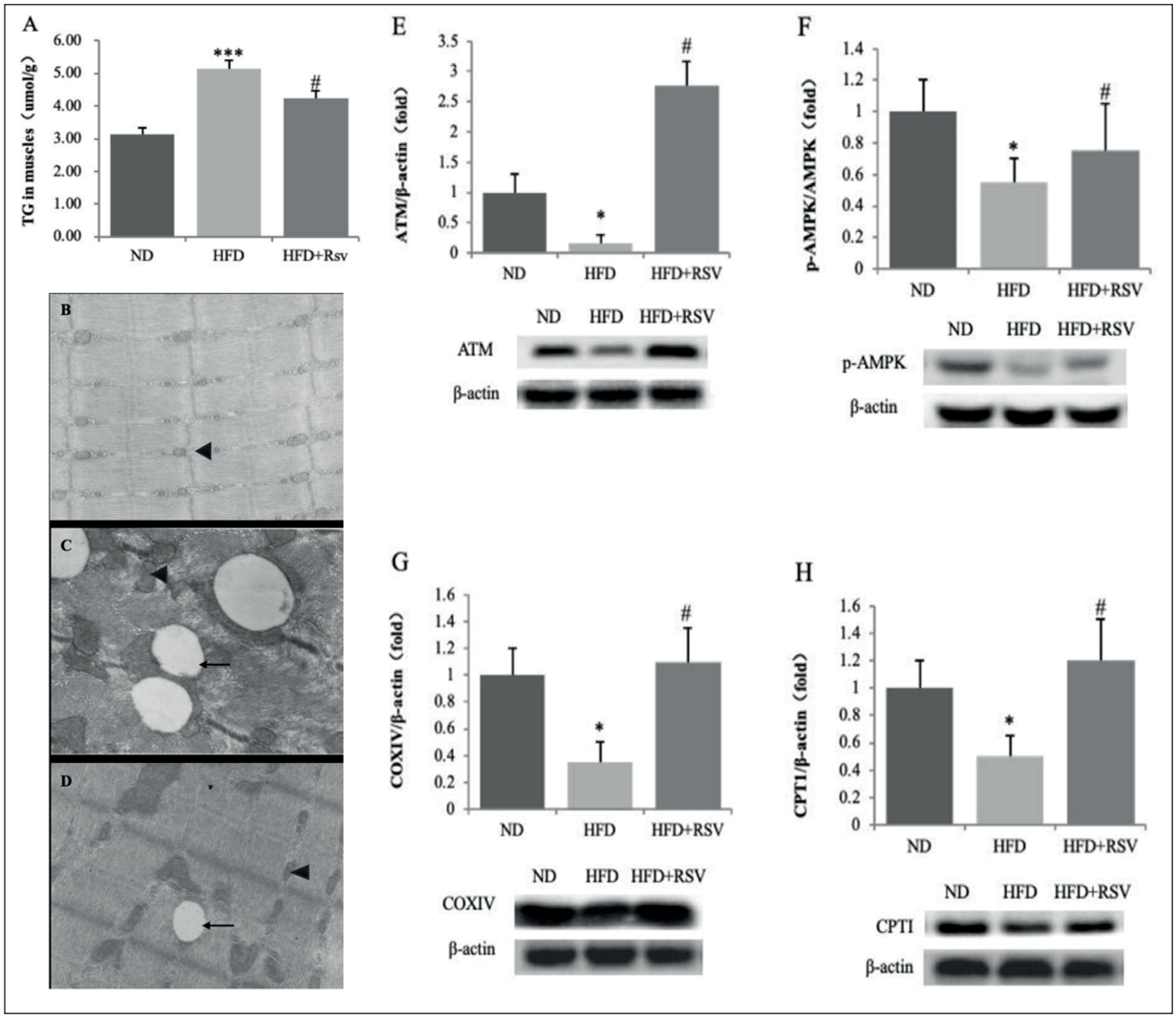

Figure 2. Effects of HFD and 100 mg/kg feed/d resveratrol on (**A**) triglyceride (TG) content and (**B-D**) lipid accumulation in skeletal muscle, and the expression of (**E**) ATM, (**F**) p-AMPK, (**G**) COXIV and (**H**) CPT1 in skeletal muscle (n = 10). *$p < 0.05$ *vs.* ND group; $^{\#}p < 0.05$ *vs.* HFD group. All results are presented as mean ± SEM. The magnification of electron microscope was 15k. Triangles indicate mitochondria and arrows indicate lipid droplets.

