Dihydromyricetin improves skeletal muscle insulin resistance by inducing autophagy via the AMPK signaling pathway

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A B S T R A C T

Skeletal muscle insulin resistance (SMIR) plays an important role in the pathogenesis of type 2 diabetes. Dihydromyricetin (DHM), a natural flavonoid, exerts various bioactivities including anti-oxidative and hepatoprotective effects. Herein, we intended to determine the effect of DHM on SMIR and the underlying mechanisms. We found that DHM increased the expression of phosphorylated insulin receptor substrate-1, phosphorylated Akt and glucose uptake capacity in palmitate-treated L6 myotubes under insulin-stimulated conditions. The expression of light chain 3, Beclin 1, autophagy-related gene 5 (Atg5), the degradation of sequestosome 1 and the formation of autophagosomes were also upregulated by DHM. Suppression of autophagy by 3-methyladenine and bafilomycin A1 or Atg5 and Beclin1 siRNA abolished the favorable effects of DHM on SMIR. Furthermore, DHM increased the levels of phosphorylated AMP-activated protein kinase (AMPK) and Ulk1, and decreased phosphorylated mTOR levels. AMPK inhibitor compound C (CC) and AMPK siRNA abrogated DHM-induced autophagy, subsequently suppressed DHM-induced SMIR improvement. Additionally, DHM inhibited the activity of F1F0-ATPase thereby activating AMPK. Finally, the results of in vivo study conducted in high fat diet-fed rats were consistent with the findings of in vitro study. In conclusion, DHM improved SMIR by inducing autophagy via the activation of AMPK signaling pathway.

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1. Introduction

Diabetes has become a major public health concern, and its incidence is expected to increase to 439 million, a prevalence of 7.7%, by 2030 (Wild et al., 2004). Type 2 diabetes is the most common form and is characterized by insulin resistance, which often begins with decreasing insulin sensitivity in skeletal muscle (Bajpey et al., 2011). Insulin resistance is an important target for the prevention and treatment of type 2 diabetes. Many anti-diabetic agents such as insulin secretion enhancers and insulin sensitizers have targeted this disorder to control type 2 diabetes. However, the side effects of long-term use, such as weight gain and hypoglycemia, and contraindications limit their utility (Maru et al., 2005). Therefore, the identification of hypoglycemic agents to effectively improve insulin resistance with few adverse effects is critical. Natural products, particularly flavonoids with excellent pharmacological properties, have recently been identified as potential candidates for the prevention or control of type 2 diabetes through the inhibition of insulin resistance (Babu et al., 2013). However, the underlying mechanisms remain to be elucidated.

Autophagy is a highly regulated process involved in the turnover of long-lived proteins, cytosolic components, or damaged organelles that is essential for maintaining cellular homeostasis (Kraft and Martens, 2012; Mizushima et al., 2008; Rubinsztein et al., 2011). Oxidative stress, endoplasmic reticulum stress and inflammation are associated with insulin resistance and play a role in the accumulation of dysfunctional and/or damaged components; autophagy plays an important role in the removal of these components (Quan et al., 2015). The suppression of autophagy by 3-methyladenine and bafilomycin A1 or Atg5 and Beclin1 siRNA abolishes the favorable effects of DHM on SMIR. Furthermore, DHM increased the levels of phosphorylated AMP-activated protein kinase (AMPK) and Ulk1, and decreased phosphorylated mTOR levels. AMPK inhibitor compound C (CC) and AMPK siRNA abrogated DHM-induced autophagy, subsequently suppressed DHM-induced SMIR improvement. Additionally, DHM inhibited the activity of F1F0-ATPase thereby activating AMPK. Finally, the results of in vivo study conducted in high fat diet-fed rats were consistent with the findings of in vitro study. In conclusion, DHM improved SMIR by inducing autophagy via the activation of AMPK signaling pathway.
2012; Wang et al., 2013; Yin et al., 2012). However, autophagy is suppressed in mouse models of insulin resistance, and tissue-specific autophagy knockout mice display insulin resistance (He et al., 2012; Kume et al., 2012; Liu et al., 2009; Yoshizaki et al., 2012). These results indicate that autophagy is implicated in insulin resistance and suggest that the regulation of autophagy could be a valuable strategy for the prevention and treatment of type 2 diabetes.