12 and 16 h, and $p < 0.001$ at 24 h post-treatment; Figure 3C). TG content in L6 cells also increased upon 24 h PA treatment ($p < 0.05$; Figure 3D), while the effect was reversed by the addition of RSV ($p < 0.05$). Moreover, PA treatment reduced the expression of ATM ($p < 0.05$; Figure 4A), p-AMPK ($p < 0.05$; Figure 4B), COXIV ($p < 0.05$; Figure 4C), and CPT1 ($p < 0.05$; Figure 4D) in L6 cells compared to the cells cultured in the regular culture medium. Supplementation of 150 μmol/L RSV in PA-treated cells for 3 h increased the expressions of all these proteins (all $p < 0.05$) in L6 cells. Supplementation of 100 μmol/L Chq, an ATM agonist, to the PA-treated L6 cells for 3 h also increased ATM expression to the same level as treated by RSV. However, supplementation with 10 μmol/L Ku, an ATM inhibitor, to the PA-treated L6 cells for 1 h did not reverse the effect of PA treatment on any of the protein expression.

Discussion

HFD is a key factor that induces insulin resistance, which is characterized by hyperinsulinemia, hyperlipidemia, hyperglycemia, as well as mitochondria dysfunction[3]. It is reported that RSV can improve HFD-induced insulin resistance. However, the specific mechanism is not yet

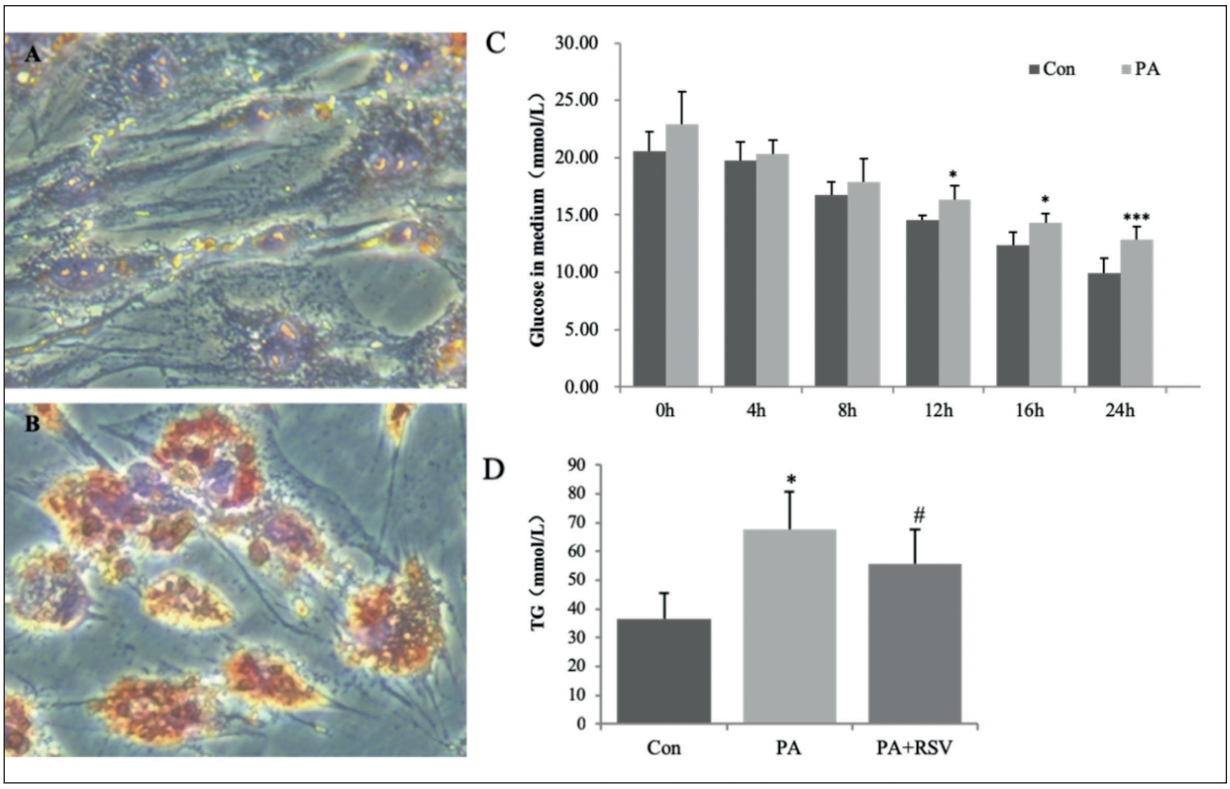

Figure 3. Effect of 24 h treatment of 200 μmol/L palmitic acid (PA) on differentiated L6 cells (n = 3 replicates per treatment group). (**A-B**) Oil red O staining of cytoplasmic lipid droplets in control cells (**A**) and PA-treated cells (**B**) under the magnification × 400. (**C**) Glucose concentration in the medium of control cells (Con) and PA-treated cells. (**D**) Triglyceride (TG) concentration in Con, PA-treated, and PA + 20 μmol/L RSV treated cells at 24 h post-treatments. *$p < 0.05$ *vs.* Con group; #$p < 0.05$ *vs.* PA group. All results are presented as mean ± SEM.

clear. In this study, we established both HFD-induced insulin resistance model *in vivo* and PA-induced insulin resistance model *in vitro* to determine the mechanism through which RSV attenuates insulin resistance. Our data indicated that RSV efficiently reversed the HFD-induced lipid over-accumulation and improved glucose sensitivity and insulin resistance in skeletal muscle and L6 cells. Such effects were likely mediated through ATM-AMPK pathway.