The regulation of autophagy involves several signaling pathways, among which the AMP-activated protein kinase (AMPK) pathway plays an important role. AMPK is a major metabolic energy sensor that regulates energy homeostasis and metabolic stress by controlling several homeostatic mechanisms, including autophagy and protein degradation (Hardie, 2007; Vingtedoux et al., 2011). AMPK serves as a positive regulator of autophagy mainly by inhibiting the mammalian target of rapamycin (mTOR) complex and phosphorylating unc-51-like kinase 1 (ULK1, the ortholog of Atg1 in mammals) (Kim et al., 2011). Recent studies have indicated that AMPK may be an important target for the prevention and treatment of type 2 diabetes because of its reported protective properties, including improving insulin resistance, decreasing serum glucose, and reducing lipid accumulation (Yu et al., 2010). In fact, several therapeutic agents against diabetes, such as metformin, may exert their protective effects by activating AMPK (Boyle et al., 2010). However, whether the regulation of autophagy by AMPK-mediated signaling pathways contributes to the beneficial effects of AMPK activators such as metformin on insulin resistance remains unclear.

Ampelopsis grossedentata is a medicinal and edible plant that is widely distributed in Southern China. Its tender stem and leaves have been consumed as a health tea (Tengcha) for hundreds of years. Dihydromyricetin (DHM), also named ampelopsin, is the main flavonoid component of Ampelopsis grossedentata (Wang et al., 2002). DHM possesses biological and pharmacological activities, including anti-oxidative, anti-inflammatory, hepatoprotective and anti-cancer effects (Xu et al., 2007, 2008; Zhou et al., 2014). A previous study from our group showed that DHM supplementation improves physical performance under acute hypoxic conditions partially by activating AMPK in skeletal muscle (Zou et al., 2014). Moreover, several flavonoids with similar chemical structure to DHM, such as quercetin and naringin, improve insulin resistance through AMPK activation (Dai et al., 2013; Pu et al., 2012). These findings indicate that DHM may exert anti-insulin resistance effects by activating the AMPK signaling pathway.

Given the close association between AMPK, autophagy, and insulin resistance, we hypothesized that DHM protects skeletal muscle from insulin resistance by inducing autophagy via the activation of the AMPK signaling pathway. To verify this hypothesis, we tested the effects of DHM on insulin resistance and autophagy induction in models of skeletal muscle insulin resistance (SMIR), and determined their relationship in vitro and in vivo. The potential involvement of the AMPK signaling pathway was also evaluated. We showed, for the first time, that DHM attenuated SMIR through the induction of autophagy in vivo and in vitro, and that autophagy induction was, at least in part, mediated by the activation of the AMPK signaling pathway.

2. Methods and materials

2.1. Reagents

3-Methyladenine (3-MA; M9281), bafilomycin A1 (BafA1; B1793), compound C (CC; P5499), rapamycin (RPA; 117), palmityl (PA; P5585), antibodies against light chain 3 (LC3; L7543) and minimum essential medium eagle (MEM; M4526) were purchased from Sigma-Aldrich. 2-[1,2-3H] -deoxy-D-glucose (NET328A001MC) was purchased from PerkinElmer. 2-[(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG; N13195) and Lipofectamine™ 2000 transfection reagent (11668-019) were purchased from Invitrogen. Fetal bovine serum (FBS; SH30370.03) was purchased from Hyclone Laboratories. Antibodies against phosphorylated AMPKα1/2 Thr172 (p-AMPKα1/2 Thr172; sc-3524), AMPKα1/2 (sc-74461), IRS-1 (sc-599) and phosphorylated IRS-1 Ser307 (p-IRS-1 Ser307; sc-3956), siRNAs for autophagy-related gene 5 (Atg5; sc-41445), Beclin 1 (sc-29797) and AMPK (sc-45312), control (sc-44230) were obtained from Santa Cruz Biotechnology. Green fluorescent protein-LC3 (GFP-LC3) plasmid was kindly provided by ADDGENE. Antibodies against Beclin 1 (3495), Akt5 (8540), Akt (9272), phosphorylated Akt Ser473 (p-Akt Ser473; 9271), sequestosome 1 (p62; 5114), phosphorylatedULK1 Ser757 (p-ULK1 Ser757: 6888), ULK1 (8054), phosphorylated mTOR Ser248 (p-mTOR Ser248; 2971), mTOR (2972), phosphorylated p70S6K (Thr389; 9202) and p70S6K (9205) were obtained from Cell Signaling Technology. DHM (msat-120131108, HPLC ≥ 98%) was purchased from Chengdu MUST Bio-Technology Co., LTD. Rat insulin RIA kit was bought from Millipore (PI-13K). ATP synthase Enzyme Activity Microplate Assay Kit (ab109714) was obtained from Abcam. D-Fructose was purchased from Shanghai Biological Engineering Company.

2.2. Cell culture and treatments

Rat skeletal muscle L6 myoblast cells were maintained in MEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO2. For differentiation into myotubes, the medium was replaced with MEM containing 2% FBS for 5 days. These differentiated L6 myotubes were used for the different treatments.

2.3. PA preparation

PA was dissolved in 40 mL of 0.1 M NaOH at 70 °C. BSA solution (1%) was prepared in distilled water. Different concentrations of palmitate were used for treatment of L6 myotubes after conjugation with 1% BSA on a magnetic stirrer set at 40 °C.

2.4. Cell transfection

Control non-targeted siRNA or siRNA against ATGS, Beclin1 or AMPK and Lipofectamine 2000 were diluted in reduced serum MEM according to the manufacturer’s protocol. The final siRNA concentration was 100 nM and plasmid concentration was 4 μg. L6 myotubes were incubated with the transfection mixture for 10 h and then supplemented with fresh medium for an additional 24 h, after which they were exposed to the indicated treatments. The same method was used for transfection of GFP-LC3 plasmids.

2.5. HPLC assay

HPLC analysis was performed as previously reported (Parvaneh et al., 2010). Acid-soluble extracts of cell suspensions were prepared by adding ice-cold 70% perchloric acid to a final concentration of 7% (wt/vol). Acid-insoluble fractions were removed by centrifugation for 10 min at 13,000 g at 4 °C. Analyses were performed with a Waters HPLC system. Samples were kept at 4 °C, and aliquots of the supernatant were applied to a 4.6 mm × 15 cm reversed-phase column (Waters, SunFire).

2.6. ATP synthase enzyme activity measurement

The ATP synthase enzyme activity was detected using a microplate assay kit according to the manufacturer’s protocol. After extraction of mitochondria from L6 cells, 50 μL of each diluted sample
was added into individual wells on the plate and incubated for 3 h at room temperature. After immobilization of the enzyme by the bound monoclonal antibody, wells were washed with buffer twice and 40 μL of LIPID MIX was added to each well and incubated for 45 min. The absorbance of each well was read in a plate reader at 340 nm at 30 °C.

2.7. Transmission electron microscopy

L6 myotubes were fixed in 3% glutaraldehyde in 0.1 M MOPS buffer (pH 7.0) for 8 h at room temperature, followed by incubation in 3% glutaraldehyde/1% paraformaldehyde in 0.1 M MOPS buffer (pH 7.0) for 16 h at 4 °C. Myotubes were then post-fixed in 1% osmium tetroxide for 1 h, embedded in Spurr’s resin, sectioned, double stained with uranyl acetate and lead citrate, and analyzed using a Zeiss EM 10 transmission electron microscope.

2.8. 2-NBDG glucose uptake assays

At the end of treatment, L6 myotubes were washed with glucose free Krebs–Ringer phosphate HEPES (KRPH) buffer containing 0.1% fatty acid-free BSA (w/v) followed by incubation in the same buffer for 30 min at 37 °C. L6 myotubes were stimulated with insulin (10 nM) for 15 min followed by the addition of 0.5 μCi/well of 3H-2-deoxyglucose in KRPH buffer for 45 min at 37 °C, washing with KRPH and lysis using 0.1% SDS. The lysates were analyzed in a scintillation counter.

2.9. 2-NBDG uptake assays

L6 myotubes were exposed to the indicated treatments for 24 h and 100 mM 2-NBDG was added to the media for 2 h, followed by washing with PBS three times. The collected myotubes were analyzed using a laser confocal microscope and flow cytometry detection analyzer.

2.10. Western blotting

The protein samples extracted from L6 myotubes or skeletal muscle of high fat diet-fed rats were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with TBST containing 5% milk and incubated with the different primary antibodies as indicated overnight at 4 °C. The membranes were then incubated with horseradish peroxidase conjugated secondary antibodies and visualized using the enhanced chemiluminescence system. Densitometric analysis was performed using Scion Image software.