Mice fed with HFD for 8 weeks showed increased BW, plasma TG, TC, FFA, and fasting plasma glucose and insulin levels, as well as reduced insulin sensitivity compared to the ND fed counterparts, indicating the successful establishment of HFD-induced insulin resistance model. Ingestion of HFD for weeks constantly elevates plasma FFA than normal physiological level, leading to increased mitochondria biogenesis in the skeletal muscle to enhance energy disposal in the form of ATP through fatty acid β-oxidation to adapt to such changes[25]. Persistent over-activation of mitochondria leads to over-accumulation of ATP, which sends signal to the energy sensor AMPK and thus downregulate the activity of AMPK to halt further production of ATP as a self-protective mechanism. A reduction in the AMPK activity sequentially results in decreased glucose uptake and oxidation induced by insulin, thus leading to insulin resistance[4,23,25]. It is found that insulin resistant subjects have less mitochondria compared to the insulin sensitive counterparts. Such dysfunction leads to reduced β-oxidation which further increases the accumulation of intramuscular lipids[3]. This is in line with our observation that HFD treatment decreased the expression of CPT1 – a rate-limiting enzyme in β-oxidation, and COXIV – the last enzyme in respiratory electron transport chain, which together with the abnormally enlarged mitochondria containing excessive lipid droplets, indicated that HFD impaired mitochondria function and sequential fatty acid oxidation.

RSV, like other insulin-sensitizing agents, is reported to improve insulin sensitivity through prohibition of mitochondria over-activation upon