2.11. Animals and treatments

Male Sprague–Dawley rats weighing 180–200 g were obtained from the Center of Experimental Animals of the third affiliated hospital of the Third Military Medical University (TMMU), Daping Hospital (Chongqing, China). These rats were maintained in an air-conditioned room with 12 h light–dark cycle. All procedures on animals followed the Institutional Animal Care Committee at the TMMU.

Rats were allowed 1 week to adapt to the laboratory environment followed by random assignment into two groups, namely control and high-fat diet groups. The control group was fed with standard laboratory normal chow. The high-fat diet group was fed a high-fat diet consisting of 55% energy derived from fat, 29% from carbohydrate and 16% from protein for 8 weeks. High-fat diet rats were further divided into five subgroups (n = 10) as follows: 1, high-fat diet; 2, high fat-diet plus metformin (150 mg/kg BW.d); 3, 4 and 5, high fat-diet and DHM (10, 50 and 100 mg/kg BW.d), respectively. Animals were gavaged with DHM or metformin per day.

2.12. Glucose tolerance test

A glucose tolerance test was performed after rats were fasted overnight. Briefly, rats were injected intraperitoneally with 1 g/kg BW glucose. Blood glucose was quantified in tail blood samples collected at 0 (prior to glucose administration), 30, 60, 90 and 120 min after glucose administration.

2.13. Insulin tolerance test

An insulin tolerance test was performed 3 days after the glucose tolerance test. All rats with free access to water and food were injected intraperitoneally with insulin (1 IU/kg BW) and blood glucose was quantified in tail blood samples collected at 0 (prior to insulin administration), 30, 60, 90 and 120 min after the administration of insulin.

2.14. Statistical analysis

Quantitative data are presented as means ± standard deviation (SD) of three experiments. Statistical analyses were performed with the t-test and one-way analysis of variance using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA). A p-value < 0.05 was considered statistically significant and the Tukey–Kramer post-hoc test was applied if p < 0.05.

3. Results

3.1. PA-induced insulin resistance and glucose uptake impairment in skeletal muscle myotubes

PA was used to induce insulin resistance in L6 myotubes as described previously (Parvaneh et al., 2010). L6 myotubes were incubated with different concentrations (0.25, 0.5 and 0.75 mM) of PA for 24 h or with 0.75 mM PA for different time intervals (2, 4, 8, and 16 h), then stimulated with or without 100 nM insulin for 20 min. As shown in Fig. 1A and B, PA decreased glucose uptake in a time- and dose-dependent manner in L6 myotubes under insulin-stimulated conditions. Furthermore, PA dose-dependently downregulated the concentration of pIRS-1 and pAkt in L6 myotubes under insulin-stimulated conditions (Fig. 1C–F). Treatment of insulin-stimulated L6 myotubes with PA (0.75 mM) for 16 h significantly decreased glucose uptake and the concentration of pIRS-1 and pAkt. Therefore, 0.75 mM PA and 16 h were the conditions selected for the induction of the SMIR model in vitro in subsequent experiments.

3.2. DHM improved SMIR in vitro and in vivo

As shown in Fig. 2A and B, DHM could not markedly increase the concentration of p-IRS1 and p-Akt under basal condition in PA-treated myotubes. Moreover, we found that DHM pretreatment significantly reversed the PA-induced decrease of glucose uptake and the phosphorylation of IRS-1 and Akt in a dose- and time-dependent manner in L6 myotubes under insulin-stimulated conditions.

In rats fed a high-fat diet to induce insulin resistance in vivo (Perreault and Marette, 2001) a notable downregulation of the phosphorylation of IRS-1 and Akt was observed in skeletal muscle tissues after 8 weeks. Treatment with DHM at 10, 50 and 100 mg/kg per day markedly upregulated these two insulin resistance signaling components, similar to the effect of metformin (100 mg/kg per day) treatment (Fig. 2I and J). A notable increase of fasting glucose levels
in the glucose tolerance test was observed in the high-fat diet groups (the model group) compared with the control group, and this increase was markedly attenuated by administration of DHM (50 and 100 mg/kg per day) in a similar manner to the upregulation observed following metformin (100 mg/kg per day) treatment (Fig. 2K). Simultaneously, improved insulin sensitivity was also observed in DHM-fed rats as measured by the insulin tolerance test (Fig. 2L). These results suggested that DHM improved SMIR in vitro and in vivo.

3.3. DHM improved SMIR by inducing autophagy in vitro and in vivo

The effect of DHM on autophagy in L6 myotubes was examined by measuring the following four autophagy-related markers by western blotting: LC3-II, Beclin1, Atg5, and the degradation of p62. L6 myotubes were pretreated with different concentrations (0.1, 0.5, and 1 μM) of DHM for 2 h followed by treatment with 0.75 mM PA for another 16 h. DHM dose-dependently increased the conjugated form of LC3 (LC3-II), the expression of Beclin1 and Atg5, and increased the degradation of p62 (Fig. 3A and B). Moreover, assessment of autophagosome formation by transmission electron microscopy (TEM), which is the most valid method for both the qualitative and quantitative analyses of autophagy, revealed that DHM induced the formation of vacuoles in PA-treated L6 myotubes (Fig. 3C and D). As shown in Fig. 3E and F, L6 myotubes cotreated with DHM (1 μM) and PA (0.75 mM) displayed a significant increase in the number of autophagic structures (GFP-LC3 dots) compared with L6 myotubes treated with PA alone.

We next assessed the effect of DHM on autophagy in high-fat diet-fed rats. DHM treatment markedly upregulated the level of LC3-II, the expression of Beclin1 and Atg5, and increased the degradation of p62 in skeletal muscle tissues compared with the model group (Fig. 3G and H). These results suggested that DHM supplementation also induced autophagy in skeletal muscle tissues of insulin resistant rats.

The role of autophagy in DHM-mediated SMIR improvement was determined using chemical inhibitors including 3-MA and Baf A1 or genetic approaches using ATG5 and Beclin1 siRNA to inhibit autophagy. Immunoblotting showed that suppression of autophagy significantly abrogated the increase of p-IRS-1 and p-Akt in response to DHM in PA-treated L6 myotubes under insulin-stimulated conditions, whereas the autophagy activator RAPA further promoted the DHM-induced upregulation of the expression of the two insulin resistance markers (Fig. 4A–F).