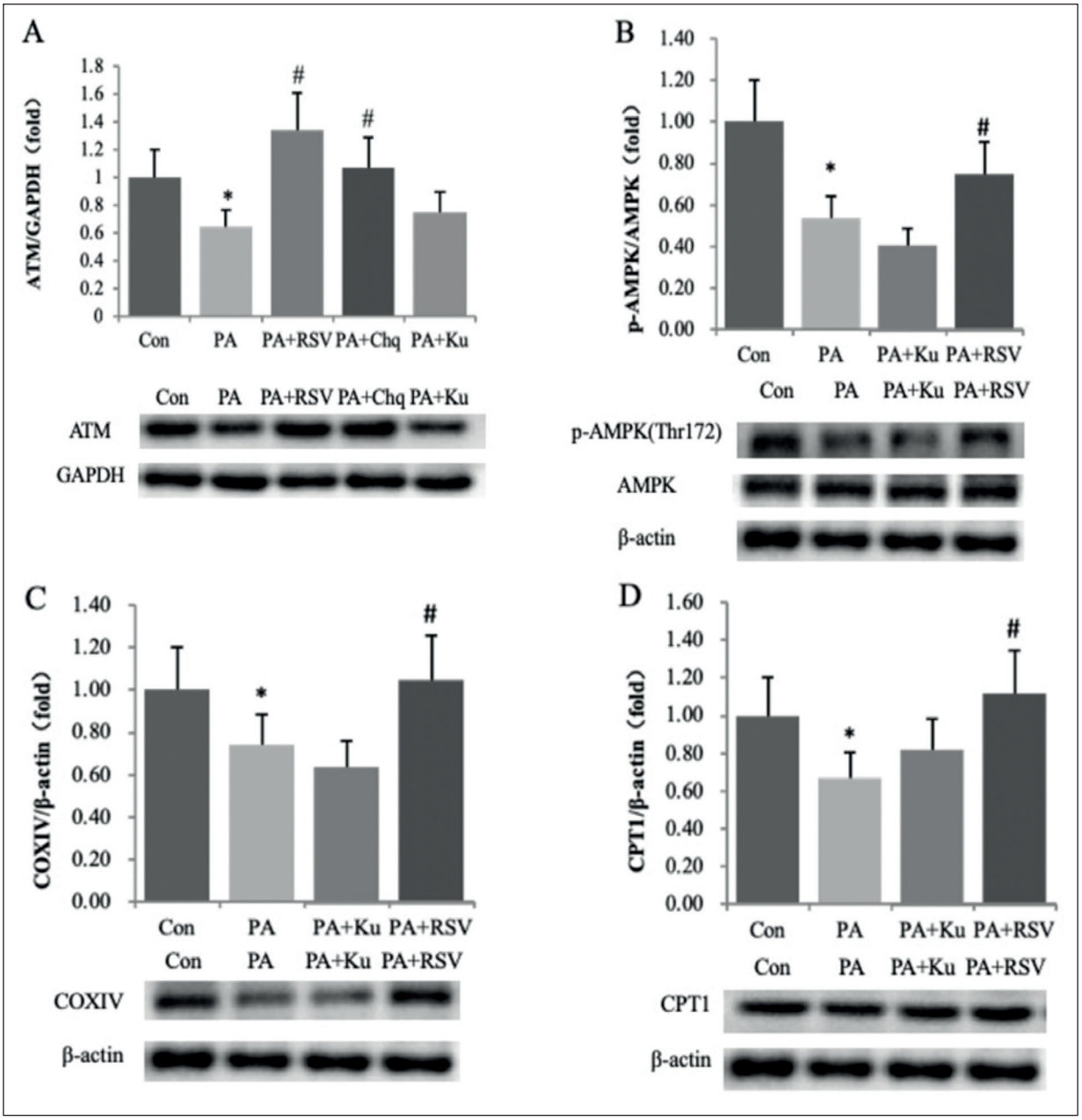

Figure 4. Effect of palmitic acid (PA) and PA + resveratrol (RSV), PA + chloroquine (Chq) and PA + Ku55933 (Ku) on protein expression of (**A**) ATM, (**B**) p-AMPK, (**C**) COXIV and (**D**) CPT1 in L6 cells (n = 3 replicates per treatment group). *$p < 0.05$ *vs.* Con group; $^{\#}p < 0.05$ *vs.* PA group. All results are presented as mean ± SEM.

HFD treatment[3] and thus resumes mitochondria biogenesis, increases lipid oxidation and decreases intramuscular lipid accumulation[26]. Since RSV is a sirtuin 1 (SIRT1) agonist, it was postulated that the effect of RSV on insulin resistance was mediated through SIRT1[26]. However, Um et al[23] reported that AMPK, but not SIRT1, is the major target of RSV as AMPKα1$^{-/-}$ or AMPKα2$^{-/-}$ mice on a HFD showed no response to RSV treatment. AMPK is the downstream effector of ATM in the cancer cells in response to chemotherapy or radiation therapies[27]. ATM regulates the activation of AMPK and, in turn, AMPK enables and stabilize ATM signaling maybe through a feedback loop[27]. Studies have found that homozygous mice lacking ATM have abnormal glucose metabolism and severe insulin resistance, while the activated ATM kinase promotes phosphorylation of AMPK

and corresponded downstream cascades[28]. We currently found that HFD reduced the expression of ATM and its sequential activation of AMPK and RSV supplement reversed this effect. Moreover, in this study, we for the first time determined the effect of RSV on PA-treated L6 cells. PA is a saturated fatty acid capable of inducing a high-fat model and insulin resistance in various cells including HepG2 hepatocytes and L6 skeletal muscle cells. Indeed, PA treatment decreased the glucose uptake while increased TG content in L6 cells, and RSV supplementation successfully decreased TG content in L6 cells. This effect is likely mediated through ATM-AMPK pathway as RSV increased the expression of ATM and AMPK decreased by PA treatment. The impact of RSV on ATM expression in PA-treated L6 cells were the same as that from the ATM agonist Chq, while adding ATM inhibitor Ku reversed the effect of RSV. ATM is a DNA double-strand break, cell cycle signal, and DNA repair sensor, which has major regulatory functions in DNA damage response[29]. Bakkenist and Kastan[30] have found that Chq activates ATM when DNA double-strand breaks are damaged. Chq also improves glucose tolerance in high-fat fed $ATM^{+/+}$ $ApoE^{-/-}$ mice, but does not increase glucose tolerance in $ATM^{-/-}$ $ApoE^{-/-}$ mice[31]. Meanwhile, the *in vitro* study also confirmed the *in vivo* result that RSV attenuated the reduction in fatty acid oxidation induced by PA treatment. It is reported[28] that the reactive oxygen species (ROS) activates ATM. This indicates that RSV may ameliorate HFD-induced insulin resistance through ATM-AMPK, due to the increase in fatty acid oxidation efficiency and sequential reduction in ROS production, which can cause oxidative stress and induce inflammation to further exacerbate insulin resistance[3,23,32].

Conclusions

ATM is a key factor to improve HFD-induced lipid metabolism and insulin resistance in skeletal muscles. The effects of RSV on ameliorating HFD-induced abnormal lipid metabolism and insulin resistance mediated through the ATM-AMPK pathway may occur due to its improvement in fatty acid oxidation efficiency and sequential reduction in ROS production in skeletal muscle. These results provide important theoretical evidence for the application of RSV in the prevention and treatment of diabetes and related metabolic diseases.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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