The autophagy inhibitor 3-MA aggravated the PA-induced decrease in glucose uptake, whereas the autophagy activator RAPA had the opposite effect. Furthermore, the protective effects of DHM against PA-induced inhibition of glucose uptake under insulin-stimulated conditions were abolished by pretreatment with 3-MA or siRNA against Beclin1 or Atg5, whereas they were enhanced by pretreatment with RAPA (Fig. 4G and H). These results suggested that autophagy induction was required for the protective effects of DHM against SMIR.
DHM attenuated SMIR in vitro and in vivo. (A) Differentiated L6 myotubes were pretreated with different concentrations (0.1, 0.5 and 1 μM) of DHM for 2 h, followed by incubation with PA (0.75 mM) for another 16 h. IRS-1, p-IRS-1, Akt, and p-Akt were detected by western blotting. (B) The bar graphs show the quantification of the indicated proteins. (C) Differentiated L6 myotubes were treated as described in (A), then were stimulated with 100 nM insulin for another 20 min. The indicated proteins were detected by western blotting. (D) The bar graphs show the quantification of the indicated proteins. (E) Differentiated L6 myotubes were pretreated with 1 μM DHM for the indicated times (0.5, 1 and 2 h) and then incubated with 0.75 mM PA for an additional 16 h before stimulation with 100 nM insulin for 20 min. IRS-1, p-IRS-1, Akt, and p-Akt were detected by western blotting. (F) The bar graphs show the quantification of the indicated proteins. (G) Differentiated L6 myotubes were treated as described in (A), then were stimulated with or without 100 nM insulin for 20 min. 3H-2-deoxyglucose uptake by L6 myotubes was assessed as indicated in Section 2. (H) Differentiated L6 myotubes were pretreated with 1 μM DHM for the indicated times (0.5, 1 and 2 h) and then incubated with 0.75 mM PA for an additional 16 h before stimulation with or without 100 nM insulin for 20 min. 3H-2-deoxyglucose uptake by L6 myotubes was assessed as indicated in Section 2. Rats were fed a high fat diet for 8 weeks and treated with or without different doses of DHM (10, 50, and 100 mg/kg BW/d) or metformin (100 mg/kg BW/d) (n = 10 per group). (I) Total tissue lysates from skeletal muscle were immunoblotted with anti-IRS-1, anti-p-IRS-1, anti-Akt, anti-p-Akt, and anti-β-actin antibodies. (J) The bar graphs show the quantification of the indicated proteins. (K) Glucose tolerance and (L) insulin tolerance were assessed as indicated in Section 2. Values are presented as means ± SD. n = 3. *p < 0.05, **p < 0.01 versus the model group (the PA and insulin-stimulated myotubes were set as the model group for in vitro studies; and for in vivo studies the high-fat diet fed animals were set as the model group). A.U., arbitrary units.
Fig. 3. DHM induced autophagy in skeletal muscle myotubes in vitro and in vivo. (A) Differentiated L6 myotubes were pretreated with different concentrations (0.1, 0.5, and 1 μM) of DHM for 2 h followed by incubation with 0.75 mM PA for an additional 16 h. Total L6 cell lysates were immunoblotted with anti-p62, anti-Atg5, anti-Beclin1, anti-LC3, and anti-β-actin antibodies. (B) The bar graphs show the quantification of the indicated proteins. (C) Representative TEM depicting the ultrastructure of myotubes incubated with or without DHM (1 μM) for 2 h followed by treatment with PA (0.75 mM) for another 16 h. Arrows indicate autophagosomes. (D) The bar graphs show the number of autophagosomes. (E) Representative confocal images of GFP fluorescent puncta in L6 myotubes transfected with GFP-LC3 plasmids for 24 h and then treated as described in (A). (F) The number of GFP-LC3 dots in each cell was counted. Metformin (100 mg/kg BW/d) or different doses of DHM (10, 50, and 100 mg/kg BW/d) were administered to rats fed a high fat diet for 8 weeks (n = 10 per group). (G) Muscle tissue lysates were then immunoblotted with the antibodies described in (A). (H) The bar graphs show the quantification of the indicated proteins. Values are presented as means ± SD. n = 3, *p < 0.01 versus the vehicle-treated control group; **p < 0.05, ***p < 0.01 versus the model group. A.U., arbitrary units.
AMPK signaling pathway played a key role in DHM-mediated autophagy induction in skeletal muscle myotubes

The AMPK-mTOR signaling pathway plays an important role in the regulation of autophagy. Therefore, the possible role of AMPK-mediated signaling in DHM-induced autophagy was further investigated. As shown in Fig. 5A–D, DHM treatment significantly upregulated the concentration of p-AMPK and p-Ulk1, and downregulated the phosphorylation of mTOR and mTOR downstream target phosphorylation of p70S6K (Fig. S1A–D) in a dose- and time-dependent manner in PA-treated L6 myotubes. Similar results were observed in skeletal muscle tissues of high fat diet-fed rats (Fig. 5E, F and S1E, F). These results indicated that DHM activated AMPK-mediated signaling pathways involved in autophagy in vitro and in vivo.

The correlation between the activation of AMPK signaling and autophagy induction by DHM was further evaluated using the AMPK specific inhibitor CC or AMPK siRNA. As shown in Fig. 6A–D, inhibition of AMPK abrogated DHM-induced increase of LC3-II in PA-treated L6 myotubes. These results suggested that the activation of AMPK signaling played a role in DHM-induced autophagy. Furthermore, suppression of AMPK significantly abolished DHM-induced upregulation in the phosphorylation of IRS-1 and Akt (Fig. 6E and F), and attenuated the DHM-induced increase in glucose uptake in vitro.
PA-treated L6 myotubes under insulin stimulated conditions (Fig. 6I and J). Taken together, these data suggest that AMPK activation plays a key role in DHM-mediated prevention of SMIR by inducing autophagy.

3.5. Activation of AMPK by DHM is mediated by the inhibition of mitochondrial F1F0-ATP synthase activity in skeletal muscle myotubes

AMPK monitors the cellular energy status mainly by sensing increases in the AMP/ATP ratio, which is usually stimulated by inhibition of mitochondrial ATP synthesis (Hardie et al., 2012). Therefore, the cellular contents of ATP and AMP were quantified by HPLC, which showed that the AMP/ATP ratio increased gradually in response to DHM (0.1, 0.5, and 1 μM) treatment in PA-stimulated L6 myotubes (Fig. 7A). Analysis of the ATP synthase (F0F1-ATPase) activity of mitochondrial fractions prepared from L6 myotubes showed that DHM significantly inhibited mitochondrial F0F1-ATPase activity in a concentration-dependent manner in PA-stimulated L6 myotubes (Fig. 7B). These results suggested that DHM activated AMPK by increasing the AMP/ATP ratio through the inhibition of F0F1-ATPase activity.

4. Discussion

To the best of our knowledge, this study provides the first evidence that DHM improves insulin sensitivity by inducing autophagy, and that autophagy is induced via the activation of AMPK by an increase in the cellular AMP/ATP ratio resulting from F0F1-ATPase inhibition.

Improving insulin resistance is an important strategy for the prevention and treatment of type 2 diabetes. Naturally occurring plant compounds, particularly flavonoids, are attractive candidates because of their abundance in nature, cost-effectiveness and fewer side effects than those associated with pharmaceutical agents currently under clinical use. *Ampelopsis grossedentata* is a medicinal and edible plant...
Fig. 6. DHM-induced autophagy through AMPK activation in skeletal muscle myotubes in vitro and in vivo. (A) Differentiated L6 myotubes were treated with CC (10 μM) for 1 h before the addition of DHM (1 μM) for 2 h, followed by incubation with PA (0.75 mM) for another 16 h. Total L6 myotube lysates were then immunoblotted with anti-p-AMPK, anti-LC3, and anti-β-actin antibodies. (B) The bar graphs show the quantification of the indicated proteins. (C) Differentiated L6 myotubes were transfected with AMPK siRNA and treated with DHM (1 μM) for 2 h followed by incubation with PA (0.75 mM) for a further 16 h. The indicated proteins were detected by western blotting. (D) The bar graphs show the quantification of the indicated proteins. (E) Differentiated L6 myotubes were treated with CC (10 μM) for 1 h before the addition of DHM (1 μM) for 2 h, followed by incubation with PA (0.75 mM) for another 16 h. Total L6 myotube lysates were immunoblotted with anti-p-AMPK, anti-p-IRS-1, anti-IRS-1, anti-Akt, anti-p-Akt, and anti-β-actin antibodies. (F) The bar graphs show the quantification of the indicated proteins. (G) Differentiated L6 myotubes were transfected with AMPK siRNA and treated with DHM (1 μM) for 2 h, followed by incubation with PA (0.75 mM) for a further 16 h. The indicated proteins were detected by western blotting. (H) The bar graphs show the quantification of the indicated proteins. (I) Myotubes were treated as described in (E) and glucose uptake was determined by flow cytometry. (J) Myotubes were treated as described in (G) and glucose uptake was determined by flow cytometry. Values are presented as means ± SD. n = 3, "p < 0.01 versus the insulin-stimulated control group or vehicle-treated control group; "p < 0.01 versus PA-treated group; "p < 0.01 versus DHM and PA co-treated group. A.U., arbitrary units.
that is widely distributed in Southern China. Its tender stem and leaves have been consumed as a healthy tea (Tengcha) for the prevention and treatment of the common cold, sore throat, and icteric viral hepatitis for hundreds of years. DHM is the main flavonoid component of Ampelopsis grossedentata, and its leaves contain more than 30% DHM (Wang et al., 2002). Such a high level of flavonoids in a natural plant is rare. DHM possesses biological and pharmacological activities including anti-oxidative, anti-inflammatory, hepatoprotective and anti-cancer effects (Zhou et al., 2014). However, the effects of DHM on insulin resistance remain to be investigated. In the present study, we showed that DHM treatment significantly improved SMIR in both in vitro and in vivo models. Our results are in line with those of previous studies evaluating the effects of flavonoids with similar chemical structure to DHM on insulin resistance (Dai et al., 2013; Pu et al., 2012), and suggest that DHM could be developed as a novel therapeutic agent for the prevention and treatment of type 2 diabetes.

Autophagy is a lysosome-dependent process for the turnover of dysfunctional or damaged intracellular organelles and molecules in response to several stresses, including oxidative stress and endoplasmic reticulum (ER) stress. Insulin resistance is characterized by the retention of dysfunctional or damaged components leading to cellular stresses and/or inflammation, which in turn exacerbates insulin resistance (Codogno and Meijer, 2010). Therefore, removal of these accumulated components by autophagy is beneficial to overcome insulin resistance. Despite controversy regarding the suppression or activation of autophagy under insulin resistance conditions, a number of reports have shown that autophagy is suppressed in many tissues including the liver, adipocytes, muscles, and the kidney (Kume et al., 2012; Meng and Cai, 2011; Yoshizaki et al., 2012). In the present study, we showed that autophagy was triggered in PA-treated L6 myotubes and skeletal muscle tissues of high fat diet-fed rats after treatment with DHM, and inhibition of autophagy significantly abolished the DHM-induced improvement of insulin resistance. These results provide new insight into the potential mechanism underlying the anti-insulin resistance effects of flavonoids including DHM, in which autophagy may play an important role.

In the present study, we examined potential mechanisms of DHM-induced autophagy. Previous studies indicated that AMPK is a key player in the regulation of energy metabolism and is considered as a new therapeutic target for type 2 diabetes because of its reported anti-insulin resistance properties (Zhang et al., 2013). In our previous work, we showed that DHM supplementation could improve physical performance under simulated high altitude conditions partially through the activation of AMPK in skeletal muscle (Zou et al., 2014). In the present study, we investigated the role of AMPK-mediated signaling in DHM-induced autophagy in skeletal muscle. Our results showed that DHM activated the AMPK/mTOR/Ulk1 pathway, as evidenced by the upregulation of p-AMPK and p-Ulk1, and decreased p-mTOR expression in both in vitro and in vivo models of skeletal muscle insulin resistance. In addition, the AMPK inhibitor compound C (CC) and AMPK siRNA transfection abolished DHM-induced inhibition of mTOR and activation of Ulk1, thereby downregulating autophagy. These results suggest that the AMPK/mTOR/Ulk1 pathway is involved in DHM-induced autophagy in skeletal muscle myotubes.

AMPK, a sensor of cellular energy status, is a heterotrimeric protein with a catalytic α subunit that is activated by phosphorylation at Thr-172 by upstream kinases, including the liver kinase B1 (LKB1), the Ca2+/calmodulin-dependent kinase kinase-β (CaMKKβ), and transforming growth factor-β-activated kinase 1 (TAK1) (Hong et al., 2003; Momcilovic et al., 2006), and regulatory (β and γ) subunits. AMPK can be allosterically activated through the binding of AMP to one of four Bateman domains in the γ subunit, resulting in allosteric activation of the associated α subunit. Additionally, AMP activates AMPK by preventing the dephosphorylation of Thr-172 in the α subunit (Suter et al., 2006; Xiao et al., 2007). Therefore, any cellular stress that depletes cellular ATP would cause an increase in the AMP/ATP ratio, and consequently activate AMPK. ATP is synthesized by the ATP synthase F0F1-ATPase; therefore, inhibition of F0F1-ATPase activity results in the activation of AMPK caused by the decrease in cellular ATP production. Our data showed that DHM significantly inhibited F0F1-ATPase activity, decreased the ATP content and increased the AMP/ATP ratio in L6 myotubes, suggesting that inhibition of mitochondrial F0F1-ATPase activity contributes to DHM-induced AMPK activation. Previous studies showed that several groups of polyphenols including resveratrol, curcumin, quercetin, kaempferol and morin could inhibit the enzymatic activity of F0F1-ATPase (Hawley et al., 2010; Zheng and Ramirez, 2000). A possible explanation for these results is that polyphenols as secondary plant metabolites protect plants from microbial infection through the inhibition of mitochondrial function, which is consistent with the findings that many polyphenols possess antibacterial activity (Kurek et al., 2011).

In conclusion, the results of the present study indicate that DHM improves SMIR by inducing autophagy via the activation of AMPK caused by F1F0-ATPase inhibition (Fig. 8). Our findings suggest a potential role for DHM in the prevention and treatment of type 2 diabetes as well as other insulin resistance-related metabolic diseases such as nonalcoholic fatty liver disease.
Fig. 8. Proposed mechanisms of DHM-induced autophagy in skeletal muscle myotubes. DHM improves SMR by inducing autophagy via the proposed pathways: DHM activates the AMPK signaling pathway by decreasing the ATP/AMP ratio through the inhibition of FIF0-ATPase, thereby increasing ULK1 and decreasing mTOR activity, and ultimately inducing autophagy.

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Appendix: Supplementary material

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References


